Petition to THE AMERICAN BOARD OF VETERINARY SPECIALTIES

For PROVISIONAL RECOGNITION

Of a RECOGNIZED VETERINARY SPECIALITY

In VETERINARY BOTANICAL MEDICINE

Under the

AMERICAN COLLEGE OF VETERINARY BOTANICAL MEDICINE (ACVBM)

This document is submitted to petition the American Board of veterinary Specialties (AVBS) and the American Veterinary Medical Association (AVMA) for provisional recognition of Veterinary Botanical Medicine as a Registered Veterinary Speciality (RVS) under the auspices of the American College of Veterinary Botanical Medicine (ACVBM), a Recognized Veterinary Speciality Organization (RVSO).

The ACVBM was formally established in 2014 following an inaugural meeting regarding its development in 2004 with a sub-committee of the Veterinary Botanical Medicine Association. The Veterinary Botanical Medicine Association has been providing an annual examination and process for industry recognition of veterinary botanical knowledge and skills since 2003. The exam is independent, as VBMA does not offer their own courses or course content. The ACVBM has been incorporated under the laws of the state of Delaware as a non-profit organization. See appendix 1(BYLAWS). The College will serve the international veterinary community and veterinarians from all countries will be invited to apply for board certification through the American College of Veterinary Botanical Medicine.

The American College of Veterinary Botanical Medicine was officially formed to meet the demands of veterinarians practicing and advising on the use of botanical medicine. It reflects the growing public demand for botanical options, increasing use by pet food companies incorporating botanicals, increasing availability of botanicals for animal use, significant research and use by the production animal industries (aquaculture, dairy, swine and poultry) and increasing use and teaching of students by university veterinary colleges faculties. Within the various special interest groups there is also an internal recognition of individual veterinarians having expertise providing teaching, referral services and consultations to other veterinarians. The growth in veterinary interest reflected by the presence of veterinary botanical medicine lectures at most major veterinary conferences and veterinary colleges represents an opportune time to take the ACVBM to the AVBS for consideration.

Our Vision

The American College of Veterinary Botanical Medicine will provide a means to reach diplomate status through maintaining a standard base of post-graduate instruction and examination, offer educational opportunities designed to advance experience and proficiency, and enhance the integration of scientific, clinical, and traditional knowledge into veterinary medicine practice for the greater benefit of the health and well-being of animals.

Our Mission

The mission of the American College of Veterinary Botanical Medicine is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine.

Statement of Objectives of ACVBM

The ACVBM shall promote the advancement of veterinary practice by identifying to professionals and the public those veterinarians who have voluntarily sought and obtained certification in Veterinary Botanical Medicine.

To accomplish such purposes the ACVBM shall be operated:

1) To establish and maintain credentialing, certification and ethical standards for veterinary practitioners who excel in botanical medicine and who shall be titled Diplomates.

2) To identify, develop, provide and maintain professional botanical programs, to include but not limited to phytochemistry, phytopharmacology, pharmacognosy, ethnopharmacology, and ethnoveterinary medicine.

3) To examine and certify veterinarians as specialists in veterinary botanical medicine and facilitate the continued professional development of the Members of the College through development and administration of continuing education programs

4) To promote the improvement of professional practice standards, scientific inquiry and research into the safe and effective use of botanical medicines for prevention, treatment and control of animal diseases to promote a high quality of life for companion animals and to enhance the wellbeing and productivity of livestock and other production animals.

5) To collaborate with veterinary colleges and other educational institutions that relate to veterinary medicine to encourage and promote the development of graduate veterinary botanical medicine programs, especially in regard to residency training for clinical practice.

6) To identify Diplomates to the public, professionals, other professional organizations and government agencies and other appropriate individuals and bodies.

Scientific Basis of Proposed Specialty

The scientific basis of veterinary botanical medicine includes, but is not limited to,

- The pharmacological basis of herbal medicines.
    - The practice of botanical medicine incorporates complex substances, some of which are well understood in pharmacologic terms, and some of which are more nutritional in action. In addition, the science of pharmacognosy recognizes the inspiration for research into the actions of plant drugs as arising from traditional use, rather than from the evolving knowledge of molecular structure and function.
    - There are over 143000 published journal articles on plant extracts of which there are over 7000 Systematic Reviews or reviews. An example of the extensive work done in one relevant area of animal agriculture can be found in a 2016 review entitled *Herbal Remedies for Coccidiosis Control: A Review of Plants, Compounds, and Anticoccidial Actions. ……*". This paper details recent advances in the use of anticoccidal phytoextracts and phytocompounds in poultry and the review coveres 32 plants and 40 phytocompounds, their biologic actions, mechanisms and prophylactic/therapeutic potential of the compounds[1].
      Another example can be found in a 2015 review of the possible pharmacologic basis for the action of herbal medicines in the treatment of epilepsy, which indicates that hundreds of plants have been screened for anti-convulsant activity, with dozens of compounds exhibiting anticonvulsant activity equivalent to that of anticonvulsant drugs. These actions have been well characterized at the receptor level. In addition many complex extracts and single plant-derived compounds exhibit

[1] Muthamilselvan T, Kuo TF, Wu YC, Yang WC Herbal Remedies for Coccidiosis Control: A Review of Plants, Compounds, and Anticoccidial Actions. Evid Based Complement Alternat Med. 2016;2016:2657981

antiinflammatory, neuroprotective, and cognition-enhancing activities that may be beneficial in the treatment of epilepsy[2].

Veterinarians need to be aware of herb drug interactions and safety of herbs supported by published literature, including toxicology

- Manufacture, quality control and regulatory aspects of botanical medicines, standardization of products via the United States Pharmacopeia (USP) and National Formulary (NF) guarantees that the active constituent found in a botanical medicine remains at uniform concentration from batch to batch.
- Pharmacognosy
    - Each plant medicine has multiple constituents, each constituent can bind to different targets; this branch of herbal medicine is the systematic science of morphological, chemical, and biological properties along with the history, cultivation, collection, extraction, isolation, bioassaying, quality control and preparation of crude drugs of natural origin. Approximately 25% of the prescription drugs dispensed in the USA contain at least one active ingredient of plant origin[4]; empirical herbal medicine use in veterinary medicine has led to research which can produce new treatments including herbal formulas for common conditions such as atopy[5].
- Zoopharmacognosy, Ethnobotanical and Ethnoveterinary medicine
    - Veterinary herbalists must be aware of the traditional uses of plant medicines as these uses are in the public domain and remain the basis for use. In addition, there is clear evidence of "medicine-seeking" by animals in the wild (zoopharmacognosy), which may assist veterinarians who treat wild animals.
    - Principles of botanical medicine respect tradition as a source of empirical evidence which frequently informs research. The study of traditional use of plant medicines within a culture is termed ethnobotany, and the study of traditional medicines is known as ethnomedicine, or ethnoveterinary medicine. Research may validate traditional uses. An example of a traditional principle is that of synergy- combining herbs into formulas rather than using single herbs to achieve better patient outcomes[6]. A recent 2016 Review[7] titled *Favorable results from the use of herbal and plant products in inflammatory bowel disease: evidence from experimental animal studies* stated that in the majority of studies herbal therapy reduced the inflammatory activity of experimental colitis and diminished the levels of many inflammatory indices, including serum cytokines and indices of oxidative stress. The herbs examined in the studies were ones derived from ethnomedical use in humans but applied to animal models where mechanisms of action could be understood.

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[2] Sucher N, Carles M A pharmacological basis of herbal mediicnes for epilepsy Epilepsy Behav 2015 Nov 52(PtB) 308-18

[4] Ahmad I, Aqil F, Owais M. Modern Phytomedicine: Turning Medicinal Plants into Drugs. Weinheim: Wiley-VCH. 2006.

[5] Schmidt V[1], McEwan N, Volk A, Helps J, Morrell K, Nuttall T. The glucocorticoid sparing efficacy of Phytopica in the management of canine atopic dermatitis. Vet Dermatol. 2010 Feb;21(1):96-105.

[6] Yang Y, Zhang Z, Li S, Ye X, Li X, He K. Synergy effects of herb extracts: pharmacokinetics and pharmacodynamic basis. Fitoterapia. 2014 Jan;92:133-47.

[7] Triantafillidis JK, Triantafyllidi A, Vagianos C, Papalois A. Favorable results from the use of herbal and plant products in inflammatory bowel disease: evidence from experimental animal studies. Ann Gastroenterol. 2016 Jul-Sep;29(3):268-81.

- Detailed knowledge of plant medicines
    - Veterinary herbalists are skilled in plant taxonomy and identification. This skill is unique to herbalists and important to ensure that the correct species of plant is used in herbal products.
    - Veterinary herbalists have in-depth knowledge of combinations of plant species with their expected therapeutic actions, contraindications, herb drug interactions and dosing. This includes the species, constituents, actions, uses, research, doses and application of each botanical medicine or formula.
    - Veterinarian herbalists understand the dosing of herbs because of the variability of the plant medicines and clinical and patient related factors affecting dosing of botanical medicines. The herbal medicines are variable in form and concentrations and bioavailability; knowing how to relate research to the form of herb is important for efficacy and safety. This is distinct from use of drugs with more consistent formulations, but also distinct from the science of clinicall nutritionwhere food ingredients are generally safer than herbal drugs.
- Principles of botanical medicine
    - This is the application of plant based medicine (supported by science and/or traditional use) to conventional diagnosis, multiple diagnoses and complex cases and to address individual signs and problem lists; or for prevention of disease by protecting organ health and /or optimizing animal health through actions not available with conventional drugs. An example is the use of *Sylibum marianum* as a hepato-protective herb and the source of silibin[8]. Another is C*oriolus versicolor* as a source of immune regulating polysaccharides to potentially improve quality of life in canine patients with hemangiosarcoma[9];
    - The term 'rational phytotherapy' has come into use over the last two decades- this is a scientific, evidence-based system that encourages *in vitro* and *in vivo* studies to explain mechanisms of action, pathophysiology, pharmacokinetics and bioavailability as well as efficacy.
- Ethical and regulatory aspects of practicing botanical medicine including conservation and avoiding endangered species; and avoidance of use of botanical medicines which can be detected in drug testing in performance animals

Relationship to Veterinary Medical Curricula

The basic principles underlying herbal medicine including anatomy, physiology, pathophysiology and pharmacology are taught in the professional curriculum however herbal medicine is often left to electives. A survey of the AVMA-accredited veterinary institutions (Memon and Sprunger, 2011) reported that 16 (47%) out of 34 respondent colleges offered a complementary and alternative medicine (CAVM) course and herbal therapy was one of the most common topics included in the curricula. The 18 colleges without any course in CAVM or Integrative Veterinary Medicine (IVM) reportedly addressed these topics in other courses, and 4 indicated plans to offer a dedicated CAVM course within the next 5 years[11]. The same

[8] Webster CR, Cooper J. Therapeutic use of cytoprotective agents in canine and feline hepatobiliary disease. Vet Clin North Am Small Anim Pract. 2009 May;39(3):631-52.
[9] Brown DC, Reetz J. Single agent polysaccharopeptide delays metastases and improves survival in naturally occurring hemangiosarcoma. Evid Based Complement Altern Med Vol 2012, Article ID 384301 8pages
[11] Memon, M.A. and Sprunger, L.K. 2011. Survey of colleges and schools of veterinary medicine regarding education in complementary and alternative veterinary medicine. J. Am. Vet. Med. Assoc. 239, 619-623.

survey noted that two-thirds of one school's veterinary graduates encountered clinical situations involving these therapies at least monthly and over 25% experienced them on a weekly or daily basis[12].

Since then the University of Florida now includes an integrative veterinary medicine focus; Washington State University has included invited speakers in its CAVM course to expand its training in this area. Louisiana State University has recently hired a faculty member to offer clinical services and teaching in CAVM, and the University of California-Davis expanded a rehabilitation service to include integrative medicine. Colorado State University continues to provide training in CAVM. Faculty members at a number of other accredited institutions without dedicated clinical programs have obtained training including botanical medicine[13].

In addition Cornell University provides the DeeDee Arrison Holistic and Integrative Wellness lectures each year to faculty and students that include herbal medicine; North Caroline State University, Texas A&M University, University of Minnesota, University of Montreal, University of Pennsylvania, University of Prince Edward Island and Auburn Veterinary School have specifically included some herbal medicine training or electives for students. It has recently been proposed in 2016 consensus guidelines for an integrative veterinary medicine curriculum within veterinary colleges that botanical medicine including the origins and major systems of herbal therapy, selected evidence based interventions, adverse events, herb drug interactions and supplement evaluation and regulation form part of the curricula for veterinary students[14].

Recently a Retrospective Analysis of 5,195 Patient Treatment Sessions in the Integrative Veterinary Medicine Service at the College of Veterinary Medicine, University of Florida demonstrated that 8.3% (431) of patients received herbal medicine in a 400 day period[15].

Members of the Veterinary Botanical Medicine Association and AHVMA were surveyed by the ACVBM organizing committee 2016 and those members nominated they provided the following exernships for veterinary students from local veterinary schools:

- Cornell University 3 students per year
- Ross Mississippi State 8 students per year
- Ohio State 4 students yearly
- Blue Pearl Georgia Veterinary Specialists 10 interns per year
- Kansas State University 1 student per year
- Virginia Maryland 1 every few years
- University Florida 1 per year
- Iowa State 1 per year
- Ontario Veterinary College 2 per year
- UC Davis 6 students per year
- Oregon State University 2-3 per year

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[12] Memon, M.A. and Sprunger, L.K. 2011. Survey of colleges and schools of veterinary medicine regarding education in complementary and alternative veterinary medicine. J. Am. Vet. Med. Assoc. 239, 619-623.

[13] Memon MA, Shmalberg J, Adair HS etal Integrative veterinary medical education and consensus guidelines for an integrative veterinary medicine curriculum within veterinary colleges *Open Veterinary Journal*, (2016), Vol. 6(1): 44-56

[14] Memon MA, Shmalberg J, Adair HS etal Integrative veterinary medical education and consensus guidelines for an integrative veterinary medicine curriculum within veterinary colleges *Open Veterinary Journal*, (2016), Vol. 6(1): 49

[15] Shmalberg J, Memon MA A Retrospective Analysis of 5,195 Patient Treatment Sessions in an Integrative Veterinary Medicine Service: Patient Characteristics, Presenting Complaints, and Therapeutic Interventions Veterinary Medicine International Volume 2015, Article ID 983621, 11 pages

- Purdue and Ross University 1-2 students per year
- Western University 1-2 students per year
- University of Guelph 4th year students 20 per year
- Oklahoma State University 2 students per year
- Mid Western University Arizona 10-12 students per year
- Washington State 1-3 students per year
- Atlantic Veterinary College 1-3 students per year

While the training is basic for veterinary students, advanced training is usually acquired after graduation through the availability of industry recognised courses and post graduate qualifications (see Continuing Education Programs) in herbal medicine or through attendance at conferences offering in-depth coverage of these areas.

Employment of Diplomates

It is estimated that about 100 diplomates will be employed in specialty private practices and integrative practices in the US. In academic institutions, it is estimated that 30 diplomates will be employed in teaching and research positions. In industry, it is anticipated that large pharmaceutical companies and pet food companies may employ diplomates as consultants, providing jobs for up to 30 diplomates.

Continuing Education Programs

Current Programs available in Veterinary Herbal Medicine specifically
VIN

- 16 hours Intermediate Herbal Medicine
- 12 hours Introduction to Herbal Medicine
- 10 hours Introduction to Veterinary Chinese Herbal Medicine
- 6 hours Using herbs for Liver and Kidney Disease

Chi

- 150 hour Chinese Veterinary Herbal Medicine Program approved by a majority of state boards, Provides training Veterinary Chinese Herbalist and this contributes towards Masters TCVM.

IVAS

- 165 hours Veterinary Chinese Herbal Medicine training
- 500 hours Advanced Veterinary Chinese Herbal Medicine training

CIVT

- 1305 hours Graduate Diploma Veterinary Chinese Herbal Medicine (Grad Dip VCHM) accredited post graduate degree competency based training
- 945 hours Graduate Diploma Veterinary Western Herbal Medicine (Grad Dip VWHM) accredited post graduate degree competency based training
- 500 hours Foundation Course in Western Veterinary Herbal Medicine
- 445 hours Advanced Western Veterinary Herbal Medicine
- 120 hours Foundation Course Veterinary Chinese Herbal Medicine
- 40 hours Getting Started Veterinary Chinese Herbal Medicine Introductory course
- 24 hours Essentials of Western Veterinary Herbal Medicine

There are conferences and sections within conferences devoted to botanical medicine (The Chi Institute, Veterinary Botanical Medicine Association, AHVMA and American College Veterinary Botanical Medicine). In addition, there are scientific sessions devoted to botanical medicine at the major veterinary medical meetings, such as NAVC, WSAVA, Atlantic Coast Vet Conference, Wild West Veterinary Conference, Western Veterinary Conference, American College Veterinary internal Medicine Forum and American Association of Feline Practitioners. As well Diplomates from many different specialties speak on the use of herbal medicine within their specialties including Oncology, Sports Medicine, Nutrition, Behavior, Dermatology, Exotics, Avian, Internal Medicine, Hospice and Palliative Care, Dairy Cattle and others.

A Sample of Conference titles by Diplomates or academics in the last 5 years that have herbal therapies included in the speakers proceedings:

- **NAVC 2016** Justin Shmalberg, DVM DACVSMR DACVN College of Veterinary Medicine University of Florida, Gainesville, FL Acupuncture and Herbs in Sports Medicine and Rehabilitation

- **NAVC 2016** Theresa DePorter, BS, DVM, DECAWBM, DACVB Use of Nutraceutical and Botanical Therapies To Modify Behavior

- **NAVC 2016** Gary Landsberg BSc, DVM, DACVB, DECAWBM A Behaviorist's Guide To Natural Therapeutics

- **NAVC 2015** Gregory K. Ogilvie, DVM, DACVIM (Internal Medicine, Oncology), DECVIM-CA (Oncology) Top Ten Natural Cancer Therapies: Banerji and Beyond

- **NACV 2015** Douglas J. DeBoer, DVM, DACVD Global Innovations in Dermatology includes a discussion on herbal oils.

- **NAVC 2015** David C. Twedt, DVM, DACVIM Colorado State University Fort Collins, CO Do Supplements Have a Role in the Support of Gastrointestinal and Hepatic Health?

- **Pacific Veterinary Conference 2015** Leah A. Cohn, DVM, PhD, DACVIM (SAIM) Respiratory Diagnostic Testing: Aspirates, Lavage, and More discusses the adjunctive use of Yunnan baiyao.

- **Pacific Veterinary Conference 2015** Peter G. Fisher, DVM, DABVP (Exotic Companion Mammal) Exotic Companion Mammal Dermatological Disease includes the use of herbal therapy.

- **Pacific Veterinary Conference 2015** M. Christine Zink, DVM, PhD, DACVP, DACVSMR, CVSMT Integrative Medicine for the Canine Athlete includes use of herbal therapy.

- **Pacific Veterinary Conference 2015** Michael D. Willard, DVM, MS, DACVIM (SA) Chronic Hepatic Diseases in Dogs discusses silymarin.

- **Atlantic Coast Veterinary Conference 2015** Beth Overley-Adamson, VMD, DACVIM (Oncology) Updates in Oncology discusses reasonable use of integrative medicine.

- **ACVIM 2014** K. Loyd DACVIM etal *In Vitro* Effects of Yunnan Baiyao (Yb) on Coagulation College of Veterinary Medicine, University of Missouri, Columbia, MO, USA, states that YB is a complex herbal remedy used in veterinary medicine to promote hemostasis.

- **ACVIM 2013** S M Boothe DVM, PhD, DACVIM, DACVCP Nutraceuticals and Drug Interactions provides examples of potential herb drug interactions.

- **International Association of Animal Hospice and Palliative Care 2013** Bonnie Wright, DVM, DACVA, cVMA, CVPP, CCRP Pain Management in Hospice and Palliative Care includes herbal supplements.

- **Central Veterinary Conference 2013** Professor Pamela L. Ruegg, DVM, MPVM University of Wisconsin, Madison, WI, USA Treatments Used on Organic Dairy Herds presented information on the use of alternatives including herbal medicines in organic dairy farms.

- **Central Veterinary Conference 2013** - Kansas City Michael O. Childress, DVM, MS, DACVIM Complementary and Alternative Medicine for Veterinary Cancer Patients: What You and Your Clients Should Know discusses herbal medicines.

- **Central Veterinary Conference 2013** - Washington DC Kendall Taney, DVM, DAVDC Fundamentals of Oral Oncology discusses adjunctive therapy with herbs.

- **Veterinary Cancer Society Conference 2013** Dr. Brian Husbands, DVM, Diplomate ACVIM, CVA East Meets West, an Introduction to Integrative Veterinary Oncology

- **ABVP 2013** Kevin Wright, DVM, DABVP Liver Disease in Reptiles discusses herbs as options for treatment

- **NAVC 2013** Gary P. Oswald, DVM, MS, DACVIM (Internal Medicine) rug and Nutraceutical Therapy of Chronic Inflammatory Liver Disease

- **NAVC 2013** Justin Shmalberg, DVM, DACVN College of Veterinary Medicine University of Florida, Gainesville, FL Beyond Yunnan Baiyao: The Safety, Nutritional Considerations, and Efficacy of Chinese Herbal Products

- **NAVC 2013** Assoc. Professor R.M. Clemmons, DVM, PhD SACS/College of Veterinary Medicine University of Florida, Gainesville, FL TCVM Treatment of Intervertebral Disk Disease and Spinal Cord Neoplasia

- **NAVC 2013** Assoc. Professor R.M. Clemmons, DVM, PhD SACS/College of Veterinary Medicine University of Florida, Gainesville, FL CVM Treatment of Megaesophogus

- **ACVIM 2012** Frank M. Andrews, DVM, MS, DACVIM Gastric Ulcers in Horses: Pharmacologic and Management Strategies included a discussion on research on Sea Buckthorn berry extract for the treatment and prevention of gastric ulcers in horses.

- **IAAAM 2012** Bethany M. Doescher etal College of Veterinary Medicine, University of Georgia, Athens, GA, USA Serenin Vet™, A Natural Alternative Supplement, Used as an Adjunct for

Marine Mammal Behavior Modification. This supplement includes three major medicinal herbs used in veterinary herbal medicine.

- **64th Convention of the Canadian Veterinary Medical Association, 2012** Assoc Professor Art Ortenburger, DVM Department of Health Management, University of Prince Edward Island, Charlottetown, PE, Canada Integrative Medicine Approach to Common Clinical Problems in Large Animals

- **Western Veterinary Conference 2012** Jacquelin J. Boggs, DVM, MS, DACVIM (LAIM) Complementary, Not Alternative? A Practical Review of Non-Western Medicine

- **Atlantic Coast Veterinary Conference 2011** Kristina R. Vygantas, DVM, DACVO Frequently Asked Questions: Conjunctiva and KCS includes *Calendula officinalis* for conjunctivitis.

- **AAV 2011** Vanessa L. Grunkemeyer, DVM, DABVP (Avian) Avian Integumentary Wound Healing and Management Techniques Discusses Yunnan Bai Yao

- **ACVIM 2011** E. Carothers etal Comparison of a Proprietary Herbal Topical Cream to Silver Sulfadiazine in an Equine Wound Model Department of Clinical Sciences, College of Veterinary Medicine, Mississippi State, MS, USA, in which the topical treatment was effective.

- **ACVIM 2011** Mark E. Peterson, DVM, DACVIM Methimazole, Carbimazole & Alternative Medical Therapies for Feline Hyperthyroidism

- **Western Veterinary Conference 2011** Meri Stratton-Phelps, DVM, MPVM, DACVIM (LAIM), DACVN Making Nutrition A Profitable Part of Your Practice horse food and herbs.

- **Western Veterinary Conference 2011** Francis W.K. Smith Jr., DVM, DACVIM (SAIM & Cardiology) Complementary Therapies for Cardiac Diseases discusses a group of herbal therapies for heart disease.

- **Atlantic Coast Veterinary Conference 2010** Emily D. Levine, DVM, MRCVS, DACVB Noise Fears, Sensitivities, and Phobias includes reference to herbal therapies.

- **Atlantic Coast Veterinary Conference 2010** Michael Willard, DVM, MS, DACVIM Chronic Hepatobiliary Diseases of Dogs includes reference to Milk Thistle.

A Sample of Conference titles discussed by veterinary herbalists in the last 5 years that have herbal therapies included in the speakers proceedings:

- **Western Vet Conference : WVC 2016.** Ihor Basko Medical Mushrooms to Treat Cancer
- **Western Vet Conference : WVC 2016.** Nancy Scanlan Herbs for Immune System and Cancer
- **Wild West Veterinary Conference 2016** Rob Silver Applications of Cannabinoid Therapies for Geriatric Conditions
- **Wild West Veterinary Conference 2016** Rob Silver Chronic Kidney Disease- an Integrative Approach
- **Wild West Veterinary Conference 2016** Rob Silver Cannabis for Cancer?
- **Wild West Veterinary Conference 2016** Gregg Todd Applying Traditional Chinese Veterinary Medicine in Respiratory Disease Treatment
- **Wild West Veterinary Conference 2016** Gregg Todd The Treatment of Musculoskeletal Disease Utilizing Traditional Chinese Veterinary Medicine.
- **Wild West Veterinary Conference 2016** Gregg Todd How to Approach Dermatological Cases with Traditional Chinese Veterinary Medicine.
- **Wild West Veterinary Conference 2016** Gregg Todd Common behavior patterns: Their Diagnosis and Treatment with Traditional Chinese Veterinary Medicine.
- **NAVC 2016** Huisheng Xie A Severe Atopic Dog, A TCVM Approach
- **NAVC 2016** Huisheng Xie Non-controlled Seizure Dog: A TCVM Approach
- **NYSVC 2016** Barbara Fougere The Evidence Base for Veterinary Herbal Medicine
- **NYSVC 2016** Barbara Fougere Herbal Oncology I
- **NYSVC 2016** Barbara Fougere Herbal Oncology II
- **NAVC 2015** Steve Marsden Integrative Management of Systemic Lupus in a Dog
- **NAVC 2015** Steve Marsden Integrative Management of Musculoskeletal Disorders
- **NAVC 2015** Steve Marsden Integrative Neurology: Disorders of the Back and Hind Limbs
- **WSAVA 2015**. X. Huisheng Traditional Chinese Veterinary Medicine for Renal Failure
- **WSAVA 2015**. S.H. Xie Traditional Chinese Veterinary Medicine for Itching Dogs
- **WSAVA 2015**. B. Fougere How Do Herbs Work? An Introduction to Herbal Modes of Action and Use
- **WSAVA 2015**. B. Fougere Veterinary Herbal Medicine - Where's the Evidence?

- **NAVC 2014** Huisheng Xie, DVM, PhD College of Veterinary Medicine University of Florida, Gainesville, FL Treatment of Icterus / Hepatitis
- **NAVC 2014** Huisheng Xie, DVM, PhD College of Veterinary Medicine University of Florida, Gainesville, FL How To Use TCVM for the Treatment of Seizures
- **NAVC 2014** Constance DiNatale, DVM Cancer and Immune Dysfunction
- **NAVC 2014** Constance DiNatale, DVM TCVM Approach To Skin Case
- **NAVC 2014** Nancy Scanlan, DVM How Some "Immune Boosters" Really Work and How Best To Use Them
- **Atlantic Coast Vet Conference : ACVC 2014**. Robert J. Silver Integrative Oncology
- **Atlantic Coast Vet Conference : ACVC 2014**. Robert J. Silver Introduction to Veterinary Nutraceuticals
- **Atlantic Coast Vet Conference : ACVC 2014**. Robert J. Silver Using Nutraceuticals to Maintain Quality of Life in Pet Hospice
- **Atlantic Coast Vet Conference : ACVC 2014**. Robert J. Silver An Overview of Integrative Veterinary Medicine
- **Wild West Veterinary Conference 2013** Gregory Todd A Traditional Chinese Veterinary Medical Approach to Neoplastic Disease
- **Wild West Veterinary Conference 2013**. Gregory Todd Immunology: A Traditional Chinese Veterinary Medicine Perspective
- **Wild West Veterinary Conference 2013**. Gregory Todd he Basic Principles of Cardiology in Traditional Chinese Veterinary Medicine
- **Wild West Veterinary Conference 2013**. Gregory Todd Diagnosis and Treatment of Common Gastrointestinal Patterns in Traditional Chinese Veterinary Medicine
- **Western Vet Conference : WVC 2013**. J. Randy Kidd Herbal Medicines for Animals
- **Am College Vet Internal Med Forum : ACVIM 2013**. Martha F. Mallicote Acupuncture and Herbal Medicine Used for Treatment of Anhidrosis
- **UC Davis 2013**. Madeline S. Yamate Managing Geriatric Patients with Traditional Chinese Medicine
- **Am Assoc Feline Practitioners : AAFP 2012**. Steve Marsden Perspectives on Feline Hyperthyroidism: Speculated Pathogenesis & Herbal Treatment
- **Am Assoc Feline Practitioners : AAFP 2012**. Steve Marsden Herbal Management of Feline Chronic Vomiting & Inflammatory Bowel Disease
- **Am Assoc Feline PractitionersAAFP 2012**. Robert J. Silver Introduction to the Clinical Use of Nutraceutical and Botanical Therapies

- **Western Vet Conference : WVC 2012.** Deborah M. Mitchell TCVM Diagnosis and Treatment of Seizures
- **Western Vet Conference : WVC 2012**. Deborah M. Mitchell Do the Symptoms Match the Picture? TCVM Approach to Skin Disease
- **Canadian Vet Med Assoc : CVMA 2012.** Deborah M. Mitchell Integrative Medical Approach to Common Clinical Problems in Small Animals
- **Western Vet Conference : WVC 2011**. Signe E. Beebe Chinese Medicine Approach to Feline Chronic Renal Failure
- **Western Vet Conference : WVC 2011.** Signe E. Beebe Chinese Herbs to Treat Endocrine Disorders I and II
- **Western Vet Conference : WVC 2011**. Signe E. Beebe Integrative Cancer Therapies I and II
- **Wild West Veterinary Conference 2011** Cindy Wallis The Use of Chinese Herbal Medicine to Treat Cancer in Dogs
- **Wild West Veterinary Conference 2011**. Cindy Wallis Chinese Herbal Medicine for Inflammatory Bowel Disease
- **Wild West Veterinary Conference 2011**. Cindy Wallis How to Use Chinese Herbal Medicine
- **UC Davis 2011.** Michael Salewski Performance: Strategies for Animal Athletes

Relationships with Existing RVSOs and the Uniqueness of Veterinary Medicine Specialty.

Botanical medicine overlaps and integrates with many specialties as herbs can fill therapeutic "holes" in the veterinarian's treatment armamentarium. For instance, there are many plants used as traditional "tonics", intended to invigorate normal organ functions. While some of these traditional tonics and alteratives have recognized activities, such as antioxidation or microbiome support, some are still not well enough understood to classify them in a physiologic or pharmacologic sense. Herbal antimicrobials are being investigated as alternatives to conventional antibiotics due to increasing antimicrobial resistance. The use of herbs is especially intriguing where clinical proof of efficacy predates our understanding of disease pathophysiology, illustrated in a recent review showing herbs and plant therapy are efficacious for IBD in human patients[16].

Specialists in canine, feline, and avian practice encounter questions about herbal therapies from their clients frequently. Among veterinarians who offer botanical medicine, herbs are recommended for patients also being concurrently treated by oncologists, internists, surgeons, physical therapy/rehabilitation and reproductive specialists, ophthalmologists, cardiologists, neurologists, behaviorists and dermatologists. Dairy, beef, swine, poultry and food animal specialists may depend upon herbal therapies for the treatment of animals raised for organic food. Plant-based medicines impact the

[16] Triantafyllidi A, Xanthos T, Papalois A, Triantafillidis JKHerbal and plant therapy in patients with inflammatory bowel disease. Ann Gastroenterol. 2015 Apr-Jun;28(2):210-220.

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practice of anesthesiology, pharmacology and toxicology and even comprise some of the training for these specialties. Herbal therapies hold potential in the treatment of infectious disease, and therefore impact the bacteriology/mycology, microbiology, parasitology and immunology specialties, as well.

However, while botanical medicine overlaps many other specialties, the depth of knowledge required of a specialist in phytopharmacology and phytomedicine is not covered by any of these. Even the pharmacology College, which might be the closest relation of all the specialties, would concentrate on only a subset of the mechanisms by which herbs work. There are tens of thousands of medicinal plants described in the herbal medicine literature, likely overshadowing the conventional drug pharmacopeia by orders of magnitude. Pharmacology focuses on isolation of a single constituent to characterize and use therapeutically. By contrast, herbalists advocate for the complex actions initiated when a plant or plant extract containing hundreds of compounds – both nutritional and pharmacologic – are in play. In addition, the discovery process for drugs and herbs differs widely. New drugs are more often designed through the theoretical knowledge of physiologic mechanisms as they are by accident. The effective use of herbal medicine often requires knowledge of their ethnobotanical medical uses - or familiarity with the culture and medical system that popularized the use of a medicinal plant. Ethnobotanical medicine is a specific subspecialty with increasing numbers of peer reviewed publications, and could be supported and explored adequately only if herbal medicine stands alone as a specialty.

No other specialty possesses the unique set of skills and knowledge required by herbal medicine practice. If herbal medicine became a subspecialty of pharmacology or any other RVS, too much additional knowledge and experience would be required of diplomat. An even larger concern is the dilutional effect that training in the other RVS plus herbal medicine would have on the practice of herbal medicine.

There are relationships and commonalities between veterinary botanical medicine and the following RVSOs:

American Board of Veterinary Practitioners: general practitioners may treat patients with chronic, recurrent disease where conventional therapies have been exhausted or are contraindicated. The herbal medicine specialist may act as a consultant by virtue of his or her detailed knowledge of herbal medicine principles, medicines and therapeutic strategies that can used in an integrative manner or instead of conventional drugs , and to help prevent disease.

American Board of Veterinary Toxicology: Toxicologists are familiar with toxic principles of plants. Many of the plants that are considered toxic by toxicologists are also considered therapeutic by botanical medicine specialists, who are necessary to provide this complementary information on plant use and abuse. Herbal medicine specialists will be more informed on dosing that "makes the medicine or the poison". The botanical medicine diplomate can also provide information on botanical support for animals that are poisoned such as *Silybum marianum* for amanita poisoning and also provide input into reducing the toxicity of certain drugs- such as *Silybum marianum* providing a protective effect against gentamycin induced toxicity.

American College of Laboratory Animal Medicine: There exists a large body of literature examining the effects of herbal medicines in experimental animals, and research in animal models is fundamental to human studies in herbal medicine. The botanical medicine diplomate can advise on suitable dosages of herbs and plant constituents and appropriate herbs the conditions being investigated based on herbal medicine principles and ethnobotanical information.

American College of Poultry Veterinarians: There is increasing research on the use of herbs and plant extracts in poultry production although most poultry veterinarians do not identify with herbal medicine

use. The botanical medicine diplomate can act as an interface between industry research and clinical medicine and advice on potential treatment of diseases, prevention of disease and also in treatment of backyard chickens.

American College of Theriogenologists: Reproduction and fertility is an area of specific research in human herbal medicine and increasing publication in veterinary medicine. This is represented by Dr Reed Holyoak DVM, PhD, DACT Professor of Theriogenology, OK State Univ who incorporates botanical medicine in the treatment of reproductive and fertility issues in horses. He teaches veterinarians in the use of plants, but as in every other facet of medicine, this is a mere introduction to a larger field that requires additional training in order to make competent use of it.

American College of Veterinary Anesthesia and Analgesia. Some herbs have the potential to interact with anaesthesia. With increasing use of herbs by the public the botanical medicine diplomate can advise on use, research topics and the benefits of using some herbs in conjunction with anesthesia as well as input into the potential for herbs and plant extracts such as cannabis for analgesia. The botanical medicine diplomat would serve as a resource to anesthesiologists and veterinarians as pet owners continue to find and administer new over the counter herbal supplements to their animals.

American College of Veterinary Behavior: Because herbal remedies are already available to the public, veterinarians are frequently consulted about the value of herbal anxiolytics, sedatives, and antidepressants even while lay dog trainers recommend them routinely. The botanical medicine diplomate can support veterinary behaviourists in having more in-depth knowledge about these over-the-counter remedies and how they will interact with pharmacologic interventions. The veterinary botanical specialist may also advise or be involved in research on herbs and plant extracts that have potential use in a number of clinical conditions associated with behavioural issues. Veterinarians are already utilising herbal training to inform research directions for companies investigating herbal alternatives to drugs for companion animals.

American College of Veterinary Clinical Pharmacology: An important basis of herbal medicine is the pharmacology and mechanisms of actions of plants and plant extracts, however they differ from drugs in their complexity, and in the underlying ethnomedical knowledge base used to prescribe them.. The botanical medicine diplomate can provide context to client and referring veterinarian inquiries, both ethnobotanical and scientific. The botanical medicines specialist might also collaborate with veterinary clinical pharmacologists on research design, herb-drug-nutrient interactions and other clinical issues.

American College of Veterinary Dermatology: Studies have shown that people seek complementary and alternative therapies for (among other things) relief of chronic discomfort, and chronic dermatologic disorders (especially allergies) that prove difficult to resolve comprise a major proportion of cases seen by general practitioners and Dermatology specialists. Veterinary herbalists are presented with a large proportion of chronic dermatological conditions that are poorly responsive to conventional treatment or where pet owners seek other options. Many herbs and formulas have the potential to improve patient quality of life and the herbal medicine diplomate can be involved in directing research in this area and working together on challenging cases. Some diplomate dermatologists have been trained in herbal medicine.

American College of Veterinary Internal Medicine: Metabolic diseases are common in equine and small animals. The herbal medicine diplomate will be trained to apply herbal medicine strategies and integrative medicine for chronic metabolic disorders where conventional treatment may not be successful or where herbal medicine can augment medication or protect from side effects or sequelae of the disorder.

Botanical medicine diplomats would act as important consultants in the design of clinical trials that incorporate herbal therapies.

American College of Veterinary Nutrition: Functional foods including herbal medicines are being employed in therapeutic diets and products in veterinary medicine. The herbal medicine diplomate will be able to advise on suitable herbs to meet therapeutic needs in product development in the pet food industry and also provide advice on the addition of herbs to diet plans and critical care nutrition to benefit the patient therapeutically.

American College of Veterinary Ophthalmology: Diseases which are refractory to conventional medicine or progressive in nature can be attenuated by herbal medicines and plant extracts. A botanical medicine diplomate can advise on research and treatment on challenging cases and product development. Some therapeutic products containing herbs have been developed by a ACVO Diplomate Dr Carmen Colitz, DVM, PhD.

American College of Veterinary Preventive Medicine: In the field of production animals, environmental health, and antibiotic resistance, the botanical medicine diplomate can provide advice on research, potential for residues and appropriate herb use to improve public health and food safety especially in the areas of organic production.

American College of Veterinary Sports Medicine and Rehabilitation: The botanical medicine diplomate can advise on appropriate herbal medicine use to support rehabilitation of patients and avoiding herb and plant extract doping of performance animals.

American College of Zoological Medicine: The botanical medicine diplomate can advise on appropriate treatment strategies for challenging cases in zoo medicine. Herbal medicine diplomats will be familiar with the zoopharmacologic, ethnoveterinary and ethnobotanical research related to the animal's geographic and cultural background, providing potentially more scientifically supported herbal therapies.

American College Veterinary Emergency & Critical Care: The botanical medicine diplomate can provide advice on integrative treatment of critical care cases where options may be limited such as acute liver and kidney failure which may benefit from the addition of botanical medicine. The formulaYunnan baiyao is already being used in critical care practice and warrants further research[17].

American Veterinary Dental College: A number of veterinary dental products contain essential oils and herb extracts and research in human dentistry includes research on herbs often using dogs as models. Having the link between botanical medicine and dentistry might accelerate preventative medicine in one of the most common disorders of small animals.

INDUSTRY AND PROFESSIONAL SUPPORT

A brief internet search will show that the number of companies offering herbal remedies for every animal condition is rapidly expanding. The vast majority of these companies, owned by lay entrepreneurs, are supported by no veterinary knowledge at all. The establishment of a veterinary botanical medicine

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[17] Murphy LA, Panek CM, Bianco D, Nakamura RK Use of Yunnan Baiyao and epsilon aminocaproic acid in dogs with right atrial masses and pericardial effusion. J Vet Emerg Crit Care (San Antonio). 2016 Sep 26

specialty will serve to highlight the role of veterinarians in the medical treatment of animals, whether the medication is OTC or prescription. The establishment of this specialty will address the growing interest, demand and use of alternatives and adjuncts to conventional drugs in production animals, equines and small animals coupled with the recognition that herbal medicine offers a substantial scope of pharmacological actions and benefits in addition to conventional medicine for challenging medical issues. The herbal medicine diplomate must have an expanded understanding in the areas including, but not limited, to principles of herbal medicine (whether ethnomedical or scientific principles are being used in decision-making);, the herbs (pharmacology, the correct portion of the plant that has medicinal properties, preparation, manufacture, indications, palatability, application, compatibilities with other herbs, herb drug interactions, contraindications, dosing), therapeutic strategies, nutrition, pharmacology and this is in addition to basic knowledge of traditional medical and surgical veterinary problems.

In a survey conducted in 2016 by the ACVBM organising committee to approximately 800 members of AHVMA and 240 members of VBMA, 103 veterinarians responded (9.9%).

- 86% had been referred a clinical case from a veterinarian in the past 12 months.
- 72% of respondents had been referred between 10 and more than 50 cases over the past 12 months.
- 45% of respondents had been referred between 1 and more than 50 clinical cases from a board certified specialist in various fields for herbal medicine treatment in the past 12 months.
- 96% of veterinary herbalists had provided advice to a veterinarian on the use of herbs in the past 12 months.
- 40% had consulted to between 10 and 50 veterinarians over the past 12 months.
- 70% of the advice to other veterinarians covered case management, herb choice, herb dose and herb drug interactions.
- 34% of respondents provided a training service or internship for veterinary students.
- 55% of veterinarians had formal training in Western or Chinese herbal medicine.
- 22% of respondents had provided advice/ consulting to industry and pharma on use of herbs, product formulation or safety.

The ACVBM Organising committee has received support for this petition from a selection of academics at veterinary schools; diplomates in private specialist practices, veterinary students, organic dairy industry boards and members of the general public (See Appendix II & III).

ACVBM BYLAWS

The Bylaws (Appendix I) were prepared according to the guidelines in the ABVS *Criteria for Establishment of a Veterinary Specialty Organization*. Specific entities required in the Bylaws include information on the following:
A. Name of the organization is the American College of Veterinary Botanical Medicine (ACVBM). The ACVBM has been incorporated under the laws of the State of Delaware as a not-for-profit, tax-exempt organization for the purposes set forth herein, and in the Certificate of Incorporation. The corporation has no members.

B. The statement of objectives is included in the *Policies and Procedures* document.

C. Election procedures, titles, and duties are detailed in the Bylaws.

D. Membership categories, including duties, privileges and methods of selection for each category, are detailed in the Bylaws. A list of current officers of the ACVBM and their curriculum vitaes are attached (Appendix IV & V). Charter diplomates will be selected at a later date based on acknowledged expertise

in veterinary botanical medicine and as recognised educators and practitioners of botanical medicine and contributions to the organization of the specialty.

E. The details on eligibility for candidacy are found in the *Policies and Procedures* document.

F. A description of approved methods for an alternative pathway is found in the *Policies and Procedures* document.

G. Description of the scope and nature of certifying exams:

The veterinary botanical medicine written examination will consists of four (4) parts.

I. General botanical medicine
II. Principles and practices
III. Clinical botanical medicine
IV. Botanical identification of medicinal plants

Description of the scope and nature of certifying exams will be developed by the Credentials and Education Committee and Administered by the Examination Committee.

H. Procedures for establishing and modification of dues and fees are the responsibility of the Board of Directors and will be determined by a majority vote.

I. Loss of licensure and/or conviction of a felony would be cause for censuring or suspending diplomates or canceling certification. The Board of Directors is responsible for reviewing documentation and selecting the appropriate form of disciplinary action by a vote of two-thirds majority. The diplomate will be notified by mail within fourteen days of the decision of the Board.

J. The procedures for appealing adverse decisions are detailed in Article XI section E of the Bylaws.

K. Governance is described in Article VIII in the Bylaws.

Organization of ACVBM Specialty

The organizing committee has selected the following committee structure and membership to undertake its functions.

Executive Committee

1. Will be composed of Co-chairs and Members

2. Duties: The Executive Committee shall procure funding for the corporation, develop a time line according to ABVS submission guidelines, appoint a secretary/treasurer for the ACVBM, develop a meeting schedule for the Board. The Executive Committee may act for the Board of Directors between meetings of the Board of Directors, or as otherwise authorized by the Board of Directors. The Executive Committee shall not, however, have the power to: remove a Regent or Officer; fill vacancies in the Board of Directors or in any committee; determine and fix compensation for any individuals for serving on the Board of Directors or any committee; sell corporate assets; amend, repeal, or adopt Bylaws; or, amend or repeal any resolution of the Board of Directors.

Nominating/Membership Committee
1. Will be composed of Co-chairs and Members

2. Duties: The Nominating Committee shall oversee and supervise the nominating process for members of the Board of Directors, and shall establish appropriate procedures and rules for the selection and presentation of qualified candidates to active ACVBM diplomates for election. Among other duties, the Nominating Committee shall: establish qualifications for charter diplomate status, nominate charter diplomats, perform surveys to identify potential members of the ACVBM; raise awareness of the establishment of ACVBM among potential members; review and study the credentials of candidates; and develop a slate of qualified candidates. Under the direction of the Board of Directors, the Nominating Committee shall perform its duties and annually report its findings to the Board of Directors.

Credentials Committee

1. Will be composed of Co-chairs and Members

2. Duties: The Credentials Committee, shall be responsible for: establishing the candidate credential eligibility criteria in accordance with these By Laws; reviewing ACVBM certification applications; approving applicants who meet the certification eligibility criteria; identifying to the Board of Directors those applicants who are deemed eligible and ineligible for examination; establish criteria for residencies in veterinary botanical medicine and oversee the establishment and development of new residency programs in veterinary botanical medicine and inform candidates of committee decisions.

Examination Committee
1. Will be composed of Co-chairs and Members

2. Duties: The Examination Committee shall: define the examinations for diplomate status in both subject matter and distribution of questions and type of exam (is multiple choice, short answer, practical etc); will write the knowledge tests; administer, monitor and grade the Diplomate Certification Examination. It shall make recommendations to the Board regarding competence of those candidates who have completed the Diplomate Certification Examination.

3. The Examination Committee has determined that examination questions will be submitted by all members of the organizing committee in multiple-choice format and written under the guidelines of the National Board of Medical Examiners (NBME).

4. The examination will cover core knowledge of botanical medicine, botanical identification and principles and practices of botanical medicine, as well as a concentration in the area of clinical application across species (Appendix VI). Components of the examination will test Western or Chinese botanical medicine. The examinations will be paper or computer-based and will be administered at a site and on a date determined by the ACVBM Board of Directors. The examinations will be administered and monitored by the Curriculum/Education/Examination Committee. Test questions will be marked and evaluated by the Curriculum/Education/Examination Committee.

5. Training programs are available (Appendix VII). It is estimated that 250 veterinarians each year for the last ten years have undertaken botanical medicine training. The objectives of these programs are to prepare veterinarians to provide botanical medicine services. In addition many veterinary conferences provide basic to advanced training in botanical medicine. The success of these programs will be evident

by growth in the number registrants. At the present time, existing educational programs are well attended and attendance is increasing.

6. A list of current officers of the ACVBM is attached (Appendix IV). Charter diplomates will be selected at a later date based on contributions to the organization of the specialty.
7. At the present time funding is based on an initial donation, membership dues and conference dues. Future funding will be based on application fees, examination fees, conference fees and membership dues.

Appeals Committee
1. Will be composed of Co-chairs and Members
2. Duties: The Appeals Committee shall consider whether correct administrative procedures have been followed in the decisions made by the Credentials Committee and Examination Committee. The Chair of this committee will call a meeting to review an appeal and notify the Chair of the Board and the Executive Director of the results of that review within thirty (30) days of notification of the appeal. The decision of the Appeals Committee shall be final and there shall be no appeals thereafter.

Training Program Evaluation Committee
1. Will be composed of Co-chairs and Members

2. The Training Program Evaluation Committee shall be responsible for establishment of minimum criteria for Standard and Alternate Training Programs and review of Standard and Alternative Training Program applications and recommendations to the Board regarding acceptability of programs. The committee will define the knowledge base for the college and assist candidates in accessing this information; develop list of organizations and associations that have an interest in and/or provide educational opportunities in the areas of veterinary botanical medicine; develop and inform diplomats of continuing education opportunities relevant to veterinary botanical medicine; develop a list of publications and encourage new initiatives that will provide advanced training in veterinary botanical medicine.

American College of Veterinary Botanical Medicine Policies and Procedures

Establishment of an organizing committee

Eligibility criteria for members of the organizing committee were chosen as having acknowledged expertise in veterinary botanical medicine and as recognised educators and practitioners of botanical medicine. According to the bylaws of the AVMA, committee members had to fulfill at least one of the following criteria:

- Be a professor of this specialty in a college or department of veterinary medicine
- Be an author of important publications resulting from research or practice in this specialty
- Have at least 10 years experience in this specialty and, by teaching, research, or practice, have contributed substantially to the development of the specialty
- Have advanced training in the specialty and have demonstrated competency through teaching, research, or practice in the specialty to which more than fifty (50%) or more of the individual's professional time is devoted

Potential committee members were identified by nomination, which generated the names of 22 candidates. Candidates were discussed and contacted. The consensus was that 16 individuals would be

sufficient to provide a diverse range of expertise in small and large animal, Western and Chinese botanical medicine. It was also determined that the organizing committee should include both practicing and academic veterinarians and diplomates who practice botanical medicine from diverse geographical areas.

Upon establishment of the specialty, charter diplomate status will be conferred upon some, but not necessarily all of the members of the organizing committee. The determination will be based on contributions of individuals to the organizing process.

Members of the organizing committee

The following 16 veterinarians were nominated to the organizing committee (full details and curriculum vitae for each member are included in Appendix IV & V):

Specialty Organising Committee Members

Signe Beebe

Ihor Basko

Shauna Cantwell

Cynthia Lankenau

Rob Silver

Steve Marsden

Hubert Karreman

Constance DiNatale

Barbara Fougere

Susan Wynn

Joyce Harman

Huisheng Xie

Nancy Scanlan

Richard Palmquist

Donna Raditic

Carmen Colitz

Criteria for recognition of ACVBM

1. Improvement in veterinary services provided to the public

Veterinary Botanical Medicine is concerned with the diagnosis, treatment and health maintenance, or health improvement of animals using specialized knowledge of botanical medicines and principles. Botanical medicines include plant preparations (dried, powdered, tinctures, glycetracts, vinegars, wines, pills, salves, oils, teas, granules) and extracts such as essential oils, or constituents like silybum from *Silybum marianum* as well as combinations or formulas. These are applied to conditions and health maintenance across the production animal industry and companion animals for a number of reasons including:

- alternatives to growth promoters and antibiotics and anthelmintics in the face of resistance issues;
- growing consumer demand for organic produce;
- human health and environmental concerns about chemical and drug use and residues in livestock, in aquaculture and poultry and on companion animals;
- treatment options for animals that are refractory to conventional care, suffering drug related side effects or where conventional options have a low evidence base or safety concerns;
- the rising demand by companion animal owners and some organic producers for botanical medicines as a preferred method of treatment.
- the need for palliative treatments in the growing field of animal palliative care and hospice

Expertise in veterinary botanical medicine meets a growing consumer demand from animal owners and producers. It has a substantial evidence base compared to some other integrative and complementary therapies as well as some veterinary specialties admitted to the ABVS. The benefits of botanical medicines for improving animal health and providing evidence of these reasons are emphasised under the following section.

Companion Animals

Trends identified in the ACVBM White Paper 2016[18], from data supplied by the National Animal Supplement Council (NASC) show an enormous growth in the popularity of herbs used on the treatment of companion animals:

- **Canine Data**: Total administrations of the top 25 herbal ingredients for dogs was 42,087,369 in 1999, the first year of tabulating this data. The number of administrations grew over the 17 years recorded to 244,797,878 administrations, estimated for the year 2015. This represents 500% growth.
- **Equine Data**: Total administrations of the top 25 herbal ingredients for horses was 8,385,566 for 1999, the first year of recording this data. This value increased in 17 years of data recording to 42,476,440 administrations estimated by the end of 2015. This is also a 500%

[18] Silber R, Bookout B, Karreman H Documentation of Trends in the Public Acceptance of Botanical Therapies: Consumer Buying Patterns, Patterns of Use and Industry Correlates 2016 January Organizing Committee Report- SeeAppendix X

growth in number of administrations.

- **Feline Data**: Total administrations of the top 25 herbal ingredients for 1999 for cats was 5,638,172 and was expected to increase to 81,495,270 by the end of 2015. Although cats are experiencing a down turn in number of visits to their veterinarians, the number of administrations of the top 25 herbal ingredients increased 14.5 times during this 17 year period being monitored.
- **Combined Data**: Total administrations of the top 25 herbal ingredients for Dogs, Cats and Horses combined for 1999 was 56,111,107. This value increased over 17 years to an estimated 368,769,588 by the end of 2015. This is a 650% increase in the administrations of these top 25 herbal ingredients, which implies a similar increase in use of these herbal supplements in all species measured.

This 2016 white paper suggests that with the growth of interest in herbal medicine among veterinarians and consumers, most animal supplement companies are in need of scientifically-derived information regarding the safety, herb-drug interactions, and clinical applications regarding herbal therapies in veterinary species, which could be facilitated by the establishment of the ACVBM.

Bovine Practice and Organic Production

The growth in interest of bovine (AABP) practitioners toward complementary and alternative medicine was investigated between 2006 and 2010 using surveys[19]. Approximately 80% of the veterinarians were interested in evidence-based alternative therapies, and in particular for the treatment of mastitis. From 2006 to 2010 interest increased significantly ($p<0.01$) in alternative treatment approaches for calf diarrhea, metritis, infertility, pneumonia and digital dermatitis/foot rot. In general veterinarians with organic clients were more interested in these alternative non-drug therapies than those veterinarians without organic clients. They were particularly interested in treatments with a rational pharmacological basis.

The majority of evidence-based complementary and alternative therapies are based on botanical remedies. This survey of the growth of bovine veterinarians' interest in CAVM between 2006 and 2010 supports the need for the establishment of a College of Veterinary Botanical Medicine as a resource for these veterinarians to better establish the science that underlies these therapies. For industry, the graduation of Board-certified veterinarians with scientific and clinical expertise in botanical medicine and phytopharmacology will be invaluable as companies develop and bring evidence-based botanical products to the marketplace to address the needs of these bovine practitioners and their clients.

Letters (Appendix III) from the Midwest Organic Dairy Producers Association and the Northeast Organic Dairy Producers Alliance (representing 836 organic dairy producers in the Eastern US) express their enthusiastic support for the creation of the ACVBM. They also express that many of their producers are not served by veterinarians with interest or skills in botanical/ holistic medicine. They state that the ACVBM could provide information and experience to support regular veterinarians.

Trends in Consumer Use of Herbal Therapies for Pets

The American Pet Products Association National Pet Owners Survey 2015- 2016

The APPA survey was mined for data regarding the trends in the use of herbal supplements by pet owners of dogs, cats and horses.

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[19] Sorge US, Bastan A, Karreman H Interest of Bovine Practitioner in Complementary and Alternative Veterinary medicine in 2006 and 2010) College of Veterinary Medicine, Dept. Veterinary Population Medicine, University of Minnesota, Saint Paul, MN 55108 Advance Research in Agriculture and Veterinary Science vol2(1&2)2015

- **The percentage of dogs and cats given medications** of any kind increased to 77% over the past 12 months. 10 years ago this was 52%, indicating a trend toward better acceptance of administration of medications, and better palatability strategies. More than 90% of horses have been administered medication or supplements this past year.
- **The percentage of pets receiving dietary supplements** excluding vitamins was 12% for dogs (9.3 million dogs), 6% for cats (5.15 million cats), and 5% for horses (375,000 horses).

Consumers source their dietary supplements from the following outlets: *(Multiple response question, therefore total may exceed 100%)*

1. **Dogs**: from Veterinarian (28%), Internet (22%), Pet chain superstore (16%), Pet store independent (13%), Discount/Mass marketing (16%) Hardware store (6%), Other (6%)
2. **Cats**: from Veterinarian (17%); Pet chain super store (25%); Internet (17%); Grocery store (17%); Discount Mass marketing (25%)
3. **Horses**: from Internet (33%); Veterinarian (25%); Feed store (17%) Tack shop (8%) Other (17%)

Consumers source their information about dietary supplements from the following outlets: (*Multiple response question, therefore total may exceed 100%)*

1. **Dogs**: from Veterinarian (65%), Internet (44%), Friends & Relatives (28%); past experience (32%); Pet store personnel (16%), Television (12%); Groomer (15%)
2. **Cats**: from Veterinarian (47%); Internet (42%); Friends & Relatives (33%); past experience (35%); Pet store personnel (13%); Television (10%); Other (8%)
3. **Horses**: from Veterinarian (73%); Internet (48%); past experience (60%); Breed club and societies (17%); Feed store personnel (25%), Farrier/Trainer (50%) Other

Analysis of Survey Data

- Veterinarians are the major source of supply to pet owners for herbal supplements
- Veterinarians are the major source of information about using herbal supplements to pet owners
- The market share that herbal supplements have, in comparison to more commonly used products like food, bedding, tack, collars is relatively small (average 11% for dogs, cats and horses).

The data from the above white paper concludes that the market share for herbal remedies and other alternative therapies has been growing sufficiently over the years since 1990 that this survey has been conducted, such that the APPA has now created a specific category in this survey to measure the trends in this growing segment.

Since 1990 there has been a steady growth in consumer demand for dietary supplements that contain herbal ingredients. Most consumers consult with their veterinarian regarding supplements, and most consumers purchase their dietary supplements from their veterinarian.

Thus, veterinarians are in a unique position of providing evidence based information to their clients about products they believe, based on the best information available, will augment their existing clinical protocols.

It is known that herbs can interact adversely with pharmaceutical therapies, and that not all herbs are

safe or effective. Currently we lack an adequate body of evidence-based information, or Board certified veterinarians, to guide the use of herbal therapies concurrent with conventional therapies. In some cases, herbal therapies can serve as complete substitutes, where appropriate, for conventional therapies. Without these safeguards and without a College of Veterinary Botanical Medicine to accredit board-certified specialists in herbal therapies, the consumer is left without adequate protections that would provide safe and effective options for the use of the botanical therapies that they are requesting and are currently using anyway.

The consumer, the marketplace and the Veterinary profession are ready for the establishment of the ACVBM for all of the reasons stated above. A specialty board would gather the diverse sources of information into a cohesive package; establish post-graduate training, fellowship and research opportunities; and determine criteria for, and administration of the process for certification. The overall goal of the specialty is to encourage and facilitate advanced training of veterinarians in botanical medicine to support the veterinary profession, industry (including aquaculture, dairy, swine, poultry, companion animals, equine and other production species) consumers and industry bodies such as NASC.

2. Necessary number of potential diplomates

The uniqueness of this new specialty is that it is using treatments outside of the established pharmacological research model, yet still having a strong rational basis in pharmacology. The field is rapidly growing and interest among veterinarians and students is high. For example, current membership of the Veterinary Botanical Medicine Association has doubled in less than 5 years to 260 members. More than 3000 veterinarians are trained in Chinese or Western Botanical Medicine by IVAS, Chi or VBMA. The fact that the Delphi process identified 44 veterinarians who fulfilled the AVMA criteria for membership of the organizing committee is evidence that a pool of qualified potential diplomates already exists.

3. The specialty of veterinary botanical medicine and its base of scientific knowledge and practice

There is a very large base of scientific knowledge to support this specialty including proceedings of several RVSO meetings and symposiums, textbooks, journals and journal articles.
A further indication of the growth of the discipline has been the establishment of :

- Grants have supported research in botanical medicine (for example by http://www.ahvmf.org have supported Dr Ronald Koh BVM MS Certified Veterinary Chinese Herbalist Assistant Professor LSU School of Veterinary Science currently involved in a research project: The efficacy of acupuncture and Chinese herbal medicine on survival and quality of life in dogs with multicentric lymphoma receiving CHOP chemotherapy: a randomized controlled trial. - See more at: http://www.lsu.edu/vetmed/vcs/people/faculty/faculty/koh.php#sthash.hnPgAPMT.dpuf).
- Scholarships (such as through the University of Tennessee scholarship in Integrative Medicine underway 7/15/2014-12/14/16 which involves substantial herbal medicine training- the student is completing a Graduate Diploma Chinse Veterinary Herbal Medicine) and
- Academic positions that support botanical medicine services, research or teaching in several universities including:
    - Dr Keum Hwa Choi DVM, MS, PhD, CVA, MSOMD, LAc, Diplomate NCCAOM Associate professor Department of Veterinary Clinical Studies University of Minnesota one of her current research areas is herbal medicine for managing cancer cases (http://www.vetmed.umn.edu/bio/veterinary-clinical-sciences/keum%20hwa-choi);

- Dr Mushtaq Memon BVSc, MSc, PhD Diplomate, American College of Theriogenologists Trained in Chinese Veterinary Herbal Medicine (http://vcs.vetmed.wsu.edu/people/faculty/h-o/memon).
- Dr Justin Shmalberg BA DVM Diplomate, American College of Veterinary Nutrition and American College of Veterinary Sports Medicine and Rehabilitation Clinical Associate Professor and Service Chief Integrative Medicine Department of Small Animal Clinical Sciences College of Veterinary Medicine University of Florida (http://www.vetmed.ufl.edu/about-the-college/faculty-directory/justin-shmalberg/)
- Dr Christine Egger DVM MVSc Diplomate of the American College of Veterinary Anesthesia Trained in Veterinary Herbal Medicine Professor College of Veterinary Medicine The University of Tennessee and coauthor of Pain Management in Veterinary Practice which has a section on herbal medicine (http://www.vet.utk.edu/faculty/egger.php).
- Dr Dwight Bowman Professor of Parasitology Cornell University College of Veterinary medicine, who published Kato S, Bowman DD, Brown DL. Efficacy of Chenopodium ambrosioides as an antihelmintic for treatment of gastrointestinal nematodes in lambs. J. Herbs, Spices, Med. Plants 7:11-25. 2000
- Dr Dorothy Brown American College of Veterinary Surgeons, Diplomate Professor of Surgery, Department of Clinical Studies University of Pennsylvania School of Veterinary Medicine who investigated Coriolus versicolor in dogs Brown Dorothy Cimino, Reetz Jennifer Single agent polysaccharopeptide delays metastases and improves survival in naturally occurring hemangiosarcoma. Evidence-based complementary and alternative medicine : eCAM 2012: 384301, 2012.
- Dr Reed Holyoak DVM, PhD, DACT Professor of Theriogenology, OK State Univ Department head. Incorproates herbal approaches in the treatment of reproductive issues in horses. Published on the use of Chinese herbs in paraparesis of dogs J Trad Comp med Dec 2015.
- Dr Manuel Roberto Cortinas Assistant Professor Practice University of Nebraska Lincoln School of Veterinary Medicine has a special interest in ethno veterinary and botanical methods of parasite control and organic livestock approaches as a tool to address anthelmintic and insecticide resistance.

Current Research

Among the colleges of veterinary medicine in the U.S., Canada and Mexico, there are 3 projects recruiting or recently completed:

University of Florida: Acupuncture and Herbal Medicine for treatment of side effects during CHOP therapy. http://research.vetmed.ufl.edu/clinical-trials/small-animal/acupuncture-and-herbal-medicine-for-treatment-of-side-effects-during-chop-therapy/

University of Pennsylvania: Novel Therapeutic for Dogs with Cataracts. http://www.vet.upenn.edu/research/clinical-trials/penn-vet-clinical-trials/clinical-trial/novel-therapeutic-for-dogs-with-cataracts

Washington State University: Clinical Effectiveness of Phycox in Elbow Osteoarthritis in Dogs. http://vcs.vetmed.wsu.edu/research/clinical-studies/elbow-arthritis

Presentations involving herbal medicine and plant extracts at RVSOs and Symposiums 2013-2015

ACVIM

Effect of a Supplement (SmartGut® Ultra) on Gastric Ulcer Scores and Gastric Juice pH. F. Andrews; P. Lofton; G. Gammon; P. Camacho; J. Cartmill; F. Garza Jr; M. Keowen; M. Kearney Proceedings of the ACVIM Forum, 2013, Seattle WA.

In Vitro Effects of Yunnan Baiyao (Yb) on Coagulation. K. Loyd; L.A. Cohn; S.A. Smith. Proceedings of the 2014 ACVIM Forum, Nashville TN.

ACVO

Kim SH, Lee ER, Park SW, Park S, Noh H, Seo K. Effect of Cepae extract on corneal haze after applying the modified big bubble technique in dogs. Proceedings of the ACVO, 2014, Fort Worth, TX.

Chen Y, Lin CT. The retinal protective and antioxidative effects by nutritional antioxidant supplelents in high intraocular pressure induced retinal ischemia in rats. Proceedings of the ACVO, 2013, Puerto Rico.

Kado F, Guou C, Kawada H, Blessinng K. Nutraceutical Optixcare EH ameliorates oxidative stress in rats. Proceedings of the ACVO, 2013, Puerto Rico

Miller EJ, Gemensky-Metzler AJ, Wilkie DA, Colitz CMH. Effects of grape seed extract, lutein, and omega-3 fatty acids on lens epithelial cell behavior in vitro and ex vivo. Proceedings of the ACVO, 2013, Puerto Rico

VCS

Pondugula S, Ferniany G, Ashraf F, Abott K, Flannery P, Smith B, Mansour M, Colemana E, Bird R, Smith Annette. A plant-based dietary fatty acid inhibits the growth of canine and human B-cell lymphoma cells by downregulating the activity of multidrug transporters. Proceedings of the Veterinary Cancer Society, Oct 17-19, 2013, Minneapolis, MN.

Levine C. Effects and synergy of feed ingredients on canine neoplastic cell growth. Proceedings of the Veterinary Cancer Society, 2015, Tysons VA.

ACVB

Pike AL, Horwitz DF. An open label prospective study of the use of L-theanine (anxitane) in storm sensitive client owned dogs. Proceedings of the Veterinary Behavior Symposim, July 25, 2014, Denver, CO.

WINSS

Combalin E, Sanchez C, Lambert C, Serister S, Henrotin YE. Synergistic beneficial effects of curcuma extract, green tea extract and hydrolysed collagen in bovine chondrocytes in monolayer culture. The Waltham International Nutritional Sciences Symposium, October 1-4, 2013, Portland OR.

Clero D, Feugier A, Grandjean D. Interest of a pre-exercise nutritional supplementation on working dogs serum inflammation and oxidative stress markers evolution during a standardized mid-intensity exercise. The Waltham International Nutritional Sciences Symposium, October 1-4, 2013, Portland OR.

Martineau AS, Leray V, Talbot C, Breniaux M, Ouguerramm K, Nguyen P. Effect of simultaneous omega 3 PUFA and curcumin supplementation on insulin sensitivity and plasma lipids in obese dogs. The Waltham International Nutritional Sciences Symposium, October 1-4, 2013, Portland OR.

Veterinary Text Book Titles from the last 10 years in Veterinary Herbal Medicine include:

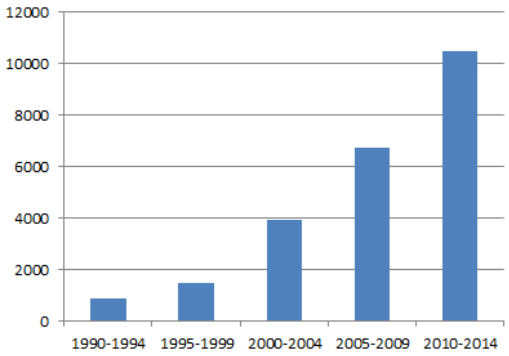
- 2016 Practical Guide to Traditional Veterinary Chinese Medicine Equine Practice Xie H Chi Institute of Chinese Medicine
- 2014 Practical Guide to Traditional Chinese Veterinary Medicine Dr H Xie Redwing Books
- 2014 Essential Guide to Chinese Herbal Formulas Dr Steve Marsden CIVT
- 2012 Chinese Herbal Formulas for Veterinarians Drs Beebe & Salewski, Chen & Chen Redwing Books
- 2012 Chinese Veterinary Herbal Handbook Xie H, Frank L, Preast V Chi Institute of Chinese Medicine
- 2010 Xie's Chinese Veterinary Herbology Wiley-Blackwell
- 2010 Ethnoveterinary Botanical Medicine: Herbal medicines for Animal Health CRC Press
- 2007 Integrating Complementary Medicine into Veterinary practice Goldstein R Ed
- 2006 Veterinary Herbal Medicine Dr Susan Wynn, Dr B Fougere Elsevier
- 2006 Clinical Handbook of Chinese Veterinary Herbal Medicine Beebe, S., Salewski, M Herbal Medicine Press, 2006
- 2005 Psychoactive Herbs in Veterinary Behaviour Medicine Schwartz S Wiley

Samples of Text Book Chapter Titles from the last 5 years in Veterinary Herbal Medicine include:

- 2016 Chapter in Principles and Practices of Veterinary Technology Sirois M Chapter 19 pp 494:507
- 2014 Chapter in Veterinary Clinics of North America: Small Animal Practice Lisa Pesch Holistic Pediatric Veterinary Medicine Pages 355-366
- 2014 Chapter in Veterinary Clinics of North America: Small Animal Practice Advances in Veterinary Oncology Evidence Based Integrative Medicine in Clinical Veterinary Oncology pp831-855
- 2014 Chapter in BSAVA manual of canine and feline neurology Treatment of neurological disorders with traditional Chinese veterinary medicine. Chrisman, C. L. Pages: 496-507
- 2014 Chapter in Pain management in veterinary practice. Egger, C. M. ; Love, L. ; Doherty, T. Chapter 15 Traditional Chinese Herbal Medicine and Homeopathy in Pain Management practice Pages: xiii + 447 pp. Wiley Blackwell
- 2014 Chapter in Anthelmintics: Clinical Pharmacology, Uses in Veterinary Medicine and Efficacy Quick W. pp,89-108 ,pp,155-176
- 2013 Chapter in Veterinary Clinics of North America: Small Animal Practice Jeanne B. Budgin, Molly J. Flaherty Alternative Therapies in Veterinary Dermatology Pages 189-204
- 2013 Chapter in Behaviour Problems of the Dog Landsberg G, Hunthausen W, Ackerman L Chapter 9 Complementary and Alternative Therapies for behaviour problems. Saunders Elsevier
- 2011 Chapter in Complementary Medicine for Veterinary Technicians and Nurses Scanlan N Chapter 10 Wiley Blackwell

Journal Publications.
Further, over the last 15 years there has been an increasing publication of research on plant extracts in animals, with more than 10000 studies published from 2010 to 2014 alone.

Table 1. Publications in pub med, plant extracts and animals for each 5 year period since 1990.



Publications since 1990 include over 2000 Systematic Reviews and Meta-analyses using animal models. Recent examples in 2016 include the use animal models and herbal and plant products in:

- Inflammatory bowel disease[20]
- Asthmatic inflammation[21]
- Ischemic stroke[22]
- Neurodegenerative diseases[23]

The scientific evidence and publications regarding veterinary herbal medicine is growing. The scientific basis of *in vivo* studies that elucidate mechanisms of action of botanical medicines and their constituents coupled with *in vitro* studies provides a solid scientific basis and supports the claim that this speciality is distinct and identifiable. Included in this petition is a list of 12 selected whole papers (Appendix VIII) demonstrating a broad range of research in the use of botanical plants and extracts in veterinary medicine

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[20] Triantafillidis JK, Triantafyllidi A, Vagianos C, Papalois A. Favorable results from the use of herbal and plant products in inflammatory bowel disease: evidence from experimental animal studies. Ann Gastroenterol. 2016 Jul-Sep;29(3):268-81.
[21] Liu F[1], Xuan NX[1], Ying SM[1], Li W[1], Chen ZH[1], Shen HH[2]. Herbal Medicines for Asthmatic Inflammation: From Basic Researches to Clinical Applications. Mediators Inflamm. 2016;2016:6943135.
[22] Seto SW, Chang D, Jenkins A, Bensoussan A, Kiat H. Angiogenesis in Ischemic Stroke and Angiogenic Effects of Chinese Herbal Medicine. J Clin Med. 2016 Jun 6;5(6)
[23] Solanki I, Parihar P, Parihar MS.Neurodegenerative diseases: From available treatments to prospective herbal therapy. Neurochem Int. 2016 May;95:100-8.

and published abstracts from USA, European and Australian journals from the last ten years (Appendix IX). These pertain directly to *in vivo* studies and a few selected *in vitro* studies in veterinary medicine (such as a recent one on yunnan baiyao in canine hemangiosarcoma cells[24]) and we have excluded the many thousands of laboratory animal studies forming a strong scientific case for pocket pets. This sample of published materials demonstrates the broad extent and application of science related to veterinary herbal medicine. Journals publishing articles are outlined for each veterinary medicine area following:

Veterinary botanical medicine and Aquaculture

Journals publishing peer reviewed studies on plant medicine for fish disease and health

- Aquaculture
- J Applied Ichthyology
- Veterinary Parasitology
- Fish and Shellfish Immunology
- Fish Physiology and Biochemistry
- Journal of Fish Diseases
- Veterinary Pharmacology and Therapeutics
- North American Journal of Aquaculture

A recent (2015) review in Veterinary Pharmacology and Therapeutics reports promising effects of many Western and Chinese herbal medicines for treating parasitic diseases caused by protozoa and metazoan, and broad activity against bacteria and fungi. The review lists the main findings and methodologies of the latest research on herbal medicines to stimulate and accelerate research recognizing the current issues regarding indiscriminate use of chemicals and antibiotics in aquaculture[25]. For example Valladao et al. (2015) were successful in treating severe cases of ichthyophthiriasis in *Piaractus mesopotamicus* fish using two-hour daily baths of the essential oil of *M. alternifolia* for five days, which shows that this has great potential for use in aquaculture[26]. Likewise a review of the use of immunostimulant herbs in aquaculture as an alternative to drugs, chemicals, growth promoters and antibiotics concludes they can be used as alternatives to these agents primarily because they are easily obtained, inexpensive, act against a broad range of pathogens and are biodegradable[27].

Appendix IX-A outlines the abstracts of 14 recent journal articles demonstrating the importance of botanical medicines in aquaculture and supported by a further 35 journal titles published since 2010 from a possible 350 articles published in the last 5 years on plant extracts and fish.

Veterinary botanical medicine and Small Animals

Journals publishing peer reviewed studies on plant medicine for canine and feline medicine

- American Journal Veterinary Research

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[24] Wirth KA, Kow K, Salute ME, Bacon NJ, Milner RJ. In vitro effects of Yunnan Baiyao on canine hemangiosarcoma cell lines. Vet Comp Oncol. 2014 Jun 29.

[25] Valladao G, Gallani S, Pilarski F Phytotherapy as an alternative for treating fish disease J Vet Pharmacol Therap 38 417-428 2015.

[26] Valladão GM, Gallani SU, Ikefuti CV, da Cruz C, Levy-Pereira N, Rodrigues MV, Pilarski F. Essential oils to control ichthyophthiriasis in pacu, Piaractus mesopotamicus (Holmberg): special emphasis on treatment with Melaleuca alternifolia. J Fish Dis. 2016 Jan 18.

[27] Galina J, Yin G, Ardo L, Jeney Z The use of immunostimulating herbs in fish. An overview of research Fish Physiol Biochem 2009 35:669-676

- Veterinary Dermatology
- The Veterinary Journal
- Journal of Feline Medicine and Surgery
- Applied Animal behaviour Science
- Journal of Veterinary internal Medicine
- Journal Applied Animal Research
- Veterinary Comparative Oncology
- Journal Veterinary Pharmacology and Therapeutics
- Veterinary Clinics of North America Small Animal

Human use of botanical medicines often informs research for veterinary use. Chou etal (2016) reported on the efficacy of cranberry extract for urinary tract conditions in dogs. An *in vivo* and *in vitro* component of the study on 12 dogs with a history of recurrent UTI demonstrated that oral administration of cranberry extract in 6 dogs prevented development of UTI in patients and adherence of E Coli to kidney cells and compared equally to antibiotics over 6 months[28]. The potential for cranberry in the management of recurrent UTIs is promising in the era of antibiotic resistance.

Some of the challenging clinical cases such as atopic skin disease may benefit from traditional botanical medicine. Marsella etal (2010) reported in Veterinary Dermatology on a multicentre trial undertaken with the University of Florida, the Animal Dermatology Clinic (San Diego, CA), Colorado State University and the Veterinary Referral Center of Colorado. It involved the use of a powdered formulation of the fruit *Actinidia argute* (hardy kiwi). This fruit has been traditionally used for health in traditional Asian medicine. In this randomized, placebo controlled trial involving 76 dogs, the preparation was found to be a promising treatment for mild to moderate canine atopic dermatitis when used for 8 weeks. While the molecular mechanism underlying the therapeutic effects remains to be elucidated it was well tolerated and no clinical adverse effects were reported[29].

Similarily another proprietary formula consisting of three herbs used in both Chinese and Western botanical medicine, *Rehmannia glutinosa*, *Paeonia lactiflora* and *Glycyrrhiza uralensiswas* investigated by Ferguson etal (2006) and reported in Veterinary Dermatology for treatment of atopic dermatitis in 120 dogs in a randomized, double-blind, placebo controlled, parallel group design, large-scale study. The efficacy of the combined formula was demonstrated to be at least as good as, and in many cases superior to, that reported for other systemic steroid-sparing agents that are administered to dogs with AD, such as antihistamines, pentoxifylline, arofylline, leukotriene inhibitors and misoprostol. This study was a follow up to a previous study on the same combination that demonstrated reduced erythema and pruritus in canine AD with mild diarrhoea and flatulence observed in a small minority of dogs supporting the safety of the formula[30] .

Further in 2010, Schmidt etal reported in Veterinary Dermatology a double-blind randomized placebo-controlled trial including 22 dogs that demonstrated that the same formula containing *Rehmannia*

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[28] Chou HI, Chen KS, Wang HC, Lee WM. Effects of cranberry extract on prevention of urinary tract infection in dogs and on adhesion of Escherichia coli to Madin-Darby canine kidney cells. Am J Vet Res. 2016 Apr;77(4):421-7.
[29] Marsella R, Messinger L, Zabel S, Rosychuck R, Griffin C, Cronin PO, Belofsky G, Lindemann J, Stull D. A randomized, double-blind, placebo-controlled study to evaluate the effect of EFF1001, an Actinidia arguta (hardy kiwi) preparation, on CADESI score and pruritus in dogs with mild to moderate atopic dermatitis. Vet Dermatol. 2010 Feb;21(1):50-7.
[30] Nagle TM, Torres SM, Horne KL et al. A randomized, double-blind, placebo-controlled trial to investigate the efficacy and safety of a Chinese herbal product (P07P) for the treatment of canine atopic dermatitis. Veterinary Dermatology 2001; 12: 265–74

glutinosa, *Paeonia lactiflora* and *Glycyrrhiza uralensiswas* can be an effective glucocorticoid sparing agent in canine atopic dermatitis[31].

More recently in The Veterinary Journal Blaskovic etal (2014) reported on 48 dogs diagnosed with atopic dermatitis that were included in a randomized, double-blinded, placebo-controlled, multicentre clinical trial. Dogs were treated with a topical formulation containing PUFAs and essential oils including neem oil, rosemary extract, lavender oil, clove oil, tea tree oil, oregano extract, peppermint extract and cedar bark extract or placebo once weekly for 8 weeks. There was significantly more improvement in pruritus scores in the treatment group than in the placebo. No adverse reactions were observed. The study concluded it was safe and beneficial in ameliorating the clinical signs of atopic dermatitis[32].

In recognition of some of the limitations of antibiotics and antifungals in small animal conditions, essential oils from plants have also been investigated for their potential use in veterinary medicine, including a randomised controlled trial (Kim etal 2009) in 11 dogs with otitis externa, where essential oils were compared favourably to antibiotics[33]; Nardoni etal (2016) reported in the Journal of Feline Medicine and Surgery a comparative open field study involving 14 cats with *Microsporum canis.* The study compared an essential oil based shampoo containing *Thymus serpyllum* (2%), *Origanum vulgare* and *Rosmarinus officinalis* (5% each) and oral itraconazole was as effective as a conventional treatment (oral itraconazole + 2% miconazole/2% chlorhexidine shampoo) concluding that the natural treatment would seem an interesting, natural alternative to conventional topical treatment[34].

Likewise essential oils of Citrus aurantium 1%, Lavandula officinalis 1%, Origanum vulgare 0.5%, Origanum majorana 0.5%, Mentha piperita 0.5% and Helichrysum italicum var. italicum 0.5%, in sweet almond oil and coconut oil in a commercial product Malacalm was investigated by Nardoni etal (2014) for the treatment of *Malassezia pachydermatis*. The treatment was compared to placebo and to conventional therapy (based on ketoconazole 10 mg/kg/day and chlorhexidine 2% twice a week for 3 weeks) in a randomised controlled trial. At the end of the treatment trial, treatment groups both improved significantly without adverse events. In follow up on day 180 recurrence of clinical signs was observed in all the subjects treated conventionally, but none of those dogs treated with the essential oil formula had recurrence. The study concluded that the formula appeared to be a safe tool for limiting recurrences of this condition[35].

Essential oils have also been investigated for their effects on small animal behaviour. In the Journal of the American Veterinary Medical Association, Wells (2006) reported on the use of lavender essential oil to reduce travel induced excitement in dogs associated with travel. 32 dogs were exposed to car travel without lavender oil and compared to travel with lavender oil and their behaviour quantified. Dogs spent significantly more time resting and sitting and less time moving and vocalizing during the experimental

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[31] Schmidt V[1], McEwan N, Volk A, Helps J, Morrell K, Nuttall T. The glucocorticoid sparing efficacy of Phytopica in the management of canine atopic dermatitis. Vet Dermatol. 2010 Feb;21(1):96-105.

[32] M. Blaskovic , W. Rosenkrantz , A. Neuber , C. Sauter-Louis , R.S. Mueller , The effect of a spot-on formulation containing polyunsaturated fatty acids and essential oils on dogs with atopic dermatitis The Veterinary Journal 199 (2014) 39–43

[33] Sang-Hun Kim1, Suk Kim2, Hyung-Kyou Jun1, Duck-Hwan Kim1,* Efficacy of aromatherapy for the treatment of otitis externa in dogs Korean J Vet Res(2009) 49(1) : 85~89

[34] Nardoni S, Costanzo AG, Mugnaini L, Pisseri F, Rocchigiani G, Papini R, Mancianti F. An open-field study comparing an essential oil-based shampoo with miconazole/chlorhexidine for hair coat disinfection in cats with spontaneous microsporiasis. J Feline Med Surg. 2016 Jan 18.

[35] S. Nardoni , L. Mugnaini , L. Pistelli , M. Leonardi , V. Sanna , S. Perruccia, F. Pisseri , F. Mancianti Clinical and mycological evaluation of an herbal antifungal formulation in canine Malassezia dermatitis Journal de Mycologie Médicale (2014) 24, 234—240

condition[36]. Graham etal (2005) reported in Applied Animal Behaviour Science[37] on the influence of essential oil olfactory stimulation (control, lavender, chamomile, rosemary and peppermint) on the behaviour of 55 dogs housed in a rescue shelter. It is suggested that the welfare of sheltered dogs may be enhanced through exposure to appropriate forms of olfactory stimulation. Lavender and chamomile were particularly beneficial, resulting in activities suggestive of relaxation and behaviours that are considered desirable by potential adopters. More recently Ellis and Wells (2010) identified catnip rather than lavender as being a herb that may hold potential as environmental enrichment for captive domestic cats[38].

Finding alternatives to chemical parasiticides has led to the development of a number of commerical products using botanicals. A study reported in Parasitology Research 2008 (Abdel-Ghaffar etal) discussed the concerns about indiscriminate use of parasiticides that may pollute the environment, but also adversely affects the animal and human health. In this uncontrolled study 10 dogs infested with S. scabiei mites were treated with a neem seed shampoo derived from the neem tree (*Azadirachta indica*) which was shown to be effective against sarcoptic mange in dogs. 80% of dogs had hair regrowth and no clinical signs at 2 weeks. The remaining 20% showed only moderate mite counts and improved clinical signs. Furthermore, the topical treatment with neem seed extract shampoo was well tolerated and safe[39].

More recently an interest in herbal medicines for integrative cancer care has led to studies including Yunnan Bai Yao (Chinese medicine) as a potential adjunctive therapy for canine hemangiosarcoma through the University of Florida Veterinary School[40] published in Veterinary Comparative Oncology and in 2011 at the University of Pennsylvania Veterinary School with coriolus (a fungal botanical medicine) shown to improve quality of life and outcomes in dogs with hemangiosarcoma [41].

The growing publication of studies in veterinary and allied journals attests to the recognition that botanical medicines offer the potential to improve treatment of many common conditions that affect cats and dogs. There are over 400 journal articles published on plant extracts and canine and feline research. Studies in animals have led to improvements in the treatment of human diseases as well. It should also be acknowledged that dogs are often used as models for studying the effects of botanical medicines of humans. For example *H hirsute* has been demonstrated to have cholesterol lowering effects in dogs (van Dooren etal 2015)[42]. *Calendula officinalis* has been used to treat acetic acid induced ulcerative colitis in German Shepherds as a model for the human disease. 10 dogs were randomly allocated to receive a placebo of saline or calendula via enema, and calendula was effective in resolving the ulceration[43].

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[36] Wells DL. Aromatherapy for travel-induced excitement in dogs. J Am Vet Med Assoc. 2006 Sep 15;229(6):964-7.
[37] Graham L, Wells D, Hepper P The influence of olfactory stimulation on the behaviour of dogs housed in a rescue shelter Applied Animal Behaviour Science 91 (2005) 143–153

[38] Ellis S, Wells D The influence of olfactory stimulation on the behaviour of cats housed in a rescue shelter Applied Animal Behaviour Science 123 (2010) 56–62
[39] Abdel-Ghaffar F, Al-Quraishy S, Sobhy H, Semmler M. Neem seed extract shampoo, Wash Away Louse, an effective plant agent against Sarcoptes scabiei mites infesting dogs in Egypt. Parasitol Res. 2008 Dec;104(1):145-8
[40] Wirth KA, Kow K, Salute ME, Bacon NJ, Milner RJ. In vitro effects of Yunnan Baiyao on canine hemangiosarcoma cell lines. Vet Comp Oncol. 2014 Jun 29.
[41] Brown DC, Reetz J. Single agent polysaccharopeptide delays metastases and improves survival in naturally occurring hemangiosarcoma. Evid Based Complement Alternat Med. Volume 2012, Article ID 384301, 8 pages
[42] van Dooren I, Faouzi Mel A, Foubert K, Theunis M, Pieters L, Cherrah Y, Apers S. Cholesterol lowering effect in the gall bladder of dogs by a standardized infusion of Herniaria hirsuta L. J Ethnopharmacol. 2015 Jul 1;169:69-75.
[43]Mehrabani D, Ziaei M, Hosseini SV, Ghahramani L, Bananzadeh AM, Ashraf MJ, Amini A, Amini M, Tanideh N The effect of calendula officinalis in therapy of acetic Acid induced ulcerative colitis in dog as an animal model. Iran Red Crescent Med J. 2011 Dec;13(12):884-90.

Appendix IX-B outlines the abstracts of 25 recent journal articles demonstrating the broad range of studies of botanical medicines in canine and feline medicine and supported by a further 30 journal titles published in the last 10 years on plant extracts and canine or feline.

Veterinary Botanical Medicine and Cattle including Dairy
Journals publishing peer reviewed studies on plant medicine for cattle medicine

- BMS Veterinary Research
- Research in Veterinary Science
- Journal Dairy Science
- American Journal of Animal and Veterinary Science
- Canadian Veterinary Journal
- Veterinary immunology and Immunopathology
- Animal Feed Science and Technology
- Animal Reproduction Science
- Veterinary Parasitology
- Journal Veterinary Internal Medicine
- Theriogenology
- Livestock Science
- Journal of Parasitic Diseases
- International Journal of Environmental Research and Public Health

Non antibiotic treatments are needed in organic dairy herds [44] which has prompted a plethora of studies on botanical medicines and plant compounds such as essential oils[45] and proprietary botanical formulas[46] for the treatment of mastitis. More broadly though, the use of antibiotics is being increasingly discouraged in the dairy industry because their presence in dairy milk may have potential downstream effects on population health and the agri-food chain[47].

Analysis of the published literature including the Journal of Dairy Science and a review in Natural Products Communication Journal(Mullen etal 2014; Taga etal 2012) shows that many botanical medicines including essential oils have significant antibacterial, antifungal and anti-inflammatory effects. A recent study through the Texan A & M & Florida Schools of Veterinary medicine and published in the Canadian Veterinary Journal evaluated a combination botanical product containing Western herbs for intramammary treatment of mastitis and demonstrated a faster recovery for the treatment group compared to the control group with median intervals from end of treatment to recovery of 4.6 d and 6.5 d, respectively[48]. A similar study showed a benefit with the Chinese herb *Panax ginseng* via intra mammary administration. Results indicated an immunomodulatory effect and the study concluded the beneficial effect of the extract could be used as alternative therapy in the control of mastitis at drying off, either

[44] Mullen KA, Lee AR, Lyman RL, Mason SE, Washburn SP, Anderson KL. Short communication: an in vitro assessment of the antibacterial activity of plant-derived oils. J Dairy Sci. 2014 Sep;97(9):5587-91
[45] Taga I, Lan CQ, Altosaar I. Plant essential oils and mastitis disease: their potential inhibitory effects on pro-inflammatory cytokine production in response to bacteria related inflammation. Nat Prod Commun. 2012 May;7(5):675-82. Review.
[46] Pinedo P, Karreman H, Bothe H, Velez J, Risco C Efficacy of a botanical preparation for the intramammary treatment of clinical mastitis on an organic dairy farm. Can Vet J. 2013 May;54(5):479-84.
[47] Taga I, Lan CQ, Altosaar I Plant essential oils and mastitis disease: their potential inhibitory effects on pro-inflammatory cytokine production in response to bacteria related inflammation. Nat Prod Commun. 2012 May;7(5):675-82. Review.
[48]Pinedo P, Karreman H, Bothe H, Velez J, Risco C Efficacy of a botanical preparation for the intramammary treatment of clinical mastitis on an organic dairy farm. Can Vet J. 2013 May;54(5):479-84.

alone or in conjunction with dry cow antibiotic therapy published in Veterinary immunology and Immunopathology[49].

Another area of investigation is the use of botanical medicines as alternatives to anthelmintics. A recent study published in the Journal of Parasitic Diseases demonstrated that the anthelmintic efficacy of crude neem (*Azadirachta indica*) leaf powder against strongyle infections in cattle was equivalent to fendendazole when compared to infected untreated controls. It was concluded that crude neem leaf powder has anthelmintic property and it can further be studied to isolate the active component to produce herbal anthelminthics [50]. In the International Journal of Environmental Research and Public Health study on the potential of botanical medicines to protect human health investigated the influence of neem (*Azadirachta indica*) materials (leaf, bark, and oil) on the survival of a strain of *E Coli* (Ec0157)in dairy manure : the neem leaf and bark supplements eliminated the pathogen in less than 10 d with a D-value (days for 90% elimination) of 1.3 d. In contrast, nearly 4 log CFU EcO157/g remained after 10 d in neem-free manure control. Control of this pathogen at the source (manure) are critical as produce crops are often grown in proximity to animal raising operations. The study concluded application of inexpensive neem supplements to control pathogens in manure and possibly in produce fields may be an option for controlling the transfer of foodborne pathogens from farm to fork[51].

Appendix IX-C outlines the abstracts of 25 recent journal articles demonstrating the amount of literature on botanical medicines for mastitis in dairy cattle medicine as well as a few studies on metritis and retained placenta. We have included a sample of recent literature, mainly *in vivo* and a few interesting *in vitro* studies from over 400 possible articles published in the last 10 years on plant extracts and cattle.

Veterinary Botanical Medicine and Equids

Journals publishing peer reviewed studies on plant medicine for equine medicine

- Equine Veterinary Journal
- Veterinary Parasitology
- American Journal of Veterinary Research
- Canadian Veterinary Journal
- Compendium Continuing Education
- Veterinary Journal
- Parasitology Research

Despite the 500% growth in herbal administrations to horses over the past 16 years[52] published research on equids and botanical medicine is still relatively limited. A review by Williams etal 2008 in Veterinary Journal pertinent to herbal supplementation in horses discussed several equine studies including one where *Panax ginseng* has been found to exert an inhibitory effect on pro-inflammatory cytokines and cyclooxygenase-2 expression; that equine studies have tested the anti-inflammatory effects of a single dose of ginger, post-exercise; echinacea was reported to have anti-inflammatory and antioxidant

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[49] Baravalle C[1], Dallard BE, Cadoche MC, Pereyra EA, Neder VE, Ortega HH, Calvinho LF. Proinflammatory cytokines and CD14 expression in mammary tissue of cows following intramammary inoculation of Panax ginseng at drying off. Vet Immunol Immunopathol. 2011 Nov 15;144(1-2):52-60.

[50] Jamra N, Das G, Singh P, Haque M. Anthelmintic efficacy of crude neem (Azadirachta indica) leaf powder against bovine strongylosis. J Parasit Dis. 2015 Dec;39(4):786-8.

[51] Ravva SV, Korn A. Effect of Neem (Azadirachta indica) on the Survival of Escherichia coli O157:H7 in Dairy Manure. Int J Environ Res Public Health. 2015 Jul 10;12(7):7794-803.

[52] Silver R, Bookout B, Karreman H Documentation of Trends in the Public Acceptance of Botanical Therapies: Consumer Buying Patterns, patterns of Use and Industry Correlates Jan 2016

properties; yucca contains steroid-like saponins, which produce anti-inflammatory, antioxidant and anti-spasmodic effects and horses fed garlic at >0.2g/kg per day developed Heinz body anaemia[53]. Another study suggested that Sea Buckthorn berry may be efficacious in the prevention or worsening of non-glandular gastric ulcers in horses during times of stress[54]. These point to potential benefits in equine health.

The recognition of the potential of botanical medicine to improve equine health care and expand treatment options is further exemplified by investigations into multiple plant agents for challenging conditions such *Prunella vulgaris* for equine infectious anemia virus (EIAV)[55], and a review by Tinworth etal 2010 in Veterinary Record on the potential for botanical medicines to manage insulin resistance and hyperinsulinaemia in non-obese horses based on human and laboratory animal data[56].

More recent studies have explored the use of plant medicines for the management of ecto and endo parasites, including the use of essential oils for donkey lice published in the Equine Veterinary Journal in 2016[57] and plants showing significant anthelmintic activity against strongyle nematodes published in Veterinary Parasitology 2015[58].

Chinese Herbal Medicine for equine reproductive management and respiratory disease has been discussed in the Compendium for Continuing Education (2011)[59].

Appendix IX-D outlines the abstracts of 22 recent journal articles demonstrating selected publications on botanical medicine research on equids.

Veterinary Botanical Medicine for Goats and Sheep
Journals publishing peer reviewed studies on plant medicine for caprine and ovine medicine

- Veterinary Parasitology
- Research Veterinary Science
- Parasitology Research
- Tropical Animal Health Production
- Journal Dairy Science
- Animal Science Journal

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[53]Williams CA, Lamprecht ED. Some commonly fed herbs and other functional foods in equine nutrition: a review. Vet J. 2008 Oct;178(1):21-31.

[54] Reese RE, Andrews FM, Elliott SB, et al. The effect of seabuckthorn berry extract (Seabuck Complete) on prevention and treatment of gastric ulcers in horses. Presented at the Proceedings of the 9th International Equine Colic Research Symposium, Liverpool. June 8–11, 2008.
[55] Brindley MA, Widrlechner MP, McCoy JA, Murphy P, Hauck C, Rizshsky L, Nikolau B, Maury W Inhibition of lentivirus replication by aqueous extracts of Prunella vulgaris. Virol J. 2009 Jan 20;6:8
[56] Tinworth KD[1], Harris PA, Sillence MN, Noble GK. Potential treatments for insulin resistance in the horse: a comparative multi-species review. Vet J. 2010 Dec;186(3):282-91.
[57] Ellse L, Sands B, Burden FA, Wall R. Essential oils in the management of the donkey louse, Bovicola ocellatus. Equine Vet J. 2016 May;48(3):285-9.
[58] Peachey LE, Pinchbeck GL, Matthews JB, Burden FA, Mulugeta G, Scantlebury CE, Hodgkinson JE. An evidence-based approach to the evaluation of ethnoveterinary medicines against strongyle nematodes of equids. Vet Parasitol. 2015 May 30;210(1-2):40-52.
[59] Shmalberg J, Xie H. Acupuncture and Chinese herbal medicine for treating orses.Compend Contin Educ Vet. 2011 May;33(5):E1-11.

Terrill etal 2012 highlight the research trends in ovine medicine by stating that anthelmintic resistance is reaching epidemic proportions in small ruminants in the U.S. and that non-chemical control alternatives are critically needed. They describe the generally warm, moist environmental conditions in the southern United States (U.S.) as ideal for survival and growth of the egg and larval stages of Haemonchus contortus and other gastrointestinal nematodes (GIN) of sheep and goats. Critically, infection with GIN is the greatest threat to economic small ruminant production in this region. They also highlight the emerging markets for grass-fed and organic livestock[60].

A global focus on botanical medicine- plant extracts for the management of gastrointestinal parasites is exemplified by a 2014 review by Mbaya etal, show that extracts of various species of medicinal plants have shown significant *in-vivo* and *in-vitro* pharmacological activities against ecto, endo and haemoparasites. They point out that the scientific evaluations of the use of the plants as antiparasitic agents were based on the claims of ethnoveterinary medicine. The pharmacological activities of these plants were associated with the presence of various bioactive compounds such as alkaloids, flavonoids, saponins, glycosides, allicinine, harmala, harmaline, harman, tetrahydroharman, ursolic acid, terapines, tannins, phenolic compounds, embelin and brucine. In the *in-vivo* studies, plant extracts were tested using animal models such as mice, sheep, goats, cattle and dogs[61].

Botanical medicines are also finding potential in reducing lamb mortality and improving ewe performance (Smeti etal 2015)[62]; improving ewe milk production (Giannenas etal 2011)[63] possibly through activity on the rumen microbiota in work conducted with the Department of Animal Sciences, The Ohio State University (Cobellis etal 2016)[64]; providing alternatives to blow fly control (Callander etal 2012)[65]and lice treatment (James etal 2012)[66] and ovine dermatophytosis (Mugnaini etal 2013)[67].

Appendix IX-E outlines a sample of 8 abstracts and 34 references of 446 journal articles published on the anthelmintic activity of plant extracts in sheep and goats

Veterinary Botanical Medicine for Poultry

Journals publishing peer reviewed studies on plant medicine for poultry.

- Poultry Science

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[60] Terrill TH, Miller JE, Burke JM, Mosjidis JA, Kaplan RM. Experiences with integrated concepts for the control of Haemonchus contortus in sheep and goats in the United States. Vet Parasitol. 2012 May 4;186(1-2):28-37.

[61] Mbaya AW, Ogwiji M. In-vivo and In-vitro activities of medicinal plants on ecto, endo and haemoparasitic infections: a review. Curr Clin Pharmacol. 2014;9(3):271-82. Review.

[62]Smeti S, Joy M, Hajji H, Alabart JL, Muñoz F, Mahouachi M, Atti N. Effects of Rosmarinus officinalis L. essential oils supplementation on digestion, colostrum production of dairy ewes and lamb mortality and growth. Anim Sci J. 2015 Jul;86(7):679-88.

[63] Giannenas I, Skoufos J, Giannakopoulos C, Wiemann M, Gortzi O, Lalas S, Kyriazakis I. Effects of essential oils on milk production, milk composition, and rumen microbiota in Chios dairy ewes. J Dairy Sci. 2011 Nov;94(11):5569-77.

[64] Cobellis G, Yu Z, Forte C, Acuti G, Trabalza-Marinucci M Dietary supplementation of Rosmarinus officinalis L. leaves in sheep affects the abundance of rumen methanogens and other microbial populations. J Anim Sci Biotechnol. 2016 Apr 27;7:27.

[65] Callander JT, James PJ. Insecticidal and repellent effects of tea tree (Melaleuca alternifolia) oil against Lucilia cuprina. Vet Parasitol. 2012 Mar 23;184(2-4):271-8

[66] James PJ, Callander JT. Dipping and jetting with tea tree (Melaleuca alternifolia) oil formulations control lice (Bovicola ovis) on sheep. Vet Parasitol. 2012 Oct 26;189(2-4):338-43.

[67] Mugnaini L, Nardoni S, Pistelli L, Leonardi M, Giuliotti L, Benvenuti MN, Pisseri F, Mancianti F.A herbal antifungal formulation of Thymus serpillum, Origanum vulgare and Rosmarinus officinalis for treating ovine dermatophytosis due to Trichophyton mentagrophytes. Mycoses. 2013 May;56(3):333-7.

- British Journal Poultry Science
- Research in Veterinary Science
- Parasitology Research
- Parasitology
- Avian Disease
- Canadian Journal of Veterinary Research
- Journal Applied Toxicology
- Journal of the Science of Food and Agriculture
- Animal Science Journal
- Virology Journal
- Annals of Agriculture and Environmental Medicine
- Biological and Pharmaceutical Bulletin
- Pharmaceutical Biology
- Biological Research

A recent review on botanical alternatives to antibiotics for use in organic poultry production highlights the issues of antibiotic resistance (from sub therapeutic use of antibiotics in poultry feed) and consumer concerns of residue contamination and antibiotic resistant bacterial pathogens in the USA (Diaz-Sanchez etal 2015)[68]. Similar concerns have led to a ban on antibiotic use in Europe since 2006 (Varmuzova etal 2015)[69].

In a 2010 review on dietary plant bioactives in poultry health and productivity the authors state there is increasing evidence indicating that plant medicines can be efficient in controlling poultry diseases and may also influence production parameters such as feed efficiency and product quality and replicate some of the effects of antibiotic growth promoters (Wallace etal 2010)[70]. Chinese herbal medicines have been investigated for their effects on common diseases in poultry for example common formulas are known to be immunomodulating (Li etal 2013)[71] and herbs are a rich source of lead compounds for antiviral treatments including Marek's (Sun etal 2014)[72]. Ginseng has been shown to have activity against Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV) of global economic importance in poultry. Oral administration of a ginseng extract enhances both humoral and gut mucosal immune responses to IBD vaccination and offers a better protection against virulent IBDV challenge and therefore might be a promising oral adjuvant for vaccination against infectious diseases in poultry (Zhai

[68] Diaz-Sanchez S, D'Souza D, Biswas D, Hanning I. Botanical alternatives to antibiotics for use in organic poultry production. Poult Sci. 2015 Jun;94(6):1419-30.
[69] Varmuzova K, Matulova ME, Gerzova L, Cejkova D, Gardan-Salmon D, Panhéleux M, Robert F, Sisak F, Havlickova H, Rychlik I Curcuma and Scutellaria plant extracts protect chickens against inflammation and Salmonella Enteritidis infection. Poult Sci. 2015 Sep;94(9):2049-58.
[70] Wallace RJ[1], Oleszek W, Franz C, Hahn I, Baser KH, Mathe A, Teichmann K. Dietary plant bioactives for poultry health and productivity. Br Poult Sci. 2010 Aug;51(4):461-87
[71] Li XT, Wang B, Li JL, Yang R, Li SC, Zhang M, Huang W, Cao L.Effects of Dangguibuxue Tang, a Chinese herbal medicine, on growth performance and immune responses in broiler chicks.Biol Res. 2013;46(2):183-8.
[72] Sun Y, Niu L, Song M, Zhao X, Sun N, He J, Wu C, Jiang J, Bai Y, Guo J, Li H.Screening compounds of Chinese medicinal herbs anti-Marek's disease virus. Pharm Biol. 2014 Jul;52(7):841-7.

etal 2014)[73]. Hypericum perforatum was shown to have a protective effect against unvaccinated chickens infected with IBDV with significant therapeutic efficacy (Shang etal 2012)[74].

Bozkurt etal 2013 review the use of botanical extracts in the control of coccidial infection in poultry with some plants and their respective volatile oils and extracts having the potential to alleviate coccidiosis and reduce its severity[75]. The use of plant extracts to control poultry helminths is also increasing and herbs like ginger and curcumin have been shown (*in vivo*) to have potential anthelmintic effects against *Ascaridia galli* (Bazh etal 2013)[76] . There is also a growing interest in the use of essential oils to control ectoparasites. In particular the economic impact of the poultry red mite, *Dermanyssus gallinae* as well as the lack of new acaricides and the occurrence of resistance and tighter legislation have driven research in this area (Nechita etal 2015)[77]. Aflatoxins are also a concern in poultry production and plants such as turmeric and Sea Buckthorn have been shown to have a protective effect against toxicity (Gholami-Ahangaran etal 2016, Solcan etal 2013)[78], [79].

Appendix IX-F outlines a sample of 40 abstracts of 846 journal articles demonstrating selected publications on botanical medicine research in poultry.

Veterinary Botanical Medicine for Swine

Journals publishing peer reviewed studies on plant medicine for swine.

- Veterinary Parasitology
- Journal Swine Health Production
- Research in Veterinary Science
- Animal Science Journal
- Virology Journal
- Journal of Animal Physiology and Animal Nutrition (Berlin)
- Asian Australis Journal of Animal Science
- Journal of Animal Science Technology
- Archives of Animal Nutrition
- Theriogenology
- Journal of Animal Science
- Animal Science Journal
- Journal Ethnopharmacology
- Reproduction in Domestic Animals

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[73] Zhai L, Wang Y, Yu J, Hu S. Enhanced immune responses of chickens to oral vaccination against infectious bursal disease by ginseng stem-leaf saponins. Poult Sci. 2014 Oct;93(10):2473-81.

[74] Can J Vet Res. 2012 Jul;76(3):180-5. Hypericum perforatum extract therapy for chickens experimentally infected with infectious bursal disease virus and its influence on immunity. Shang R, He C, Chen J, Pu X, Liu Y, Hua L, Wang L, Liang J.

[75] Bozkurt M[1], Giannenas I, Küçükyilmaz K, Christaki E, Florou-Paneri P. An update on approaches to controlling coccidia in poultry using botanical extracts. Br Poult Sci. 2013;54(6):713-27.

[76]Bazh EK, El-Bahy NM In vitro and in vivo screening of anthelmintic activity of ginger and curcumin on Ascaridia galli. Parasitol Res. 2013 Nov;112(11):3679-86.

[77] Nechita IS[1], Poirel MT[2], Cozma V[3], Zenner L[2]The repellent and persistent toxic effects of essential oils against the poultry red mite, Dermanyssus gallinae. Vet Parasitol. 2015 Oct 24. S0304-4017(15)30052-2.

[78] Gholami-Ahangaran M, Rangsaz N, Azizi S.Evaluation of turmeric (Curcuma longa) effect on biochemical and pathological parameters of liver and kidney in chicken aflatoxicosis. Pharm Biol. 2016 May;54(5):780-7.

[79]Solcan C, Gogu M, Floristean V, Oprisan B, Solcan G. The hepatoprotective effect of sea buckthorn (Hippophae rhamnoides) berries on induced aflatoxin B1 poisoning in chickens 1. Poult Sci. 2013 Apr;92(4):966-74.

- Animal Reproduction Science
- Immunopharmacology and Immunotoxicology
- Amino Acids
- Clinical and Vaccine Immunology
- Environmental Toxicology and Pharmacology
- Meat Science
- The British Journal of Nutrition

In the past two decades, an intensive amount of research has been focused on the development of alternatives to antibiotics to maintain swine health and performance however there is limited research validating herbs in general for their potential benefits for pigs and the perfect alternative does not exist (Thacker 2013)[80]. None the less plant derived products called botanicals, phytogenics , and also phytobiotics are used extensively in feed to improve pig performance[81]. Aside from having antimicrobial activity, these products potentially provide antioxidative effects, enhance palatability, improve gut function, or promote growth[82] Two of the most common phytogenic substances evaluated in swine include the spices oregano and thyme[83].

Three plant extracts from capsicum, turmeric and garlic have improved immune responses of pigs and feed efficiency of pigs challenged with porcine reproductive and respiratory syndrome virus (Liu etal 2013)[84] and reduced diarrhea and inflammation caused by *Escherichia coli* infection, which may be beneficial to pig health (Liu etal 2013)[85]. *Centella asiatica i*ncreases serum haematocrit and white blood cells and mycoplasma immunity to *Mycoplasma hyopnuemoniae* in swine(Maneewan etal 2014)[86]. Chinese herbs have been investigated in treating swine disease. In China Chinese patent medicines play and important role in veterinary clinical use. Treatment of swine with Wu Huang Hu can significantly inhibit pneumonia in infectious pleuropneumonia (Wang etal 2015)[87]. *Sophora flavescens* and stevioside combined have a beneficial effect on rotaviral diarhhea in pigs (Alfajaro etal 2014)[88], *Glycyrrhiza uralensis* extract cures rotaviral enteritis with both antiviral and anti-inflammatory effects in piglets

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[80] Thacker PA[1]Alternatives to antibiotics as growth promoters for use in swine production: a review. J Anim Sci Biotechnol. 2013 Sep 14;4(1):35.

[81] Windisch W, Schedle K, Plitzner C, Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. J Anim Sci. 2008;86(suppl 14):E140-E148.

[82] Jacela JY, DeRouchey JM, Tokach MD, et al. Feed additives for swine: Fact sheets – prebiotics and probiotics, and phytogenics. J Swine Health Prod. 2010;18(3):132–136.

[83] Neill CR, Nelssen JL, Tokach MD, Goodband RD, DeRouchey JM, Dritz SS, Groesbeck CN, Brown KR. Effects of oregano oil on growth performance of nursery pigs. J Swine Health Prod. 2006;14:312–316

[84] Liu Y[1], Che TM, Song M, Lee JJ, Almeida JA, Bravo D, Van Alstine WG, Pettigrew JE Dietary plant extracts improve immune responses and growth efficiency of pigs experimentally infected with porcine reproductive and respiratory syndrome virus. J Anim Sci. 2013 Dec;91(12):5668-79

[85] Liu Y[1], Song M, Che TM, Almeida JA, Lee JJ, Bravo D, Maddox CW, Pettigrew JE. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. J Anim Sci. 2013 Nov;91(11):5294-306

[86] Maneewan C[1], Mekbungwan A, Charerntantanakul W, Yamauchi K, Yamauchi KEEffects of dietary Centella asiatica (L.) Urban on growth performance, nutrient digestibility, blood composition in piglets vaccinated with Mycoplasma hyopneumoniae. Anim Sci J. 2014 May;85(5):569-74

[87] Wang G[1], Kang S[1], Yin Z[2],etal Therapeutic effect of Chinese patent medicine "Wuhuanghu" on porcine infectious pleuropneumonia and its acute and subchronic toxicity as well as evaluation of safety pharmacology. Environ Toxicol Pharmacol. 2015 Sep;40(2):388-96.

[88] Alfajaro MM, Rho MC, Kim HJ etal Anti-rotavirus effects by combination therapy of stevioside and Sophora flavescens extract. Res Vet Sci. 2014 Jun;96(3):567-75.

(Aljajaro etal 2012)[89]. *Taraxaumi mongolicum, Viola yedoensis Makino, Rhizoma coptidis,* and *Radix isatidis* were evaluated in combination on newborn piglets challenged with virulent porcine epidemic diarrhea virus and the herbs ameliorated the impaired growth performance and lesions compared to controls and the study suggested the mixture could be used as a prophylactic or therapeutic agent (Kim etal 2015)[90].
In swine reproduction, numerous herbs have been studied for their effects on preserving boar spermatoazoa , examples include *Salvia miltiorrhiza* and *Rosmarinus officinalis* which can both protect against peroxidative damage and increase sperm motility and litter size during the process of freezing-thawing (Shen etal 2015)[91], [92]. Silymarin has been shown to increase prolactin concentrations and protect against oxidative stress in gilts (Farmer etal 2014)[93].

Appendix IX-G outlines a sample of 20 abstracts of 599 journal articles demonstrating selected publications on botanical medicine research in swine.

4. Standards for admission to membership

a. The ACVBM will examine only veterinarians who:

- Are a graduate of a college of veterinary medicine accredited by the AVMA; or possess a certificate issued by the Commission for Foreign Veterinary Graduates (ECFVG) or are legally qualified to practice veterinary medicine in some state, province, territory, or possession of the United States, Canada, or other country
- Are licensed to practice veterinary medicine
- Meet the education, training, and experience requirements established by the ACVBM
- Demonstrate unquestionable moral character and ethical professional behavior

b. The ACVBM will certify only those veterinarians who have met established training and/or experience requirements and attained acceptable scores on comprehensive examinations administered by the ACVBM, their fitness and ability to practice the specialty.

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[89] Alfajaro MM, Kim HJ, Park JG etal Anti-rotaviral effects of Glycyrrhiza uralensis extract in piglets with rotavirus diarrhea. Virol J. 2012 Dec 18;9:310.
[90] Kim HB, Lee CY, Kim SJ, Han JH, Choi KH. Medicinal herb extracts ameliorate impaired growth performance and intestinal lesion of newborn piglets challenged with the virulent porcine epidemic diarrhea virus. J Anim Sci Technol. 2015 Oct 8;57:33

[91] Shen T, Jiang ZL, Liu H, Li QW. Effect of Salvia miltiorrhiza polysaccharides on boar spermatozoa during freezing-thawing. Anim Reprod Sci. 2015 Aug;159:25-30.
[92] Malo C, Gil L, Cano R, Martínez F, Galé IAntioxidant effect of rosemary (Rosmarinus officinalis) on boar epididymal spermatozoa during cryopreservation. Theriogenology. 2011 Jun;75(9):1735-41.
[93] Farmer C, Lapointe J, Palin MF. Effects of the plant extract silymarin on prolactin concentrations, mammary gland development, and oxidative stress in gestating gilts. J Anim Sci. 2014 Jul;92(7):2922-30.

5. Requirements for training and experience and prerequisites for examination

The eligibility requirements and experience prerequisities for taking the examination have been based on those of other established RVSOs.

6. Approved routes through education, training, and experience to qualify candidates for examination

Candidates must:

- Be a graduate from a college or school of veterinary medicine accredited by the AVMA; or possess a certificate issued by the Educational Commission for Foreign Veterinary Graduates (ECFVG) or by the PAVE program; or be legally qualified to practice veterinary medicine in some state, province, territory, or possession of the United States, Canada, or other country
- Be licensed to practice veterinary medicine
- Fulfill the requirements for the academic residency training path or alternative residency training path (see below)
- Demonstrate unquestionable moral character and ethical professional behavior

There will be two routes for satisfying the credentials requirements: an standard residency training path and an alternative residency training path.

1. ***STANDARD RESIDENCY TRAINING PATH***: Minimum 2 years residency under guidance of ACVBM board mentor or a PhD in a field related to botanical medicine, ACVBM case log, and one (1) peer reviewed/refereed publication.

2. ***ALTERNATIVE RESIDENCY TRAINING PATH****:* Minimum 3 years experience in practice with greater than 50% time devoted to botanical medicine, ACVBM case log, and 1 peer reviewed/refereed publication

Candidates must submit a completed application form from the ACVBM, a copy of their current veterinary license, curriculum vitae, three letters of reference, requested case medical records from the ACVBM case log and one (1) peer-reviewed/refereed publication.

7. Experience requirements relevant to the objectives of the ACVBM specialty

Candidates will not be required to have a waiting period between formal training and eligibility to sit for the examination.

8. Notification of candidates regarding deficiencies in credentials that prevent their examination or certification by the ACVBM

Candidates will be notified of any deficiencies in credentials no later than 120 days prior to the date of the examination.

9. Examination procedures

a. Written examinations will reflect the professional competence and knowledge base expected of a diplomate of the ACVBM.

b. After approval of credentials, candidate's will have a period of 120 days available for examination preparation prior to taking the certification test.
c. If a candidate's credentials are denied and an appeal is filed, the ACVBM will review this appeal and inform the candidate of their decision no later than 90 days prior to the examination date.
d. Candidates will receive an outline of the exam content (blueprint) and exam format prior to the exam.
e. Candidates will be informed prior to the examination of the passing score, or, if this is not determined in advance, the method of setting the passing score The passing score may be adjusted lower but not higher after administering the exam. The minimum passing score will be determined the Examination Committee and approved by the Board.
f. Candidates shall be sent written notification of the results of the examination no later than thirty (30) calendar days after the examination. All candidates shall be given such notification on the same day.
g. Candidates who do not successfully pass the examination, will, upon request, be provided with an explanation of the deficiencies that prevented their passing the examination. This procedure will be published by the ACVBM organization prior to the examination.
h. All candidates will be informed of their remaining eligibility and reapplication procedures.
i. ACVBM will avoid personal conflict, or the appearance of conflict, that could affect results of examinations.
j. The ACVBM will accommodate reasonable requests from applicants with documented disabilities for special test considerations in accordance with the Americans with Disabilities Act (ADA). Model language for ADA requests is provided in Appendix V.

10. Certificates attesting to diplomate status

Certificates will be issued indicating that the individual is a diplomate of the American College of Veterinary Botanical Medicine. The certificates will not be time limited.

11. Establish a formal appeal procedure for candidates in case of an adverse decision by the ACVBM

An appeals process has been established and is included in the Bylaws in Article XI Section E. A copy of the appeals procedures will be sent with the application form. A person who believes a decision by ACVBM has adversely affected them may appeal the decision by submitting a written petition for reconsideration on the grounds that the ACVBM:

a. disregarded the established criteria for certification
b. failed to follow stated procedures according to Bylaws
c. failed to consider relevant evidence and documentation presented.

The appeal of a credentials or examination determination will be subject to the policies and procedures for appeals adopted by the Board of Directors. An appeal relating to the candidate's credentials must be reviewed by the Board of Directors and the candidate will be informed of the decision 30 days prior to the exam date. An appeal relating to the examination will be evaluated by the Board of Directors and a decision made within 60 days after receipt of the appeal.

12. Provision of special training beyond the professional veterinary degree to enhance the ability of candidates to meet certification requirements and to maintain the competence of diplomates.

Botanical medicine training is available through industry recognised courses and post graduate qualifications in herbal medicine or through attendance at conferences offering in-depth coverage of these areas. The Education Committee will work toward expanding and enriching existing programs, including CE opportunities offered. The establishment of new residencies in botanical medicine will be a priority.

13. Scope of activities

The ACVBM will avoid agreements or contracts that lead to activities outside the scope of the stated objectives.

14.Contents of Constitution, Bylaws and Policies and Procedures Documents

The ACVBM will notify the ABVS of any and all changes to the Bylaws and *Policies and Procedures* documents at the time of the next annual report. Both the previous and newly accepted wording will be presented.

15. Incorporation of ACVBM

The ACVBM has legal incorporation as a not-for-profit educational organization within the state of Delaware with tax exemption under section 501 (C)(3) or 501 (C) (6) of the Internal Revenue Service Code.

Appendix 1 Bylaws

American College of Veterinary Botanical Medicine, Inc.

By Laws

ARTICLE I: NAME, NOT-FOR-PROFIT CORPORATION

The name of the corporation shall be the American College of Veterinary Botanical Medicine Inc., hereinafter referred to as ACVBM, or "the College". The ACVBM has been incorporated under the laws of the State of Delaware as a not-for-profit, tax-exempt organization for the purposes set forth herein, and in the Certificate of Incorporation. The corporation has no members.

ARTICLE II STATEMENT OF OBJECTIVES AND LIMITATIONS

Section A: Mission Statement

The American College of Veterinary Botanical Medicine advances the scope, quality, safety, professional knowledge and competency of veterinary medicine through identification and certification of veterinarians who demonstrate excellence in botanical medicine. The ACVBM shall increase the proficiency and competency of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine.

Section B: General Purposes

The ACVSMR has been founded as a not-for-profit, tax-exempt, voluntary professional certification board and credentialing program dedicated to professional, educational, and scientific purposes, within the meaning of Section 501(c)(6) of the U.S. Internal Revenue Code and regulations, the state of Delaware Not-For-Profit Corporation Law, and any other applicable successor laws. The purposes and mission of the ACVBM, are subject to the limitations set forth in these Bylaws and in the Certificate of Incorporation, are the establishment, maintenance, evaluation, and administration of professional credentialing programs in the field of veterinary medicine.

Section C: Specific Purposes

Consistent with the ACVBM Certificate of Incorporation and these Bylaws, the ACVBM shall promote the advancement of veterinary practice by identifying to the profession and the public those individuals that have obtained certification in veterinary botanical medicine in one of the following categories: Western botanical medicine or Chinese botanical medicine. To accomplish such purposes the ACVBM shall be operated:

1) To establish and maintain credentialing, certification and ethical standards for veterinary practitioners who excel in botanical medicine and who shall be titled Diplomates.

2) To identify, develop, provide and maintain professional botanical programs, to include but not limited to phytochemistry, phytopharmacology, pharmacognasy, ethnopharmacology, and ethnoveterinary medicine.

3) To examine and certify veterinarians as specialists in veterinary botanical medicine and facilitate the continued professional development of the Members of the College through development and administration of continuing education programs

4) To promote the improvement of professional practice standards, scientific inquiry and research into the safe and effective use of botanical medicines for prevention, treatment and control of

animal diseases to promote a high quality of life for companion animals and to enhance the well being and productivity of livestock and other production animals.

5) To collaborate with veterinary colleges and other educational institutions that relate to veterinary medicine to encourage and promote the development of graduate veterinary botanical medicine programs, especially in regard to residency training for clinical practice.

6) To identify Diplomates to the public, professionals, other professional organizations and government agencies and other appropriate individuals and bodies.

Section D: Limitations

The purposes and limitations of the ACVBM are restricted as follows:

1. No part of the net earnings of the ACVBM shall inure to the benefit of, or be distributed to, the Board of Directors or Officers, or other private persons, except that the ACVBM shall be authorized to pay reasonable compensation for services rendered and to make payments and distributions in furtherance of, and consistent with, the purposes set forth in these Bylaws and applicable ACVBM policies.

2. The Board of Directors is accountable for and shall have the authority and responsibility to develop, establish, approve, and enforce policies and procedures necessary to implement the goals and requirements of this Article.

ARTICLE III: BOARD OF DIRECTORS

Section A: Power, Duties and Function of the Board of Directors

1. General Authority: The ACVBM shall be governed by the Board of Directors. The Board shall have the duties, functions and power necessary to carry out the objectives and purposes of the corporation. The Board of Directors shall manage, control and supervise the business activities, property and other affairs of the ACVBM. The Board of Directors shall uphold and execute the purposes of the corporation; establish and adopt such policies, rules, and regulations for the conduct of its business, appoint and remunerate agents and employees; disburse funds of the corporation; purchase, lease, sell, transfer, and otherwise convey property; and, or any other lawful activities deemed necessary to further the purposes of the ACVBM, in accordance with the Certificate of Incorporation and these Bylaws, in their present or amended form. The Board of Directors will meet annually and will establish meeting rules, including agendas, frequency, and related procedures.

2. Specific Authority. The Board of Directors will also oversee publications concerning certification and will be responsible for staffing and management of resources to conduct the programs and activities of ACVBM. The Board of Directors will develop procedures for grievance, appeals, and disciplinary processes. The Board of Directors shall have the authority and control over all matters related to certification and other authorized, lawful activities. Standards for the development and administration of a program of certification of veterinary specialists will be established and overseen by the Board of Directors. The Board's powers and duties with respect to certification of veterinary botanical specialists shall include, without limitation, the following:

1) Examination content, administration of examinations; and the establishment of cut scores and passing points.
2) Review and approval of Certification Examinations prepared by the Examination Committee, and establishment of minimum passing standards of performance for such Certification Examinations.

3) Determination of eligibility of candidates for Certification Examination, upon consideration of the recommendation of the Credentials Committee.
4) Review and approval of Standard and Alternate Training Programs, upon consideration of the recommendation of the Training Program Evaluation Committee.
5) Review of recommendations of the Examination Committee as to the candidates who passed the Certification Examination and formal acceptance of candidates and issuance of certificates.

3. Appointment of Executive Director. An Executive Director may be appointed by the Board and their executive duties and responsibilities will be defined through a contractual agreement between the Board and the Executive Director. A performance review will be conducted annually by the Board.

Section B: Qualifications and Composition of Board of Directors

1. The number of Officers constituting the Board of Directors shall be not less than eight (8) voting members. including at least three (3) Board of Directors members known as Regents, and five (5) Officers. The Directors shall be elected by the Diplomates.
Each Officer shall be at least eighteen (18) years of age. The members of the Board of Directors shall be the following: the officers of the College, consisting of the Chair of the Board, President, President-Elect, Vice-President, Secretary/Treasurer; and three (3) Regents, (at-large Directors).

2. All voting Board of Directors members shall maintain Diplomate certification status and shall be in good standing with the ACVBM. The Board of Directors may, at its discretion determine additional qualifications for Board of Directors members, consistent with these Bylaws.

Section C. Ex Officio Members of the Board of Directors

The Board of Directors at its discretion, may appoint an Executive Director of the corporation and an ACVBM representative to the American Board of Veterinary Specialties, who shall be *ex-officio*, nonvoting members of the Board of Directors. The Board of Directors may appoint other *ex-officio*, nonvoting members of the Board of Directors, as deemed necessary, on an annual basis.

Section D: Terms of Office

1. The Directors who are Officers of the College shall serve as Directors co-extensively with their service as Officers.

2. All voting Regents, except Officers shall be elected by the Diplomates for a term of three (3) years, and shall serve until his successor has been elected and shall have qualified. During the first three (3) years the Board of Director's operate under these Bylaws, the terms of the Regents shall be staggered to ensure that approximately one half of the positions expire each year. Unless otherwise and specifically authorized by these Bylaws, no Regent shall be eligible to serve more than two (2) consecutive terms, or six (6) years whichever is greater. At each annual election Directors shall be chosen for a full term, to succeed those whose terms expire, whichever is greater.

ARTICLE IV: MEETINGS OF THE BOARD OF DIRECTORS

Section A. Annual Meeting/Regular Meetings

1. Meetings of the Board may be held at any place within or outside the State of Delaware. Such majority shall be capable of transacting business as may be provided in these Bylaws or under applicable law. Except as otherwise indicated in these Bylaws or by applicable law, the act of a majority of the Board of Directors present at a meeting at which a quorum is present shall be the act of the Board of Directors. The time and place for regular meetings of the Board shall be determined and scheduled by the Board.

2. The Annual Meeting of the Board of Directors shall be at a time and place designated by a majority of the Board of Directors for the transaction of business that comes before the Board of Directors. There shall be at least one (1) other regular meeting of the Board of Directors each year at a place designated by the Board of Directors for the transaction of business. Agendas of all items to be discussed at regular Board of Directors meetings shall be distributed at least two weeks (14) days prior to the meeting.

3. The rules contained in the most recently revised edition of *Roberts Rules of Order* shall be the parliamentary authority for the conduct of all meetings of the Board of Directors, except as otherwise provided in these Bylaws.

Section B. Special Meetings

Special meetings may be called by the Chair or President, or by a majority of the Board of Directors, or upon the filing of a written special meeting notice stating the object, location, date, and hour of such meeting. Notice of any and all special meetings will be delivered by the Secretary of the College to each Director via email to each Board of Directors member at least ten (10) days prior to the date of the meeting. The Secretary must receive confirmation that the email was received by the recipient in order for the notice to be valid.

Section C. Telephone Conference Meetings

1. The President or Chair may authorize a Board of Directors meeting via telephone conference, or similar form of telecommunications, when deemed necessary, provided that ten (10) days notice of such telephone conference is given to each Board of Directors member by the Secretary. The Secretary must receive confirmation that the email was received by the recipient in order for the notice to be valid.

2. Should an urgent item of business require immediate attention and action by the Board of Directors, a telephone conference may be called without previous notice, so long as all of the Board of Directors members have been contacted and advised of such telephone meeting and the item(s) to be reviewed or acted upon. All Board of Directors members participating in a telephone conference meeting must be able to hear, and communicate effectively with, each other. A two-thirds (2/3) roll call vote of the Board of Directors members in attendance will be necessary to carry out a resolution and to authorize Board of Directors action at a telephone conference meeting. Participation by such means shall constitute presence in person at the meeting.

Section D: Quorum and Action by the Board

a. A majority of the voting membership of the Board of Directors shall constitute a quorum for any meeting of the Board of Directors, and provided a quorum is present, the vote of a majority of the Board Members present shall be the vote of the Board.

b. Any action required or permitted to be taken by the Board or any committee thereof may be taken without a meeting if all the Members of the Board of the committee consent in writing or by electronic transmission to the adoption of a resolution authorizing the action. The resolution and the written consents or electronic transmissions shall be filed with the minutes of the proceedings of the Board of committee.

Section E: Notice and Waiver

The Secretary shall give notice of all regular meetings of the Board of Directors to all Regents and Officers no less than sixty (60) days prior to the meeting. Any notice may be waived before or after the date and time stated in the notice. Except as provided herein, the waiver must be in writing, signed by the person entitled to the notice, and delivered to the corporation for inclusion in the minutes, or for filing with the corporate records. A Board of Directors member's attendance at, or participation in, a meeting shall constitute waiver of any required notice to him or her unless the Board of Directors member shall, at the beginning of the meeting, object to the holding of the meeting or transaction of business at the meeting, and does not thereafter vote for, or assent to, any action taken at the meeting.

Section F. Email Votes

Should a matter arise that requires a vote of the Board of Directors between the Board of Directors meetings, a ballot by email, facsimile transmission, or other appropriate means authorized by the Chair or President, may be taken. A two-thirds (2/3) affirmative vote of the entire voting membership of the Board of Directors shall be necessary to carry any motion, and all members of the Board of Directors must consent, in writing, to the adoption of a resolution authorizing the action. The signed consents, or signed copies, shall be placed in the minutes book of the Board of Directors of Regents. Voting by proxy shall not be permitted.

Section G. Actions of the Board of Directors

Every decision of the Board of Directors shall be by a majority vote, unless otherwise required by law, the policies of the Board of Directors, or these Bylaws. Each Regent and Officer shall be entitled to one (1) vote on any matter coming before the Board of Directors.

Section H. Nomination of Board of Directors Members

Recommendations of qualified candidates to be nominated for election to the Board of Directors and Officer positions, consistent with the terms of this Article, shall be submitted to the Chair of the Nominating Committee at least four (4) months prior to the beginning of the fiscal year. The Nominating Committee will select and declare a slate of qualified and appropriate Directors and Officer candidates at least three (3) months prior to the beginning of the fiscal year. The candidate slate will specifically identify the Diplomate(s) nominated for each Director position and the Diplomate(s) nominated for each Officer position. Consistent with rules adopted by the Board of Directors, additional Director candidates for each practice category may be nominated by petition of two (2) Diplomates in good standing and additional candidates for Officer positions may be nominated by petition of one percent (1%) of the all Diplomates in good standing.

Section H. Election of Board of Directors Members

All voting Board of Directors members, including Officers, shall be elected by a majority vote of the voting active Diplomates in good standing. Board of Directors members shall be elected by mail ballot, or

by any other method designated by the Board of Directors, consistent with rules or procedures established by the Board of Directors. Balloting must be completed before the end of the fiscal year.

Section I. Director Resignation/Vacancy

A Director may resign at any time by providing written notice to the President and or the Executive Director. Such resignation shall take effect at the time specified therein, or, if no time is specified, at the time of acceptance as determined by the President or Board of Directors. Vacancies, as they occur on the Board of Directors by resignation, death, incapacity, or the like, shall be filled by appointment by the Board of Directors for the remainder of the term. As otherwise provided by these Bylaws, Officers may resign and Officer positions may be filled.

Section J. Removal of Directors

A Director may be removed, for cause, by a two-thirds (2/3) affirmative vote of the Board of Directors at any regular or special meeting of the Board of Directors at which a quorum of the Board of Directors is present, and under rules or procedures approved by the Board of Directors. Officers may be removed by the Board of Directors as otherwise provided by these Bylaws.

Section K. Limitations and Conduct of the Board of Directors

The Board of Directors shall be granted the authority to establish policies and procedures specifying Board of Directors conduct and limitations, including but not limited to the following:

1. Compensation for Services. Regents and Officers of the Board of Directors shall not receive any compensation, or other tangible or financial benefit for service on the Board of Directors. However, the Board of Directors may authorize payment by the ACVBM of actual, reasonable expenses incurred by Regents or Officers regarding attendance at Board of Directors meetings and other approved activities.

2. Compensation from ACVBM activities**.** Regents and Officers of the Board of Directors shall not receive any compensation, or other tangible or financial benefit from any activity of, or related to, the ACVBM, except as reimbursement for actual, reasonable expenses directly associated with such ACVBM element or activity, when authorized by the Board of Directors.

ARTICLE V: OFFICERS

Section A: Officer Titles/Authority

The Officers of the ACVBM shall consist of the Chair of the Board, President, President-Elect, Vice-President, and Secretary/Treasurer. The Officers shall be bound by, and be responsible and accountable to, the ACVBM Board of Directors for satisfying resolutions and directives of the Board of Directors, and shall have the authority and accountability conferred and granted by these Bylaws and by the Board of Directors. No individual shall hold more than one elective Officer position at any one time.

Section B: Election and Terms of Officers

The initial Officers shall be elected by the incorporator. Thereafter, the Officers shall be elected by the Diplomates. The Officers shall serve in their elected position for a term of one (1) year. A slate of candidates shall be prepared by the Nominating Committee and submitted to the Diplomates and the Board at least thirty (30) days before the annual meeting. Members may submit recommendations for

nominations (with nominee approval) to the Nomination Committee. The candidate receiving a plurality of the votes will be elected to the office. Officers shall take office immediately following the meeting at which they are elected. The Officers shall serve a term of one (1) year. It is expected, but not required, that continuity of administration will be maintained by the President moving up to Chair of the Board, the President-Elect to President, and the Vice-President to President-Elect.

Section C. Duties of the Officers

1. President. The President shall have the authority and responsibilities commonly incident to, and vested in, the corporate offices of Chief Executive Officer consistent with these Bylaws, including, but not limited to: the authority and responsibility to satisfy the directives of the Board of Directors; designation and appointment of ACVBM representatives (subject to Board of Directors approval), the role of presiding officer at all meetings of the ACVBM and the Board of Directors; the direction of other Officers, and the administration of the affairs of the corporation according to the Certificate of Incorporation, these Bylaws, and the policies adopted by the Board of Directors.
The President shall manage the day-to-day operations of the ACVBM and serve as Chair of the Nominating Committee. If an Executive Director has been retained, the President shall be responsible for day-to-day communications with the Executive Director. At the expiration of his or her term, the President shall succeed to the office of the Chair of the Board.

2. Chair of the Board. The Chair shall preside over all meetings of the Board, call meetings of the Board, act as the spokesman for the Board, perform the usual duties of the Chair and serve as the development officer for the College. If the President, President-Elect, and Vice-President are unable to perform the duties of President, the Chair (immediate past president) shall serve as acting President, shall have all authority conferred upon the office of President, and shall perform all duties for which the President is responsible for the unexpired portion of the term, or until another Officer can resume duties.

3. President-Elect. The President-Elect shall perform such other duties as the Board of Directors of Regents or the President may designate and serve as Chair for the Professional Botanical Program Committee. In the absence or disability of the President, the President-Elect shall serve as acting President, shall have all authority conferred upon the office of President, and shall perform all duties for which the President is responsible for the unexpired portion of the term, or until the President can resume duties. At the expiration of his or her term, the President-Elect shall succeed to the office of the President.

3. Vice-President. The Vice-President shall perform such other duties as the Board of Directors or the President may, from time to time, designate. In the absence or disability of the President and the President-Elect, the Vice-President shall serve as acting President, shall have all authority conferred upon the office of President, and shall perform all duties for which the President is responsible for the unexpired portion of the term, or until the President or President-Elect can resume duties. The Vice-President shall succeed to the office of President-Elect should that office be vacated, serve as Chair of the Credentials Committee, and perform such other duties as shall be assigned by the President.

4. Secretary/Treasurer. The Secretary-Treasurer shall be the Chief Financial Officer of the corporation. The Secretary-Treasurer shall have and perform all duties commonly incident to, and vested in, the offices of secretary and treasurer of a corporation, as well as all duties delegated and designated by the Board of Directors or the President including, but not limited to: supervision, safe and secure maintenance of all corporate documents, conduct the correspondence of the College, responsibility for accurate accounting of the minutes of all meetings and the books of the corporation, administration of the fiscal and financial policies of the corporation and ensure the preparation and submission of all other documents required by state or federal authorities,
and perform the usual duties of a secretary.

Section D: Officer Resignation/Vacancy

An Officer may resign at any time by providing sixty (60) days written notice to the President, or other authorized representative designated by the Board of Directors, and the Executive Director if retained. Such resignation shall take effect at the time specified therein, or, if no time is specified, at the time of acceptance as determined by the President or Board of Directors. In the event that the office of President becomes vacant, the President-Elect shall assume the office of President for the remainder of the term of office. In the event that any other Officer position becomes vacant, the President shall appoint interim officers to fill such vacant offices until a new Officer is elected by the Board of Directors to serve the unexpired portion of the term at the next scheduled Board of Directors meeting.

Section E. Removal of Officers

The Board of Directors may remove any Officer from office whenever, in its judgment, the best interests of the ACVSMR will be served thereby. An Officer of the ACVSMR may be removed by a two-thirds (2/3) affirmative vote of the Board of Directors at any regular or special meeting of the Board of Directors at which a quorum is present, and under rules or procedures approved by the Board of Directors.

ARTICLE VI: EXECUTIVE DIRECTOR

Section A. Appointment

The Board of Directors shall have the responsibility and authority to appoint an Executive Director of the ACVBM, who shall act as the Chief Operating Officer and Chief Staff Officer of the ACVBM. The Executive Director shall report to the Board of Directors, and shall be responsible and accountable for the supervision, control, and management of the ACVBM in its administrative, business, financial, and other operational affairs.

Section B. Authority and Duties

1. At the discretion of the Board, an Executive Director may be retained as a contracted employee of the College. The Executive Director's authority and responsibilities shall be defined in detail in a contract that is mutually satisfactory and agreed upon between the Executive Director and the Board of Directors. In general, the Executive Director shall be the chief administrative officer of the College with the responsibility to manage the affairs of the College in keeping with the policies, programs and budget as established by the Board. The Executive Director shall recommend and participate in the formation of new policies and shall make decisions within the existing policies approved by the Board. The Executive Director may delegate responsibilities and authority necessary to effectively manage the College but may not relinquish accountability to the Board.

2. If retained, the Executive Director shall have the authority and duty to implement all policies of the corporation, and will report to the Board of Directors. The Executive Director shall have the authority to: hire and dismiss employees and other personnel of the corporation, including consultants, contractors, counsel, and the like; legally bind the corporation and sign on its behalf contracts, checks, drafts, notes, mortgages, leases, and other legal documents, without limitation by reason of specification; receipt of correspondence to the College and distribution to appropriate Diplomates; assist the Secretary and Chair in the performance of their duties. The Executive Director shall perform such other duties as may be elsewhere specified in these Bylaws, or as may be designated by the Board of Directors.

ARTICLE VII: DIPLOMATES (Members)

Individuals certified by ACVBM shall be known as Diplomates. Individuals seeking certification must apply for membership to the ACVBM. The ACVBM will examine and certify those veterinarians that have demonstrated, by meeting established training and/or experience requirements and by attaining acceptable scores on comprehensive examinations administered by the College, their fitness and ability to practice the specialty. The benefits and procedures for acquiring each classification are governed by the policies and procedures of the Board of Directors. Active Diplomates have fulfilled the requirements set forth for certification by ACVBM and are current on all renewal fees. These members are eligible to vote and hold office.

In all of the following sections of these Bylaws, the term "Diplomate" includes both Charter Diplomate and Diplomate. Before applying for certification by the ACVBM a veterinarian must:

1. Be a graduate of a college of veterinary medicine accredited by the AVMA; or possess a certificate issued by the Commission for Foreign Veterinary Graduates (ECFVG) or are legally qualified to practice veterinary medicine in some state, province, territory, or possession of the United States, Canada, or other country.
2. Be licensed to practice veterinary medicine.
3. Meet the education, training, and experience requirements established by the ACVBM.
4. Demonstrate unquestionable moral character and ethical professional behavior.

Section A: Classification of Diplomates

There will be four classes of Diplomates and shall consist of the following: Charter Diplomates, Diplomates, Honorary Diplomates, and Emeritus Diplomates. The benefits and procedures for acquiring each classification are governed by the policies and procedures of the Board of Directors.

Section B: Charter Diplomate
Proposed Charter Diplomate status may be conferred on a person who has distinguished themselves with respect to, or contributed materially to the development of veterinary botanical medicine and to be deserving of special recognition by the College. Proposed Charter diplomates will be selected based on acknowledged expertise in veterinary botanical medicine and as recognised educators and practitioners of botanical medicine and contributions to the organization of the specialty. Proposed Charter Diplomates have fulfilled all the general requirements set forth for certification by ACVBM and are current on all renewal fees. The ACVBM Organizing Committee has chosen eight (8) Proposed Charter Diplomates to develop the educational programs of the ACVBM and to mentor potential new diplomates. These Diplomates are eligible to vote and hold office. These Proposed Chartered Diplomates will be dependent upon AVBS approval . In addition to meeting the general eligibility requirements, Proposed Charter Diplomates must have one or more the following:

- Ten (10) or more years of experience in veterinary botanical medicine, with not less than 75% of professional time devoted to the practice

- Experience of teaching/lecturing/research in the specialty for ten (10) years and have contributed substantially to the development of the specialty

- Authorship of significant publications resulting from research or practice in botanical medicine as determined by the Board or a professorship in the specialty of botanical medicine at a college or school of veterinary medicine

- Ability and willingness to mentor potential new ACVBM diplomates

- Evidence of advanced training in botanical medicine and have demonstrated competency through teaching, research, or practice the specialty to which most of the individuals professional time is devoted.

Section C: Diplomate

Diplomates have fulfilled all the general requirements set forth for certification by ACVBM and are current on all renewal fees. These members are eligible to vote and hold office.

Section D: Honorary Diplomate

Honorary Diplomate status may be conferred on a person who has contributed materially to the development of veterinary botanical medicine as to be deserving of special recognition by the College, under the following provisions:

1) A person must be nominated for Honorary Diplomate status by at least two Diplomates.
2) The nomination and supporting documentation must be in writing and submitted to the Board no later than ninety (90) days before the annual meeting of the Diplomates.
3) The name of the nominee and supporting documentation shall be distributed to voting Diplomates with a ballot no later than sixty (60) calendar days before the annual meeting.
4) Appropriately identified ballots must be delivered to the Secretary/Treasurer before or at the annual meeting, and the Secretary/Treasurer shall tally the vote.
5) The status of Honorary Diplomate must be approved by a two-thirds vote of the entire voting membership of the ACVBM. Honorary Diplomates as such shall be listed in the College Directory, and this individual shall not be required to pay dues, may not hold office, nor vote.

Section E: Emeritus Diplomate

Emeritus Diplomates have fulfilled the requirements for Diplomate status but are retired from active clinical practice in their specialty category. A Diplomate may request Emeritus Diplomate status by submitting a written application to the Board. Such application shall be granted provided the Diplomate meets the following requirements:

1) The Diplomate has been an active member of the ACVBM for at least 25 years or has reached the age of sixty five (65).

2) The Diplomate has retired from employment in which their ACVBM credentials are required for employment. Income generated from activities associated with their ACVBM credentials through activities such as part-time consulting, teaching, writing, or continuing education is acceptable and will not preclude Emeritus Diplomate status. Emeritus Diplomates are able to vote and hold office, shall be required to pay a renewal fee, but will not be required to recertify.

Section F: Inactive Diplomate

These individuals have fulfilled the requirements set forth for certification by ACVBM but are not current on renewal fees or have elected to take inactive status due to temporary discontinuation of active

professional clinical practice. Inactive Diplomates will lose the following privileges and benefits until active status is restored: (1) status as Supervising Diplomates of candidates in College programs, (2) right to vote, (3) right to hold office, (4) right to attend business meetings, (5) listing as a College Diplomate in the College Directory. Inactive Diplomates can revert to active status upon payment of annual dues plus a reactivation fee.

Section G: Disability of Diplomate

1. A Diplomate may request permanent or temporary disabled status by submitting a request therefor to the Board, with a certification that such Diplomate is permanently or temporarily disable. Such request shall be approved if the Diplomate meets the definition of permanent or temporary disability set forth herein. A Diplomate who is approved for temporary disability status must submit an annual request to the Board to maintain such status.

2. For the purposes of these Bylaws "permanent disability" means the permanent inability to engage in veterinary activity as a full-time occupation. For the purposes of these Bylaws, "temporary disability" means the inability, due to a temporary medical disability, to currently engage in veterinary activity as a full-time occupation/

3. A Diplomate who is permanently or temporarily disabled shall have all the rights and all the obligations of Diplomates except they shall be exempt from the payment of annual dues and shall not have the right to vote or be elected as an Officer or Director. A Diplomate who is no longer disabled shall notify the Board and, as of the date of such notification and payment of a portion of the current year's dues attributable to the remaining portion of the year, shall be restored to Diplomate status.

Section H. Meetings of Diplomates

1. Annual Meeting. The annual meeting of Diplomates for the election of Directors and officers and for such other business of the ACVBM shall meet annually at such a time and place as designated by the Board of Directors.
2. Special Meeting. Special meetings of Diplomates may be called by the Board in accordance with the procedures set for in the Delaware General Corporation Law ("GCL") at such a time and place as designated by the Board of Directors.
3. Professional Botanical Meeting. A professional botanical meeting of Diplomates shall be held on the date and at the time and place set by the Board. The professional botanical meeting shall be open to non-Diplomates and may be held in conjunction with the Veterinary Botanical Medicine Association (VBMA).
4. Notice of Meetings. Written notice of the annual and special meetings of Diplomates shall be given by the Board and shall state the place, date and hour of the meeting and, unless it is the annual meeting, shall indicate that it is being issued by or at the direction of the person or persons calling the meeting. Notice of a special meeting shall also state the purpose(s) for which the meeting is called. A copy of the notice shall be given, personally or by mail, to each Diplomate entitled to vote at the meeting. If the notice is given personally or by first class mail, it shall be given not less than ten (10) or more than sixty (60) days before the meeting. If mailed, notice is given when deposited in the U.S. mail, postage prepaid, directed to the Diplomate at his address as it appears on the records of the Diplomates. If a meeting is adjourned, notice of such adjourned meeting shall be given, if required, as provided in Section 22 of the GCL.
5. Notice by Electronic Transmission. Notice may be given to any Diplomate by a form of electronic transmission if such member consents in writing to such form of notice. Any such consent shall be revocable by written notice by the Diplomate to the College.

Section I: Quorum at Meeting of Diplomates

Diplomates entitled to cast one-third of the total number of votes entitled to be cast shall constitute a quorum at all meetings of Diplomates for the transaction of any business.

Section J: Termination of Diplomate Status

Before the Board takes action to terminate Diplomate status, the Board shall notify the Diplomate in writing by registered mail of its intended action and the reasons therefor. The Diplomate shall be entitled to present written evidence and to appear before the Board in person, at a date, time and place mutually acceptable to the Board and Diplomate. The Board shall consider all such evidence and shall notify the Diplomate of its decision in writing. One year after termination of Diplomate status under this Section 12, a person shall be entitled to reapply for Diplomate status under Article II Section 3 of these Bylaws. The Board shall terminate a person's status as a Diplomate in any of the following circumstances:

1. The Diplomate has violated any provision of the Certificate of Incorporation or these Bylaws, as determined by the Board.
2. The Diplomate fails to maintain an acceptable degree of competence in the practice of veterinary botanical medicine, as determined by the Board.
3. The Diplomate has brought discredit upon the ACVBM by unethical conduct, incompetence, fraud or any other reason, as determined by the Board.

Section K. Membership Dues

Annual dues shall be fixed by the Board. Annual dues for each year are due and payable by January 1st of such year. Persons who become Diplomates during a calendar year shall pay a pro rata portion of annual dues for such year, and such pro rata portion is due and payable within thirty (30) days after such person becomes a Diplomate.

ARTICLE VIII : GOVERNANCE

Section A. Autonomy

The Board of Directors shall in all respects be autonomous with respect to: ACVBM credentialing criteria and activities; administration; the conduct of meetings; policies; finances; election and appointment of Committee members and ACVBM representatives; and all other lawful activities.

Section B. Authorization to Act

Except as provided in the Certificate of Incorporation, these Bylaws, or applicable law, no Regent, Officer, employee, agent, or representative of the corporation may act on behalf of the ACVBM, or hold himself or herself out to the public as authorized to act on behalf of the ACVBM, without the prior, express, written approval of the Board of Directors.

ARTICLE XI: COMMITTEES

Standing Committees
Standing committees of the College shall consist of the Executive Committee, Nominating Committee, the Credentials Committee, the Examination Committee, the Appeals Committee, the Professional Botanical Program Committee, the Training Program Evaluation Committee, and such other committees

as may be designated by the Board. Committee members shall each serve a term of two (3) years, unless otherwise provided by the Board or these Bylaws.

Section A. Executive Committee

1. Composition. The Executive Committee shall be composed of the President, President-Elect, Vice-President, Secretary-Treasurer, and Executive Director (should one be retained). All Executive Committee members shall be voting members of the Committee, with the exception of the Executive Director.

2. General Authority, Duties and Limitations. Obtain funding for the corporation, develop a time line according to ABVS submission guidelines, appoint a secretary/treasurer for the ACVBM and develop a meeting schedule for the Board. The Executive Committee may act for the Board of Directors between meetings of the Board of Directors, or as otherwise authorized by the Board of Directors. The Executive Committee shall not, however, have the power to: remove a Regent or Officer; fill vacancies in the Board of Directors or in any committee; determine and fix compensation for any individuals for serving on the Board of Directors or any committee; sell corporate assets; amend, repeal, or adopt Bylaws; or, amend or repeal any resolution of the Board of Directors.

All proceedings and actions of the Executive Committee shall be recorded and reported to the Board of Directors at the next meeting of the Board of Directors.

3. Meetings of the Executive Committee. The Executive Committee shall meet at least two (2) times each calendar year, and otherwise at the direction of the President. Any member of the Executive Committee may request that an Executive Committee meeting be convened to conduct specific business. Such requests shall be communicated to the President, who may call a meeting if appropriate and necessary. Notice of Executive Committee meetings shall be given to all Committee members at least five (5) days prior to such meeting, unless the President determines that a shorter notice period is appropriate under the circumstances. Executive Committee meetings shall be conducted in person or via telephone conference at a date and time determined by the President, so long as all participants can communicate and effectively participate. Minutes shall be kept of all Executive Committee meetings, and such minutes shall be promptly circulated to the Board of Directors and maintained with the corporate minutes of the Board of Directors.

4. Actions by the Executive Committee. Unless contrary to applicable law or these Bylaws, the actions of the Executive Committee shall constitute the actions of the Board of Directors between meetings of the Board of Directors, unless subsequently rescinded or withdrawn by the Board of Directors.

Section B. Nominating Committee

1. Composition. The Nominating Committee shall consist of two (3) members: the President, and two members appointed by the Board, none of whom shall be current Regents or Officers. All Nominating Committee members must maintain Diplomate certification status and be in good standing with the ACVBM.

2. Appointment. The President shall appoint, with the approval of the Board of Directors, the Chair of the Nominating Committee. The Board of Directors shall appoint and replace the members of the Nominating Committee consistent with rules or procedures established by the Board of Directors.

3. Terms of Office: Nominating Committee members shall serve for a term of three (3) years, which shall be staggered to ensure that two (2) to three (3) members are replaced each year.

4. General Authority and Duties. The Nominating Committee shall oversee and supervise the nominating process for members of the Board of Directors, and shall establish appropriate procedures and rules for the selection and presentation of qualified candidates to active ACVBM Diplomates for election. Among other duties, the Nominating Committee shall: establish qualifications for charter diplomate status, nominate charter diplomats, perform surveys to identify potential members of the ACVBM; raise awareness of the establish ment of ACVBM among potential members; review and study the credentials of candidates; and develop a slate of qualified candidates. Under the direction of the Board of Directors, the Nominating Committee shall perform its duties and annually report its findings to the Board of Directors.

Section C. Credentials Committee

1. Composition: The Credentials Committee shall composed of a Chair and a representative from each practice category (Western or Chinese Botanical Medicine), none of whom shall be current Regents or Officers. All Credentials Committee members must maintain Diplomate certification status and be in good standing with the ACVBM. The Credentials Committee member from each professional practice category shall serve as the Vice-Chair of the Credentials Committee for that practice category.

2. Appointment. The President shall appoint, with the approval of the Board of Directors, the Chair of the Credentials Committee. The Board of Directors shall appoint and replace the members of the Credentials Committee, consistent with rules or procedures established by the Board of Directors.

3. Terms of Office. Credentials Committee members shall serve for a term of three (3) years, which shall be staggered to ensure that two (2) to three (3) members are replaced each year.

4. General Authority and Duties. The Credentials Committee, shall be responsible for: establishing candidate credential eligibility criteria in accordance with these By Laws; reviewing ACVBM certification applications; approving applicants who meet the certification eligibility criteria; identifying to the Board of Directors those applicants who are deemed eligible and ineligible for examination; establish criteria for residencies in veterinary botanical medicine and oversee the establishment and development of new residency programs in veterinary botanical medicine. The Board of Directors shall make the final decision in each case on eligibility to sit for the Diplomate Certification Examination. Under the direction of the Board of Directors, the Credentials Committee shall perform its duties and annually report its findings to the Board of Directors.

Section D. Examination Committee

1. Composition. The Examination Committee shall consist of three (3) members appointed by the Board of Directors, none of who shall be current Regents or Officers. All Examination Committee members must maintain Diplomate certification status and be in good standing with the ACVBM. The Examination Committee member from each professional practice category (Western or Chinese Botanical Medicine) shall serve as the Vice-Chair of the Examination Committee for that practice category.

2. Appointment. The President shall annually appoint, with the approval of the Board of Directors, the Chair of the Credentials Committee. The Board of Directors shall appoint and replace the members of the Credentials Committee, consistent with rules or procedures established by the Board of Directors.

3. Terms of Office. Examination Committee members shall serve for a term of three (3) years, which shall be staggered to ensure that two (2) to three (3) members are replaced each year.

4. General Authority and Duties. The Examination Committee shall: define the examinations for diplomate status in both subject matter and distribution of questions and type of exam (is multiple choice, short answer, practical etc); will write the basic core knowledge to be taken by all candidates and write the species and botanical medicine-specific knowledge in small animal, equine botanical medicine; administer, monitor and grade the Diplomate Certification Examination. It shall make recommendations to the Board regarding competence of those candidates who have completed the Diplomate Certification Examination. Under the direction of the Board of Directors, the Examination Committee shall perform its duties and annually report its findings to the Board of Directors.

Section E. Appeals Committee

1. Composition. The Appeals Committee shall consist of between three (3) members appointed by the Board, none of whom shall be current Regents or Officers. The senior member of the committee will serve as Chair. All Appeals Committee members must maintain Diplomate certification status and must be in good standing with the ACVBM.

2. Appointment. The committee will elect the Chair when there are two (2) or more equally senior members. An Appeals Committee member may not be a member of the Credentials Committee or Examination Committee. If a member has a conflict of interest in a specific appeal, the Chair of the Board shall appoint a temporary member to consider that appeal.

3. Terms of Office. Appeals Committee members shall serve for a term of three (3) years, which shall be staggered to ensure that two (2) to three (3) members are replaced each year.

4. General Authority and Duties. The Appeals Committee shall consider whether correct administrative procedures have been followed in the decisions made by the Credentials Committee and Examination Committee. The Chair of this committee will call a meeting to review an appeal and notify the Chair of the Board and the Executive Director of the results of that review within thirty (30) days of notification of the appeal. The decision of the Appeals Committee shall be final and there shall be no appeals there from.

Section F. Professional Program Committee

1. Composition. The Professional Program Committee shall consist of three (3) members: the President-Elect shall serve as Committee Chair, and two (2) members appointed by the Board none of who shall be current Regents or Officers. All Professional Botanical Program Committee members must maintain Diplomate certification status and must be in good standing with the ACVBM.

2. Appointment. The committee will elect the Chair when there are two (2) or more equally senior members.

3. Terms of Office. Professional Botanical Program Committee members shall serve for a term of three (3) years, which shall be staggered to ensure that two (2) to three (3) members are replaced each year.

4. General Authority and Duties. The Professional Botanical Program Committee shall determine the content of, and make all necessary plans and arrangements for the RVS Botanical Medicine conference.

Section G. Training Program Evaluation Committee

1. Composition. The Training Program Evaluation Committee shall consist of three (3) members appointed by the Board, none of whom shall be current Regents or Officers. All Professional Botanical

Program Committee members must maintain Diplomate certification status and must be in good standing with the ACVBM.

2. Appointment: All members shall be recommended by the Nominating Committee and appointed by the Board. The Board shall not be obligated to follow the recommendation of the Nominating Committee.

3. Terms of Office. Training Program Evaluation Committee members shall serve for a term of three (3) years, which shall be staggered to ensure that two (2) to three (3) members are replaced each year.

4. General Authority and Duties. The Committee shall be responsible for establishment of minimum criteria for Standard and Alternate Training Programs and review of Standard and Alternative Training Program applications and recommendations to the Board regarding acceptability of programs. The committee will define the knowledge base for the college and assist candidates in accessing this information; develop list of organizations and associations that have an interest in and/or provide educational opportunities in the areas of veterinary botanical medicine; develop and inform diplomats of continuing education opportunities relevant to veterinary botanical medicine; develop a list of publications and encourage new initiatives that will provide advanced training in veterinary botanical medicine.

5. The Examination Committee has determined that examination questions will be submitted by all members of the organizing committee in multiple-choice format and written under the guidelines of the National Board of Medical Examiners (NBME).

6. The examination will cover core knowledge of botanical medicine that is common to all species and common botanical theory including pharmacognosy, principles and pharmacology; then a concentration in the areas of veterinary botanical medicine for equine, production and small animals; and the scientific aspects of either Chinese or western botanical medicine. The examinations will be paper or computer-based and will be administered at a site and on a date determined by the ACVBM Board of Directors. The examinations will be administered and monitored by the Curriculum/Education/Examination Committee. Test questions will be marked and evaluated by the Curriculum/Education/Examination Committee.

7. Training programs are available (Appendix VII). It is estimated that 250 veterinarians each year for the last ten years have undertaken botanical medicine training. The objectives of these programs are to prepare veterinarians to provide botanical medicine services. In addition many veterinary conferences are entirely or partially devoted to advanced training in botanical medicine. The success of these programs will be evident by growth in the number registrants. At the present time, existing educational programs are well attended and attendance is increasing.

8. A list of current officers of the ACVBM is attached (Appendix IV). Proposed Charter Diplomates have been selected based on contributions to the organization of the specialty.

9. At the present time funding is based on an initial donation, membership dues and conference dues. Future funding will be based on application fees, examination fees, conference fees and membership dues.

Section H. Changes in Standing Committees and Additional Committees

The membership of the standing committees may be increased and other committees may be appointed as the need arises. The Board of Directors may authorize and supervise additional committees; from time to time to perform such functions as may be determined by the Board of Directors. The President shall annually appoint, with the approval of the Board of Directors, the Chair of all standing or special

committees, sub-committees or divisions, as may be required by these Bylaws, or as may be deemed necessary.

ARTICLE XII: ADMISSSION TO ACVBM SPECIALTY TRAINING PROGRAM

Section A. Eligibility for Admission

To be eligible to enroll in an approved ACVBM specialty training program, all applicants must meet the following criteria:

1. The applicant must be of high ethical, moral and professional standing.
2. The applicant has received a diploma from a college or school of veterinary medicine approved by the American Veterinary Medical Association ("AVMA"); a certificate issued by the Educational Commission for Foreign Veterinary Graduated ("ECFVG") or legal qualification to practice veterinary medicine in any country, or any state or province of any country, and after satisfaction of the requirements by the ACVBM Bylaws.
3. Applicants shall procure, from the Secretary/Treasurer, a copy of the application procedures, and shall submit the completed application, along with all other required documents and fees to the Secretary/Treasurer by the deadline designated in the application procedures.
4. The applicant must submit to the Credentials Committee three (3) case reports in which botanical medicine was the primary system of medicine used, and that is suitable for publication in a peer-reviewed veterinary or botanical journal. Each publication must meet acceptability criteria as determined by the Credentials Committee.
5. To be eligible to sit for the Diplomate Certification Examination, an applicant must complete a Board approved Standard Residency Training Program or an Alternate Residency Training Program.

ARTICLE XIII: ACVBM SPECIALTY TRAINING PROGRAMS

Terminology for Training Programs: the word *resident* is used when referring to veterinarians who are registered in an ACVBM Specialty approved training program. Residents include veterinarians that are registered in full-time and part-time residency training programs. A veterinarian who is applying for registration of a training program with ACVBM is called an *applicant.* A veterinarian that has completed an ACVBM approved residency training program and whose credentials have been approved is known as a *candidate* and they are then eligible to take the Diplomate Certification Examination.

Section A. Standard Residency Training Program (SR-TP)

1. Applicants must complete one (1) year of general clinical experience (e.g., internship, residency, or practice) or equivalent or have completed a minimum of two (2) years of training in a graduate level botanical training program previously reviewed and recommended as a SR-TP by the Training Program Evaluation Committee and Credentials Committee and approved by the Board of Directors. The program will include clinical, teaching and research activities, at least twelve (12) months of on-clinic time in veterinary botanical medicine under the supervision (may be long distance) of a Diplomate of the College, submission of a ACVBM case log, standards to be determined by the Credentials Committee, that was maintained throughout the residency program and one (1) peer reviewed publication.

2. An annual training report is to be submitted to the Executive Secretary to monitor the progress of an ACVBM Specialty resident by the supervising Diplomate. The report serves to review the progress and training of all residents to prepare them for the credentials application process.

3. Application for program approval must be submitted no later than ninety (90) days after starting the program. The trainee must register with the Secretary no later than ninety (90) days after beginning the training program using forms available from the Secretary/Treasurer. A response to the application for an SR-TP shall be issued no later than one hundred twenty (120) days after the application is submitted. A response may be approval, disapproval, request for further information or clarification, or indication of program changes required for approval. Upon approval of an SR-TP by the Board, the program shall not be subject to any future additional requirements that may be imposed with respect to such program by the College.

Section B. Denial of Standard Residency Training Program

1. An applicant who's SR-TP was denied may appeal this decision within thirty (30) calendar days of the postmarked date of the notification. The appeal must be made in writing to the Secretary/Treasurer and shall include a statement of the grounds for reconsideration and appropriate documentation.

2. Upon receipt of an appeal, the Secretary/Treasurer shall notify the Chair of the Board, the Chair of the Training Program Evaluation Committee, and the Chair of the Appeals Committee. The Chair of the Board shall submit to the Appeals Committee documentation indicating the reasons for denial of the SR-TP, including, but not limited to, the complete application package of the institution and all available documentation pertaining to the Training Program Evaluation Committee's review of the application and recommendations to the Board.

3. The Appeals Committee shall review the appeal and provide a recommendation to the Board no later than thirty (30) calendar days after receiving all the documentation from the Chair of the Board.

4. The Board shall render a decision on the appeal after receiving the recommendation of the Appeals Committee and shall notify the appellant of the decision no later than fifteen (15) calendar days after receipt of the recommendation of the Appeals Committee.

Section C. Alternate Resdiency Training Program (AR-TP)

1. The AR-TP, is designed for veterinarians that wish to complete training requirements while working primarily in private practice because family or financial constraints do not permit them to undertake a full-time residency program. A part-time resident may spend directly-supervised diplomate time at the residency director's facility, the resident's practice or any other facility at which appropriate veterinary botanical medicine cases are attended to with the mentorship and supervision of an ACVBM Diplomate.

2. Applicants must complete a minimum of three (3) years of training in an AR-TP, reviewed and recommended by the Training Program Evaluation Committee and Credentials Committee and approved by the Board as equivalent in training and experience to a Standard Residency Training Program. The program will include clinical, teaching and research activities, and at least twelve (12) months of on-clinic time in veterinary botanical medicine under the supervision (may be long distance) of a Diplomate of the College, submission of a ACVBM case log, standards to be determined by the Credentials Committee, that was maintained throughout the residency program and one (1) peer reviewed publication.

3. An annual training report is to be submitted to the Executive Secretary by the supervising Diplomate to monitor the progress of an ACVBM Specialty resident. The report serves to review the progress and training of all residents to prepare them for the credentials application process. Maximum time permitted to complete an AR-TP is 6 years.

4. Application for program approval must be submitted no later than ninety (90) days after starting the program using forms available from the Secretary/Treasurer. A response to the application for an AR-TP shall be issued no later than one hundred twenty (120) days after the application is submitted. A response may be approval, disapproval, request for further information or clarification, or indication of program changes required for approval. Upon approval of an AR-TP by the Board, the program shall not be subject to any future additional requirements that may be imposed with respect to such program by the College.

Section D. Denial of Alternate Residency Training Program.

1. An applicant who's AR-TP was denied, may appeal this decision within thirty (30) calendar days of the postmarked date of the notification. The appeal must be made in writing to the Secretary/Treasurer and shall include a statement of the grounds for reconsideration and appropriate documentation.

2. Upon receipt of an appeal, the Secretary/Treasurer shall notify the Chair of the Board, the Chair of the Training Program Evaluation Committee, and the Appeals Committee. The Chair of the Board shall submit to the Appeals Committee documentation indicating the reasons for denial of the AR-TP, including, but not limited to, the complete application package of the applicant and all available documentation pertaining to the Training Program Evaluation Committee's review of the application and recommendations to the Board, except that such documentation shall be redacted to preserve anonymity of the appellant.

3. The Appeals Committee shall review the appeal and provide a recommendation to the Board no later than thirty (30) calendar days after being appointed and receiving necessary documentation.

4. The Board shall render a decision on the appeal after receiving the recommendation of the Appeals Committee and shall notify the appellant of the decision no later than fifteen (15) calendar days after receipt of the recommendation of the Appeals Committee.

ARTICLE XIV: DIPLOMATE EXAMINATION AND CERTIFICATION

Section A. Diplomate Certification Examination (DCE)

1. The DCE will be offered only once per year at a time and place designated by the Board and as determined by the Examination Committee and in accordance with these Bylaws. The candidate must pass all sections of the examination no later than six (6) consecutive years after his or her eligibility to sit for the examination was determined.

2. To be eligible to sit for the Diplomate Certification Examination, an applicant must:

> a) complete one (1) year of general clinical experience (e.g., internship, residency, or practice) or equivalent or have completed a minimum of two (2) years of training in a graduate level botanical training program previously reviewed and recommended as a Standard Residency Training Program by the Training Program Evaluation Committee and approved by the Board of Directors, or;
>
> b) complete a minimum of three (3) years) of training in an Alternate Residency Training Program, reviewed and recommended by the Training Program Evaluation Committee and approved by the Board as equivalent in training and experience to a Standard Residency Training Program.

3. The applicant must submit to the Credentials Committee one (1) publication that has been published in the peer reviewed literature within the past 3 years in which botanical medicine was the primary medical treatment used. Each publication shall pass acceptability criteria as determined by the Credentials Committee.

4. Applicants deemed eligible by the Board to sit for the DCE shall be notified of the date and format of the examination no later than on hundred twenty (120) calendar days before the date of the examination.

5. The certifying examination for the ACVBM Specialty will test whether the candidate can perform at the level expected of an entry-level specialist in veterinary botanical medicine. The DCE may include but is not limited to: The history of botanical medicine in context of contemporary practice, understanding the language of botanical medicine terminology and concepts, botanical medicine resources and research evidence based approaches, philosophy and principles of botanical medicines and Materia Medica. General botanical medical principles common to all species: herbal therapeutics in practice (of the gastrointestinal system, cardiovascular system, integumentary system, respiratory, hematologic system, musculoskeletal system, nervous system, endocrine system, etc), clinical strategies, botanical medicine case analysis and diagnosis, development of therapeutic treatment plans and prognosis, integration with conventional medicine, pharmacology, drug herb interactions and adverse effects, pharmacognosy, ethnoveterinary, ethnobotanical medicine, zoopharmacognosy, manufacturing, processing and dispensing of botanical medicines, veterinary herbal pharmacy management.

6. The written examination will consists of four (4) parts.

I. A general botanical medicine section (all candidates are required to take this section)
II. Principles and Practices
III. Clinical botanical medicine
IV. Botanical identification of 20 medicinal plants.

The examination shall consist of 100 or more questions for sections I-III in a multiple choice format with one correct answer and four distractors. Three (3) minutes will be allotted for each question. Depending on the number of questions, the total examination time may vary. The contents of the examination will be determined by the Credentials and Education Committee. Components of the examination will test Western or Chinese botanical medicine.

7. Examination Dates: Candidates taking the examination for the first time shall take all parts in the same year. Candidates for the examination shall be required to submit questions for future examinations as instructed prior to the examination.

8. Minimum score. The minimum score as determined by the Examination Committee and approved by the Board must be achieved on each section in order to pass the examination. Candidates shall be sent written notification of the results of the examination no later than thirty (30) calendar days after the examination. All candidates shall be given such notification on the same day. Certificates will be issued indicating that the individual is a diplomate of the American College of Veterinary Botanical Medicine. The certificates will not be time limited.

9. Candidate failing one section of the exam. A candidate failing one section of the exam may re-take that section at the next scheduled examination without fulfilling other prerequisites. The candidate must submit a letter of intent to the Secretary/Treasurer and pay the examination fee for only that failed section of the examination. Provided, however, that if it has been more than six (6) years since the candidate was first deemed eligible to take the examination, he or she must submit a new application with all required documentation and fees, including new case reports, to the Secretary/Treasurer for review by the Credentials Committee and approval by the Board, and if deemed eligible, must re-take the entire Diplomate Certification Examination.

10. Candidate failing to pass one or more sections. A candidate that fails any section of the examination a second time must re-take the entire examination. The candidate must submit a letter of intent, updated curriculum vitae, and examination fee to the Secretary/Treasurer. Provided, however, that if it has been more than six (6) years since the candidate was first deemed eligible to take the examination, he or she must submit a new application with all required documentation and fees, including new case reports, to the Secretary/Treasurer, for review by the Credentials Committee and approval by the Board, and if deemed eligible, must re-take the entire Diplomate Certification Examination.

Section B. Failure to Pass Diplomate Certification Examination

1. Candidates failing to pass the Diplomate Certification Examination may appeal this decision within thirty (30) calendar days of the postmarked date of notification. The request for appeal must be made in writing to the Secretary/Treasurer and shall include a statement of the grounds for reconsideration and appropriate documentation. The Secretary/Treasurer shall notify the Chair of the Board, the Chair of the Examination Committee, and the Chair of the Appeals Committee.

2. The Chair of the Board shall submit to the Appeals Committee a written statement of the reasons for the failure of the candidate. The Chair of the Examination Committee shall submit to the Appeals Committee the examination and scores of the candidate, the complete list of scores of all candidates on that examination, and a statement as to the criteria used for the Committee's recommendation for success or failure, except that such documentation shall be redacted to preserve anonymity of both the appellant and the other candidates.

3. The Appeals Committee shall review the appeal and render a recommendation to the Board no later than thirty (30) calendar days after being appointed. The Board shall render a decision on the appeal upon consideration of the recommendation of the Appeals Committee and notify the appellant of the decision no later than fifteen (15) calendar days after receipt of the recommendation of the Appeals Committee.

4. If an appeal is unsuccessful and the appellant wishes to reapply to sit for the Diplomate Certification Examination, the deadline for application shall be extended three months from its designated date.

Section C. Certification by the ACVBM

1. Candidates achieving a passing score on all sections of the Diplomate Certification Examination. Candidates which pass all sections of the DCE shall be reviewed by the Examination Committee and a recommendation shall be made by such committee to the Board.

2. After consideration of the recommendation by the Examination Committee, the Board shall determine whether to grant Diplomate status to the candidate. Such determination shall be made by the Board no later than sixty (60) calendar days after a candidate sits for the examination.

3. Diplomate certificates shall be issued to successful candidates by the Board no later than ninety (90) calendar days after Diplomate status is granted.

Section D. Diplomate Certification Examination Denial of Eligibility

1. An applicant denied eligibility to sit for the Diplomate Certification Examination may appeal this decision within thirty (30) calendar days of the postmarked date of the notification. The appeal must be made in writing to the Secretary/Treasurer and shall include a statement of the grounds for reconsideration and appropriate documentation.

2. Upon receipt of an appeal, the Secretary/Treasurer shall notify the Chair of the Board, the Chair of the Credentials Committee and the Chair of the Appeals Committee.
The Chair of the Board shall submit to the Appeals Committee documentation indicating the reasons for denial of eligibility to sit for the examination, including, but not limited to, the complete application package of the applicant and all available documentation pertaining to the Credential Committee's review of the application and recommendations to the Board, except that such documentation shall be redacted to preserve anonymity of the appellant.

3. The Appeals Committee shall review the appeal and provide a recommendation to the Board no later than thirty (30) calendar days after receiving all necessary documentation.

4. The Board shall render a decision on the appeal upon the recommendation of the Appeals Committee and notify the appellant of the decision no later than fifteen (15) calendar days after receipt of the recommendation of the Appeals Committee.

Section E. Maintenance of Diplomate Status

To maintain active Diplomate status, a Diplomate of the ACVBM is required to obtain 30 hours of botanical medicine continuing education (CE) credits per year. These credits may be obtained from both veterinary and botanical medicine meetings, at least fifty (50) percent of the CE units must be obtained from veterinary botanical medicine CE . A point system will be used as outlined by AVMA where points may be accrued in a variety of ways including continuing education attendance or presentations, publications, serving on committees. An honor system of compliance requires diplomates to self-declare completion of requirements each year with membership renewal. A log book will be submitted. Each year ACVBMA will audit 5% of members, requiring specific evidence supporting requirements. Diplomates will be provided with standard CPE Log Books and Guidelines for completion.

ARTICLE XV: FISCAL YEAR and FEES

The fiscal year of the corporation shall be determined by the Board of Directors. The Board of Directors is authorized to change and fix the fiscal year as it deems appropriate from time to time. The current fiscal year of the College shall be from November 1st to October 31st.

1. All funds of the College shall be deposited from time to time in such bank or banks as the Board of Directors may select.
2. Annual dues for Diplomats are due and payable January 1 of each year.
3. Applicants shall pay the prescribed fee to the College to sit for the Diplomate Certification Examination. This fee is non-refundable and payable each time the examination is repeated.
4. The annual operating budget for the College will be prepared by the Secretary/Treasurer under the direction of the President and with the assistance of the Executive Director. The budget shall be approved by the Board of Directors.

ARTICLE XVI: INDEMNIFICATION

In accordance with and to the maximum extent permitted under the GCL, in the event any person who is or was a Regent, Officer, employee, trustee, authorized representative, or agent of the ACVBM, acting in good faith and in a manner he reasonably believed to be in the best interests of the ACVBM has been made party, or is threatened to be made a party, to any threatened, pending or completed action or proceeding by reason of being a representative, whether civil, criminal, administrative, or investigative (other than an action or proceeding by or in the right of the corporation), such representative may be

indemnified against reasonable expenses and liabilities, including attorney fees, actually and reasonably incurred, judgments, fines, and amounts paid in settlement in connection with such action or proceeding. Where the representative was successful in defending the action, indemnification is mandatory.

Section A. Determination of Proper Indemnification

Unless ordered by a court, discretionary indemnification of any representative shall be approved and granted only when consistent with the requirements of applicable law, and upon a determination that indemnification of the representative is proper in the circumstances because the representative has met the applicable standard of conduct required by law and in these Bylaws.

ARTICLE XVII: AMENDMENTS

Section A. Amendments to the Certificate of Incorporation

1. The Board of Directors shall adopt a resolution setting forth a proposed amendment to the Certificate of Incorporation and declaring its advisability. These Bylaws may amended by a vote of the Diplomates in good standing, provided that proper written notice of proposed Bylaw change(s) with recommendations by the Board of Directors is given to each eligible Diplomate at least thirty (30) days prior to the counting of the ballots.

2. A two-thirds (2/3) affirmative vote of the Diplomates voting is required in favor of such amendment and if passed a certificate thereof shall be executed, acknowledged and filed and shall become effective in accordance with the GCL.

3. Proper written notice under this Article shall be a copy of the text of the proposed amendment, including any relevant explanatory materials, whether transmitted by mail, facsimile transmission, or other appropriate means. Notice by mail shall be deemed sufficient if sent to the last Post Office address furnished to the Executive Director or Secretary-Treasurer.

ARTICLE XVIII:ADOPTION OF BYLAWS

The American College of Veterinary Botanical Medicine Inc., will organize under the laws of the incorporated State on the gate of incorporation. These Bylaws were adopted by the ACVBM Board of Directors, and became effective in 2015.

ARTICLE XIX: AMERICAN BOARD OF VETERINARY SPECIALITIES (ABVS) RESPRESENTATIVE

The duties of the ABVS Representative shall be determined by the Board of Directors and shall include, but are not limited to, the following: attend all regular and special meetings of the ABVS as the official representative of the College; inform the Board and membership of all actions of the ABVS, especially those having a direct impact on the College; assist as needed in the preparation of annual and other reports of the College to the ABVS; and provide a summary of all ABVS meetings in a timely fashion to the Secretary/Treasurer. If the ABVS Representative is unable to attend an ABVS meeting, a representative designated by the Board shall serve as the Alternate Representative to the ABVS.

Appendix 2 Industry Support:
Academics
Diplomates
Practitioners
Veterinary Students
Public

CALIFORNIA STATE UNIVERSITY | Chico

TO: American Board of Veterinary Specialties (ABVS) January 4, 2016

FROM: Cynthia A. Daley, Ph.D.,
California State University Chico, College of Agriculture

Dear ABVS members,

As a member of the consortium of University's involved in organic dairy research and education, I am wholly supportive of the efforts of the American College of Veterinary Botanical Medicine (ACVBM) to increase the proficiency and competence of health professionals in field of ethno-veterinary medicine.

The need for well trained health professionals within the field of phyto-medicine has never been greater. The organic dairy industry has been one of the fastest growing segments of the $35 billion-dollar organic industry, according to USDA ARS, and is a prime candidate for the use of phyto-medicine as an approved approach within certified herds. University support for the transition within the dairy industry has been slow. The availability of trained veterinary professionals who understand alternative medicine as a means to support dairyman in the paradigm shift to organic has also been in short supply.

Even so, the herbal supplement industry has blossomed over the course of the last three decades into a multinational, multibillion dollar industry that includes both professional and trade organizations, national and international practice and research conferences. We now have specialized integrated medicine practices and clinics in pain management and adjunctive cancer therapy. Many conventional medical colleges have introduced CAM degree-level education programs which is also quite encouraging. Finally, there are now credible sources of funding through the U.S. National Institutes of Health (NIH) National Center for Complementary and Alternative Medicine (NCCAM; http://nccam.nih.gov/), and the Australian National Institute of Complementary Medicine (NICM; http://www.nicm.edu.au), so that researchers interested in pursuing careers in TCM or CAM can do so with the knowledge that funding will be available to support their work.

The field of ethno-veterinary medicine is at a critical stage in its evolution here in the US. There currently is no recognized group of experts with in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine. For that reason, I strongly urge the AVMA to create the charter for diplomats of the ACVBM, to represent veterinarians who are recognized specialists in herbal medicine. These experts are to be consulted regularly by veterinarians and representatives of industry for information regarding appropriate use of botanicals in animals. I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,
Cynthia A. Daley, Ph.D.
Director of the Organic Dairy Education & Research Program
College of Agriculture
California State University Chico

AUBURN
UNIVERSITY

COLLEGE OF VETERINARY MEDICINE
DEPARTMENT OF ANATOMY, PHYSIOLOGY AND PHARMACOLOGY

Date: Friday, December 18, 2015
TO: American Board of veterinary Specialist (ABVS)
From: Auburn University College of Veterinary Medicine

Dear ABVS,
Thank you for the opportunity to write to you in support of botanical medicine within veterinary medicine. My name is Barbara Kemppainen, professor; Auburn University College of Veterinary Medicine (AUCVM) started working with botanical medicines in 2007. My interest in veterinary botanicals is primarily studying their effectiveness in treating diseased food animals that are being raised organically. Organic food is the fastest growing area of agriculture, and organic food animal products are the fastest growing aspect of organic food.
In 2010 and 2011, I developed an elective course for our veterinary students entitled "Complementary and Alternative Veterinary Medicine", [CAVN]. One of the junior veterinary students who completed the course wrote me a thank you note saying the course was her favorite of the courses she had completed at her
I have been active in obtaining funds to support research on the botanical medicines that potentially reduce the effect of poisons on poultry. I have attached Appendix 1, which is an abstract entitled "Investigation of the sparing effects of American skullcap, Scutellaria lateriflora on aflatoxin-contaminated feed in broiler chickens". The reason skullcap was chosen to test its ability to reduce aflatoxin's adverse effects is that aflatoxin causes its toxic effects by anti-oxidants and anti-inflammatory effects. When the ant-oxidant effects of skullcap were measured and compared to about 30 different anti-oxidants, skullcap was among the 5 strongest anti-oxidant effects. This abstract was published at the Annual Conference of the American Council for Medicinal Plants, July 2011, Huntsville, AL.
I was also the Chair person for the session entitled "Bioactives – Animal Health Benefits", at the meeting "the Annual Conference of the American Council for Medicinal Plants, July 2011, Huntsville, AL."
In the last several months, I've had several journal editors send me manuscripts to review. John Richards, Associate Editor of "Journal of Veterinary Science & Animal Husbandry (JVSAH) asked me to review the manuscript entitled "Comparative Efficacy of Neem Leaves, Pineapple Leaves, and Levamisole Against GastrointestinalNematodiasis in Sheep.".
[Continuation from page 1]
In addition, the Editor of BioMed Central Complementary and Alternative Medicine, Dr. Vivek K. Bajpai "Pharacokinetics and Bioavailability of Orthosiphon Ethanolic Extract and Nano Ethanolic Extract and its Nano Liposomes in Sprague Dawley Rats".

Please contact me if you have any questions or need further information.

109 GREENE HALL
AUBURN, AL 36849-5518

TELEPHONE:
334-844-4427

FAX:
334-844-4542

www.auburn.edu

Sincerely,

Barbara Kemppainen

Dr. Barbara Kemppainen
Professor, AUCVM
Auburn AL 36832
Phone 334 844 5415, cell 334 750-2152

Appendix 1

Investigation of the sparing effects of a native medicinal plant (American skullcap, *Scutellaria lateriflora*) on aflatoxin-contaminated feed in broiler chickens. M. Lohani[1], B. T. Akingbemi[1], E. G. Welles[1], J. Right[1], F. Hoerr[1], K. S. Joiner[1], F. W. Van Ginkel[1], J. Hess[2], W. B. Berry[2], A. Similien[2], D. A. Shannon[2], G. E. Rottinghaus[3], D. R. Ledoux[4] and B. W. Kemppainen[1], [1]College of Veterinary Medicine, [2] College of Agriculture, Auburn University, Auburn, AL, [3]Veterinary Medical Diagnostic Lab, and [4]College of Agriculture, Food, and Natural Resources, University of Missouri, MO, USA, 65205

Poultry are very sensitive to the toxic effects (poor performance, immunosuppression, and liver disease characterized by diffuse fatty changes) caused by chronic consumption of feed contaminated with low levels of aflatoxin (AF), a naturally occurring fungal toxin. American skullcap's flavonoids (bioactive compounds) have potent anti-oxidant and anti-inflammatory actions that oppose oxidative damage and inflammation caused by AF. The objective of this research was to determine if American skullcap reduces damage caused by AF fed to chickens. Three different groups of chicken were given feed amended with a range of doses of dried American skullcap (50, 250 and 1250 mg/kg, BW) for six weeks; 3 treatment groups consumed feed amended with the same doses of American skullcap for 1 week, followed by five weeks of feed amended with the same doses of American skullcap and 1.4 PPM aflatoxin. Negative and positive control groups received basal diet and basal diet amended with AF. Results showed that highest dose of American skullcap (1250 mg/kg BW) was associated with toxic effect [lower ($p<0.05$) body weight after 42 days of exposure]. However, forty-two days of exposure to AF resulted in severe liver damage [glutamate dehydrogenase (GLDH), 9.8 IU/L ±1.99] that was not observed in chicks consuming AF and the highest dose of American skullcap (4.16±0.73). Additionally, the highest dose of American skullcap partially protected chickens from AF-induced liver weight (g liver/100 g BW); and as the dose of skullcap increased, there was a linear (R^2=0.85) decrease in the AF-induced hepatic lipidosis. In conclusion, the highest dose of skullcap was associated with the adverse effect of lower body weight and beneficial effect of partially protecting chickens from AF-induced liver damage. The protective effect of skullcap on AF-induced liver damage could be due in part to skullcap's flavonoids decreasing AF metabolism to its toxic form by the liver.

Key words: American skullcap, Aflatoxin, *Scutellaria lateriflora*, Broiler chickens.

UF UNIVERSITY of FLORIDA

College of Veterinary Medicine
Small Animal Hospital

2015 SW 16th Ave
PO Box 100101
Gainesville, FL 32610-0000
352-294-4555
352-294-9888 Fax

DATE: January 4, 2015

TO: American Board of Veterinary Specialties (ABVS)

FROM: Meg M Sleeper VMD, DACVIM (cardiology)

Dear ABVS members,

I am writing to support the use of botanical medicine within veterinary medicine. I spent my sabbatical a few years ago learning about Traditional Chinese Veterinary Medicine (TCVM), specifically acupuncture and herbal treatment. I was very interested to learn how these modalities are being used in an integrative approach for an enhanced therapeutic effect. It was particularly interesting for me as someone coming from training in western veterinary medicine that many owners decline western medicine in favor or these other approaches. I firmly believe it is important to have veterinarians properly educated in the use of botanical medicines to promote safety and efficacy, avoid side effects of herbal-drug combinations and to be capable of communicating with owners about the strengths and weakness of all the treatment options that are available. Clearly TCVM and western veterinary medicine both have limitations and an integrative approach is probably the ideal.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill a much-needed role for our profession. With the rise of herbal medicines used in veterinary practice worldwide, there is a need for a recognized group of experts with in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research on herbal veterinary medicine, and for assisting pet owners in the responsible use of herbs for their pets. The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine and I support their effort to become a boarded specialty.

Sincerely,

Meg M. Sleeper VMD DACVIM (cardiology)

American Board of Veterinary Specialties
American Veterinary Medical Association
1931 North Meacham Road, Suite 100
Schaumburg, IL 60173

May 23, 2016

Dear Members of the American Board of Veterinary Specialties:

Please accept my endorsement of the proposed college of veterinary botanical medicine, which as proposed stands to recognize and elevate the scientific study of plant-based medicines and their derivatives. The prevalence of ethnopharmacologic interventions is well established in the scientific literature, and plants and their derivatives have served as the foundation of a number of important contributions to the veterinary medical pharmacopeia. The continued investigation and utilization of such substances requires a body of dedicated veterinarians with academic and experiential knowledge in the traditional uses of plants for veterinary and human health, as well as the scientific validation of their principles and therapeutic effects. The current College of Veterinary Clinical Pharmacology and the Board of Veterinary Toxicology may have some impact in these areas, but the lack of focus on the clinical utilization of these supplements and derivatives would stand in contrast to the proposed college described by the organizing committee of this particular current effort.

The petitioned college structure would allow for a repository of knowledge and qualified veterinarians which would inform the public, practicing veterinarians, and the human health field on the potential promise and toxicities of ethnopharmacologic agents. There are a number of studies which have highlighted the safety, efficacy, and adverse reactions from such substances in clinical patients. Surveys suggest that due to the unregulated nature of plant-based supplements, many of which are known to have drug-like effects, such products are often used by owners without the advisement of a veterinarian. Although the lack of appropriate queries as to whether these products are being administered by owners is likely one reason for this trend, there is a lack of suitable information or resources for veterinarians to consult. Therefore, one key advantage of the college would be an ability to disseminate and expand on the knowledge which is necessary to ensure patient safety and to identify areas where plant-based agents could influence the course of particular diseases.

A specialty in botanical medicine would easily draw on supporting knowledge from nutrition, toxicology, pharmacology, integrative medicine, and physiology. Training programs would therefore be academically rigorous in their foundation and in the application of clinical techniques. Undoubtedly, the organizing committee will submit a detailed list of charter members who can facilitate a critical mass of scientific study and training to support the goals of the college. The public would be served by an

increased number of specialists trained to offer *evidence-based* guidelines for the use of products, which are already given for a number of conditions. Ethnopharmacology holds additional promise of identifying ways to ensure the standardization or purification of products designed for animal use. Such products would be expected to be safer, their clinical effects more reliable, and the rate of contamination lower. As such compounds are also used in animal production, additional study would benefit human health as the impact of residues of such compounds in milk or meat remains largely unknown.

I strongly recommend the proposed college for approval given that it stands to provide more informed veterinary recommendations to the public, to serve a unique need in providing an organizational structure for botanical researchers and practitioners to advance scientific study, and to be distinct from the missions of the other approved specialties. The qualified founding members would be adequate to support a clear and scientifically-based training and education program in this area.

Please do not hesitate to contact me with any additional questions about my support of this endeavor.

Warm regards,

Justin Shmalberg DVM DACVN DACVSMR CVA CVFT CVCH
Clinical Assistant Professor of Integrative Medicine
Medical Director, Small Animal Hospital
College of Veterinary Medicine
University of Florida
shmalberg@ufl.edu
352.392.2235

Cornell University

Ambulatory & Production Medicine
College of Veterinary Medicine
29 Tower Road
Ithaca, NY 14853
t. 607-253-3140
f. 607-253-3982

DATE: November 23, 2015

TO: American Board of Veterinary Specialties (ABVS)

FROM: Robert B Hillman, Retired from Cornell University College of Veterinary Medicine

Dear ABVS members,

I am writing to support the use of botanical medicine within the veterinary medicine. Botanical medicine can be used to treat most conditions recognized by conventional medicine and many that are not. Botanical medicines are often prescribed to treat conditions for which there is no diagnosis or treatment available or in cases where conventional medical treatment has failed or is contraindicated.

Botanical medicines are especially helpful in the treatment of organ failure, chronic and geriatric diseases and cancer. They are often used to relieve pain, help protect and restore internal organ function, strengthen and support the immune system and reduce the dosage and frequency of conventional mediations and to reduce their side effects.

Often a botanical and conventional medical prescription will be used in an integrative approach for an enhanced therapeutic effect. It is important to have veterinarians properly educated in the use of botanical medicines to promote safety and efficacy, avoid side effects of herbal-drug combinations and promote better treatment outcomes.

Organic producers are requesting botanical medications in lieu of antibiotics due to public demand for antibiotic free meat, milk and eggs. Also, to prevent the development of antibiotic resistant organisms from antibiotic overuse.

According to surveys done by the National Center for Complementary and Alternative Medicine (NCCAM), the use of botanical medications is steadily rising in the human sector and by extension more people are requesting botanical mediations to treat their pets in addition to themselves.

Due to these demands, it is incumbent upon the veterinary profession to educate veterinarians in the proper use of botanical medications, in order to safely prescribe them as a primary therapy or in combination with conventional drugs, reduce the incidence of adverse effects, and to educate clients and the veterinary profession as a whole on the safe use of botanical medicines.

Sincerely Yours,

Robert B Hillman

Dr. Robert B. Hillman, BS, MS, DVM, DACT, CVA

Emeritus Sr Clinician

COLLEGE OF VETERINARY MEDICINE
& BIOMEDICAL SCIENCES

Department of Small Animal Clinical Sciences

TEXAS A&M UNIVERSITY

November 21, 2015

TO: American Board of Veterinary Specialties (ABVS)

Dear ABVS members,

Herbal medicine is of increasing interest to pet owners, pet professionals, and researchers. The American Botanical Council reported in September 2015 that sales of herbal supplements rose for the 11th consecutive year[1]. As the ultimate authority in the care of animals, veterinarians must become proficient in the use of botanicals where clients request it, but more importantly, to help develop alternatives in an era of antibiotic resistance and other emerging medical challenges.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill a much-needed role for our profession.

The American Veterinary Medical Association's policy on complementary, alternative, and integrative veterinary medicine (of which herbal medicine is considered a part) states that "veterinarians should have the requisite knowledge and skills for every treatment modality they consider using." The largest corporate veterinary hospital chain in the US acknowledges that herbal medicine is a part of veterinary practice, but presents outdated information from a defunct website on the VCA practice website[2]. Standard textbooks such as Ettinger's Internal Medicine as well as the Merck Manual offer a chapter on herbal medicine, acknowledging the interest in use of herbs by the profession, yet provide little practical guidance on actual clinical uses of plant medicine[3].

The field of ethnoveterinary medicine, which brings scientific scrutiny to traditional practices, is gaining credence. Traditional herbal medicine has an established place in the management of livestock and food animals in third world countries, as reviewed by the Food and Agriculture Organization of the United Nations[4]. A Medline search on the word "ethnoveterinary" yields 50 publications from 2013-2015 as compared to 30 from the period 2010-2012, and 24 from the period 2007 to 2009.

With the rise of herbal medicines used in veterinary practice worldwide, advanced training is now being offered. The College of Integrative Veterinary Medicine (online C.E. based in Australia) and the Chi Institute (Florida, USA) both offer Masters level training in specialty herb practice. Still, there is no recognized group of experts with such in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. Experts in this specialty are consulted regularly by veterinarians and representatives of industry and government for information regarding appropriate use of botanicals in animals. I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,

Debra L. Zoran, DVM, PhD, DACVIM-SAIM
Professor of SA Internal Medicine
Medical Operations Supervisor, Texas A&M VET
College of Veterinary Medicine and Biomedical Sciences
Texas A&M University

4474 TAMU
College Station, TX 77843-4474

Tel. 979.845.9053 Fax. 979.845.6978
vetmed.tamu.edu/vscs

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August 20, 2016

To Whom It May Concern:

My name is Amanda Fulmer and I am a veterinary medical oncologist currently practicing in Greenville, SC. I am currently completing my final assignments to receive a graduate diploma in Veterinary Chinese Herbal Therapy. I have been a student of the course provided by the College of Integrative Veterinary Therapies for the past two years. My coursework has consisted of reviewing lectures, course notes, and reading published scientific research articles in the field of herbal medicine. This very thorough training has provided an excellent background in this ancient form of Eastern medicine, and has prepared me for adding this valuable treatment modality to my oncology practice.

I was trained conventionally in Western veterinary medicine and knew early in my veterinary career that I wanted to pursue internship and residency training. I achieved Diplomate status in 2007 and have practiced in large multi-specialty practices since that time. Throughout my time in practice, I have received questions from owners regarding alternative forms of therapy. Owners were interested in using herbal medicine either in addition to conventional chemotherapy or radiation or as an alternative to these forms of treatment. Over the years, I have found that owners were asking about herbal medicine more often and my patients were more frequently receiving herbal supplements that owners had discovered through online research or through recommendations from friends.

I became interested in training in herbal medicine for two primary reasons. I wanted to be able to answer questions about herbal therapies and supplements intelligently for owners, and also to learn whether they were truly safe for my patients. I also wanted to have alternative treatment options available for my patients. Conventional cancer therapy often results in limited outcomes, so the opportunity to provide treatment that could improve these outcomes is appealing. There are also many owners who, for various reasons, have no interest in pursuing chemotherapy or radiation therapy for their pets, but are willing to pursue more natural forms of treatment.

Since beginning the practice of herbal medicine, I have found that herbs have not only affected cancers directly, but also lessen chemotherapy side effects and alleviate anxiety in more anxious pets. I am also providing herbal therapy for patients throughout the hospital and have had significant success in managing chronic renal failure, severe skin disease, degenerative joint disease and immune-mediated disease. My training has also provided information regarding dietary and nutritional changes that have helped many of these patients experience improved quality of life. I have several patients receiving herbs as their sole therapy who have survived significantly longer than the expected average of a patient who receives no treatment for a particular disease.

Herbal medicine provides a safe, effective treatment option either in conjunction with or instead of conventional therapies. Herbs have very few side effects and are cost effective for most owners. I would fully support the movement to make Chinese Herbal Medicine a specialty under the AVMA. There is no other discipline that exploits the properties of entire plants and other organic materials to the benefit of people and animals. Herbalism allows practitioners to take advantage of the antioxidant, anti-inflammatory, anti-neoplastic, and other properties of these natural substances that have been used for thousands of years to treat and prevent disease.

My course faculty promotes responsible, conscientious methods of practice, including responsibly sourcing our herbs. We as students are taught the importance of data collection and moving towards case collaboration and scientific publication to provide evidence that herbal medicine is a safe and effective practice. At no time are we encouraged to eschew Western veterinary practices, but to work in tandem to provide an even more desirable outcome. As alternative medicine practices become more common for people, owners will continue to utilize herbal medicine and other alternative treatments for their pets. It is important for our profession to embrace alternative therapies as valid and to promote research and collaboration between general practitioners, specialists, and practitioners of alternative medicine. Veterinarians will appreciate having more management options for certain cases, while owners will benefit from the availability of multiple treatment choices.

If you need additional information, please feel free to contact me by email at oncovet@live.com or by phone at (571) 232-1520. I appreciate your consideration and can't say enough about the value of the education I have received by pursuing this course of study.

With Regards,

Amanda Fulmer

Amanda Fulmer, DVM, DACVIM (Oncology)

Friday, January 22, 2016

TO: American Board of Veterinary Specialties (ABVS)

FROM: BC Phytoceutical Corporation

Dear ABVS members,

I am a veterinary researcher in Canada. I hold a PhD in veterinary science and a post doc in veterinary population medicine. Recently, I have received a significant grant from the veterinary phytoceutical industry to undertake research in Veterinary Herbal Medicine due to the high demand among Canadian veterinary clinics for evidence-based findings on the therapeutic use of herbal products.

In light of this growing interest in the industry, I am writing to support the mission of the American College of Veterinary Botanical Medicine (ACVBM) "to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine". ACVBM proposes to fulfill a much-needed role for the profession.

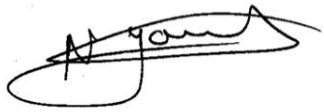
The American Veterinary Medical Association's policy on complementary, alternative, and integrative veterinary medicine (of which herbal medicine is considered a part) states that "veterinarians should have the requisite knowledge and skills for every treatment modality they consider using." The largest corporate veterinary hospital chain in the US acknowledges that herbal medicine is a part of veterinary practice, but presents outdated information from a defunct website on the VCA practice website[2]. Standard textbooks such as Ettinger's Internal Medicine as well as the venerable Merck Manual offer a chapter on herbal medicine, acknowledging the interest in use of herbs by the profession, yet provide little practical guidance on actual clinical uses of plant medicine[3].

The field of ethnoveterinary medicine, which brings scientific scrutiny to traditional practices, is gaining credence. Traditional herbal medicine has an established place in the management of livestock and food animals in third world countries, as reviewed by the Food and Agriculture Organization of the United Nations[4]. A Medline search on the word "ethnoveterinary" yields 50 publications from 2013-2015 as compared to 30 from the period 2010-2012, and 24 from the period 2007 to 2009.

In British Columbia, Canada, the number of veterinary practices offering herbal medicine is growing exponentially. Further, with the rise of herbal medicines used in veterinary practice worldwide, advanced training is now being offered. The College of Integrative Veterinary Medicine (online C.E. based in Australia) and the Chi Institute (Florida, USA) both offer Masters level training in specialty herb practice. Still, there is no recognized group of experts with such in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. These experts are consulted regularly by veterinarians and representatives of industry and government for information regarding appropriate use of botanicals in animals. I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,



Nadine Gourkow, PhD (Vet Sciences)
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November 22, 2015

American Board of Veterinary Specialties (ABVS)

Donna M. Raditic DVM, DACVN, CVA
Nutrition and Integrative Medicine
Stamford, CT

Dear ABVS members,

Herbal medicine is of increasing interest to pet owners, pet professionals, and researchers. The American Botanical Council reported in September 2015 that sales of herbal supplements rose for the 11th consecutive year[1]. As the ultimate authority in the care of animals, veterinarians must become proficient in the use of botanicals where clients request it, but more importantly, to help develop alternatives in an era of antibiotic resistance and other emerging medical challenges.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill a much-needed role for our profession.

The American Veterinary Medical Association's policy on complementary, alternative, and integrative veterinary medicine (of which herbal medicine is considered a part) states that "veterinarians should have the requisite knowledge and skills for every treatment modality they consider using." The largest corporate veterinary hospital chain in the US acknowledges that herbal medicine is a part of veterinary practice, but presents outdated information from a defunct website on the VCA practice website[2]. Standard textbooks such as Ettinger's Internal Medicine as well as the venerable Merck Manual offer a chapter on herbal medicine, acknowledging the interest in use of herbs by the profession, yet provide little practical guidance on actual clinical uses of plant medicine[3].

The field of ethnoveterinary medicine, which brings scientific scrutiny to traditional practices, is gaining credence. Traditional herbal medicine has an established place in the management of livestock and food animals in third world countries, as reviewed by the Food and Agriculture Organization of the United Nations[4]. A Medline search on the word "ethnoveterinary" yields 50 publications from 2013-2015 as compared to 30 from the period 2010-2012, and 24 from the period 2007 to 2009.

With the rise of herbal medicines used in veterinary practice worldwide, advanced training is now being offered. The College of Integrative Veterinary Medicine (online C.E. based in Australia) and the Chi Institute (Florida, USA) both offer Masters level training in specialty herb practice. Still, there is no recognized group of experts with such in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. These experts are consulted regularly by veterinarians and representatives of industry and government for information regarding appropriate use of botanicals in animals. I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,

Donna M. Raditic DVM, DACVN, CVA

References

[1]American Botanical Council; http://cms.herbalgram.org/press/2015/HerbalDietarySupplementSalesinUSRisein2014.html?ts=1448128594&signature=cadbf068182c04983449517442d9d17b. Accessed November 21, 2015

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[4]FAO, 2015. http://www.fao.org/wairdocs/ilri/x5483b/x5483b0g.htm. Accessed Novemer 21, 2015.

DATE: 12/31/15

TO: American Board of Veterinary Specialties (ABVS)

FROM: Oakland Veterinary Referral Services

Dear ABVS members,

I am writing to support the use of botanical medicine within veterinary medicine. Botanical medicine can be used to treat most conditions recognized by conventional medicine and many that are not. Botanical medicines are often prescribed to treat conditions for which there is no diagnosis or treatment available or in cases where conventional medical treatment has failed or is contraindicated.

Botanical medicines are especially helpful in the treatment of organ failure, chronic and geriatric diseases and cancer. They are often used to relieve pain, help protect and restore internal organ function, strengthen and support the immune system and reduce the dosage and frequency of conventional medications and to reduce their side effects.

Botanical medicines provide effective avenues of treatment for pets who may have no other safe or effective conventional options. For example, with the use of botanical medicines patients who are poor candidates for conventional cancer therapies routinely experience improved quality of life, disease stabilization and, in some cases, even tumor regression.

Additionally, for some botanical medicines there are no comparable pharmaceutical drugs. This is the case for botanicals such as San Qi (pseudoginseng), which is effective at controlling cancer related hemorrhage. Botanical medicines are also a promising addition to the antiangiogenic treatment approach for cancer, many of which have demonstrated antiagniogenic properties and immune modulating properties known to be important in tumor progression and metastasis.

Often a botanical and conventional medical prescription will be used in an integrative approach for an enhanced therapeutic effect. It is important to have veterinarians properly educated in the use of botanical medicines to promote safety and efficacy, avoid side effects of herbal-drug combinations and promote better treatment outcomes.

According to surveys done by the National Center for Complementary and Alternative Medicine (NCCAM), the use of botanical medications is steadily rising in the human sector and by extension more people are requesting botanical medications to treat their pets in addition to themselves.

This trend is observable in my clinical experience, where client interest and demand for integrative therapies and botanical medicines in cancer treatment has steadily increased over the past 10 years. I routinely see clients who would, without the option of consultation to advise on botanical medicine, administer these therapies to their pet without training or veterinary supervision.

1400 Telegraph Rd. • Bloomfield Hills, MI 48302-0245 • Phone: 248-334-OVRS (6877) • Fax: 248-334-3693
INTERNET: http://www.ovrs.com

Due to these demands, it is incumbent upon the veterinary profession to educate veterinarians in the proper use of botanical medications, in order to safely prescribe them as a primary therapy or in combination with conventional drugs, reduce the incidence of adverse effects, and to educate clients and the veterinary profession as a whole on the safe use of botanical medicines.

Sincerely Yours,

Dr. Erin Bannink, DVM, DACVIM (oncology), GDVCHM, CVA

1400 Telegraph Rd. • Bloomfield Hills, MI 48302-0245 • Phone: 248-334-OVRS (6877) • Fax: 248-334-3693
INTERNET: http://www.ovrs.com

Letter of Support for the American College of Veterinary Botanical Medicine

DATE: January 5, 2016

TO: American Board of Veterinary Specialties (ABVS)

FROM: Margot Mercer, fourth year veterinary student Oregon State University

Dear ABVS members,

Herbal medicine is of increasing interest to pet owners, pet professionals, and researchers. The American Botanical Council reported in September 2015 that sales of herbal supplements rise for the 11th consecutive year (ABC). As the ultimate authority in the care of animals, veterinarians must become proficient in the use of botanicals where clients request it, but more importantly, to develop alternatives in an era of antibiotic resistance and other emerging medical challenges.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill a much-needed role for our profession. As a veterinary student, and part of the future of our profession, I believe that organizations like the ACVBM will play a vital role in ensuring that veterinary practitioners stay well informed in a changing landscape of client desires.

The American Veterinary Medical Association's policy on complementary, alternative, and integrative veterinary medicine (of which herbal medicine is considered a part) states that "veterinarians should have the requisite knowledge and skills for every treatment modality they consider using." The largest corporate veterinary hospital chain in the US acknowledges that herbal medicine is a part of veterinary practice, but presents outdated information from a defunct website (altvetmed) on the VCA VCA 2015 practice website. Standard textbooks such as Ettinger's Internal Medicine as well as the venerable Merck Manual offer a chapter on herbal medicine, acknowledging the interest in use of herbs by the profession, yet provide no practical guidance on actual clinical uses of plant medicine (Merck Manual online, 2015).

It is imperative that veterinary professionals have the appropriate knowledge base to provide advice regarding recommendations and/or prescriptions about plant-based medications. As with any prescribed therapy, herbal medicine is not benign; knowledge of the physiology of how plants interface with animal systems, herb-drug interactions, herb-disease interactions and herb-herb interactions are crucial for the safety of our patients. Without proper training and the backing of a reputable diplomat organization it is difficult to ensure that veterinarians are providing the appropriate advice and care.

The field of ethnoveterinary medicine, which brings scientific scrutiny to traditional practices, is gaining credence. Traditional herbal medicine has an established place in the management of livestock and food animals in third world countries, as reviewed by the Food and Agriculture Organization of the United Nations (FAO). A Medline search on the word "ethnoveterinary" yields 50 publications from 2013-2015 as compared to 30 from the period 2010-2012, and 24 from the period 2007 to 2009. Many organic livestock producers often turn to botanical medical treatments, and again it would behoove the veterinary community to not provide confident, evidence-based guidance under the umbrella of an organization such as the ACVBM to ensure the safety of those animals and our food chain.

With the rise of herbal medicines used in veterinary practice worldwide, advanced training is now being offered. The College of Integrative Veterinary Medicine (online C.E. based in Australia) and the Chi Institute (Florida, USA) both offer Masters - level training in specialty herb practice. Still, there is no recognized group of experts with such in-depth knowledge to whom the profession

and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. These experts are consulted regularly by veterinarians and representatives of industry for information regarding appropriate use of botanicals in animals (Wynn). I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA and as a student on the cusp of joining the profession I look forward to the day where I may one day join the ACVBM as well.

Sincere thanks for your consideration,
Margot Mercer

References

ABC: http://cms.herbalgram.org/press/2015/HerbalDietarySupplementSalesinUSRisein2014.html?ts=1448128594&signature=cadbf068182c04983449517442d9d17b. Accessed November 21, 2015

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Merck Manual
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Letter of Support for the American College of Veterinary Botanical Medicine

DATE: January 4, 2016
TO: American Board of Veterinary Specialties (ABVS)

FROM: Jodie Joseph, Cornell CVM Holistic Club President

Dear ABVS members,

With Dr. Google at a client's fingertips, there are a ton of resources, both informational and misinformational available. As a veterinary student, almost veterinarian, I hope to have resources that I can turn to when I am unfamiliar with specific treatments that clients are either already using or inquiring about, or become an expert and well-versed in such treatments. The VBMA (Veterinary Botanical Medical Association) is an incredible resource that offers science and education should I choose to incorporate botanical medicine into my future practice. Most pharmaceuticals have been derived from plant materials. Having formal training in the safety, efficacy, indications, and contraindications for these plants would enable me to provide the best quality of care to my patients as possible. Rather than the stigma of "being a quack", a speciality in Botanical Veterinary Medicine will empower me to have the most scientific evidence behind herbal practice when other standard treatments yield unsatisfying results.

Herbal medicine is of increasing interest to pet owners, pet professionals, and researchers. The American Botanical Council reported in September 2015 that sales of herbal supplements rise for the 11th consecutive year (ABC). As the ultimate authority in the care of animals, veterinarians must become proficient in the use of botanicals where clients request it, but more importantly, to develop alternatives in an era of antibiotic resistance and other emerging medical challenges.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill a much-needed role for our profession.

The American Veterinary Medical Association's policy on complementary, alternative, and integrative veterinary medicine (of which herbal medicine is considered a part) states that "veterinarians should have the requisite knowledge and skills for every treatment modality they consider using." The largest corporate veterinary hospital chain in the US acknowledges that herbal medicine is a part of veterinary practice, but presents outdated information from a defunct website (altvetmed) on the VCA VCA 2015) practice website. Standard textbooks such as Ettinger's Internal Medicine as well as the venerable Merck Manual offer a chapter on herbal medicine, acknowledging the interest in use of herbs by the profession, yet provide no practical guidance on actual clinical uses of plant medicine (Merck Manual online, 2015).

The field of ethnoveterinary medicine, which brings scientific scrutiny to traditional practices, is gaining credence. Traditional herbal medicine has an established place in the management of livestock and food animals in third world countries, as reviewed by the Food and Agriculture Organization of the United Nations (FAO). A Medline search on the word "ethnoveterinary" yields 50 publications from 2013-2015 as compared to 30 from the period 2010-2012, and 24 from the period 2007 to 2009.

With the rise of herbal medicines used in veterinary practice worldwide, advanced training is now being offered. The College of Integrative Veterinary Medicine (online C.E. based in Australia) and the Chi Institute (Florida, USA) both offer Masters - level training in specialty herb practice. Still, there is no recognized group of experts with such in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. These experts are consulted regularly by veterinarians and representatives of industry for information regarding appropriate use of botanicals in animals (Wynn). I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,

Jodie Joseph
CUCVM DVM Candidate 2017

References

ABC: http://cms.herbalgram.org/press/2015/HerbalDietarySupplementSalesinUSRisein2014.html?ts=1448128594&signature=cadbf068182c04983449517442d9d17b. Accessed November 21, 2015

VCA Animal Hospitals; http://www.vcahospitals.com/main/pet-health-information/article/animal-health/veterinary-herbal-therapy/660. Accessed November 21, 2015.
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FAO, 2015. http://www.fao.org/wairdocs/ilri/x5483b/x5483b0g.htm. Accessed Novemer 21, 2015.

27943 Seco Canyon Road, Suite 501
Santa Clarita, CA 91350-3872
(855) 838-9378 Phone & FAX
www.vetimagewest.com

DATE: 04. December. 2015

TO: American Board of Veterinary Specialties (ABVS)

FROM: VetImage West, Incorporated

Dear ABVS members,

Herbal medicine is of increasing interest to pet owners, pet professionals, and researchers. The American Botanical Council reported in September 2015 that sales of herbal supplements rose for the 11th consecutive year[1]. As the ultimate authority in the care of animals, veterinarians must become proficient in the use of botanicals where clients request it, but more importantly, to help develop alternatives in an era of antibiotic resistance and other emerging medical challenges.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to Diplomate status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill a much-needed role for our profession.

The American Veterinary Medical Association's policy on complementary, alternative, and integrative veterinary medicine (of which herbal medicine is considered a part) states that "veterinarians should have the requisite knowledge and skills for every treatment modality they consider using." The largest corporate veterinary hospital chain in the US acknowledges that herbal medicine is a part of veterinary practice, but presents outdated information from a defunct website on the VCA practice website[2]. Standard textbooks such as Ettinger's Internal Medicine as well as the venerable Merck Manual offer a chapter on herbal medicine, acknowledging the interest in use of herbs by the profession, yet provides little practical guidance on actual clinical uses of plant medicine[3].

The field of ethnoveterinary medicine, which brings scientific scrutiny to traditional practices, is gaining credence. Traditional herbal medicine has an established place in the management of livestock and food animals in third world countries, as reviewed by the Food and Agriculture Organization of the United Nations[4]. A Medline search on the word "ethnoveterinary" yields 50 publications from 2013-2015 as compared to 30 from the period 2010-2012, and 24 from the period 2007 to 2009.

With the rise of herbal medicines used in veterinary practice worldwide, advanced training is now being offered. The College of Integrative Veterinary Medicine (online C.E. based in Australia) and the Chi Institute (Florida, USA) both offer Masters level training in specialty herb practice. Still,

there is no recognized group of experts with such in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

I have personally been monitoring a feline patient with hypertrophic cardiomyopathy (HCM) in conjunction with Dr. Richard Palmquist over the past 18 months. Based upon my numerous measurements on this patient's cardiac parameters, the HCM has significantly improved with herbal therapies only. This is very encouraging in my opinion knowing that feline HCM can improve significantly without the use of traditional Western medications. To date there has been no significant complications or unwanted side effects from the herbal therapies. I encourage the ABVS to seriously consider bringing the ACVBM into the specialty family of our profession.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. These experts are consulted regularly by veterinarians and representatives of industry and government for information regarding appropriate use of botanicals in animals. I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,

Sandy Sanford, DVM, DABVP (Canine/Feline)
President, VetImage West, Incorporated

References

[1]American Botanical Council; http://cms.herbalgram.org/press/2015/HerbalDietarySupplementSalesinUSRisein2014.html?ts=1448128594&signature=cadbf068182c04983449517442d9d17b. Accessed November 21, 2015

[2] VCA Animal Hospitals; http://www.vcahospitals.com/main/pet-health-information/article/animal-health/veterinary-herbal-therapy/660. Accessed November 21, 2015.
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[3]Merck Manual Online, http://www.merckvetmanual.com/mvm/management_and_nutrition/complementary_and_alternative_veterinary_medicine/herbal_medicine.html. Accessed November 21, 2015.

[4]FAO, 2015. http://www.fao.org/wairdocs/ilri/x5483b/x5483b0g.htm. Accessed November 21, 2015.

DATE: December 9, 2015

TO: American Board of Veterinary Specialties (ABVS)

FROM: Veterinary Cancer Group

Dear ABVS members,

I am writing to you in support of the need for recognition of an additional subspecialty under the umbrella of the ABVS. As an oncologist, I am faced daily with the pet owning public expecting guidance from veterinarians regarding the use of botanicals and other complementary methodologies. When we are deficient in providing this expertise, pet owners will use every and any sources to guide them.

As a board certified doctor, this troubles me. Pet owners are more and more educated regarding the existence of specialists in veterinary medicine. Yet when it comes to the use of botanicals, there currently does not exist board certified specialists to guide them. Currently, any veterinarian can hang out a "shingle" announcing they are a holistic veterinarian, without any advanced training to guarantee a standardized proficiency.

The supplement industry is a multi-billion dollar industry and growing. We cannot lose sight of the fact these products are *drugs*. There is no regulation regarding the safety and appropriate use of these products. When pet owners are unable to get guidance, they purchase these botanicals and use them, sometimes to the detriment to their pet's health. Some pet owners may be reluctant to share they are self medicating, leading to the potential for dangerous interactions of supplements and pharmaceuticals. We know the use of high doses of antioxidants will negate the effects of chemotherapy and radiation, for instance. Specialists in botanical medicine would help the pet owning public to choose manufacturers that produce pure, high quality products and use them in the safest, most appropriate ways.

Our Veterinary Oath is based on safeguarding animal health and welfare. To this end, we need to advance our scientific knowledge regarding the use of botanicals for supportive care and treatment of animals. The best way to do this is to recognize the College of Veterinary Botanical Medicine.

Please do not hesitate to contact me if you have additional questions.

Sincerely,

Mona Rosenberg, DVM, DACVIM (Oncology)
Chief of Staff
Veterinary Cancer Group
www.vetcancergroup.com

ANIMAL VISION CENTER

TO: American Board of Veterinary Specialties (ABVS)

FROM: Animal Vision Center February 23, 2016

Dear ABVS members,

I am writing to support the use of botanical medicine within veterinary medicine. Botanical medicine can be used to treat most conditions recognized by conventional medicine and many that are not. Botanical medicines are often prescribed to treat conditions for which there is no diagnosis or treatment available or in cases where conventional medical treatment has failed or is contraindicated.

Botanical medicines are especially helpful in the treatment of organ failure, chronic and geriatric diseases and cancer. They are often used with the goal to relieve pain, help protect and restore internal organ function, strengthen and support the immune system and reduce the dosage and frequency of conventional medications and to reduce their side effects.

Often a botanical and conventional medical prescription will be used in an integrative approach for an enhanced therapeutic effect. It is important to have veterinarians properly educated in the use of botanical medicines to promote safety and efficacy, avoid side effects of herbal-drug combinations and promote better treatment outcomes.

Organic producers are requesting botanical medications in lieu of antibiotics due to public demand for antibiotic free meat, milk and eggs and to prevent the development of antibiotic-resistant organisms from antibiotic overuse.

According to surveys done by the National Center for Complementary and Alternative Medicine (NCCAM), the use of botanical medications is steadily rising in the human sector and by extension more people are requesting botanical medications to treat their pets in addition to themselves.

Due to these demands, it is incumbent upon the veterinary profession to educate veterinarians in the proper use of botanical medications, in order to safely prescribe them as a primary therapy or in combination with conventional drugs, reduce the incidence of adverse effects, and to educate clients and the veterinary profession as a whole on the safe use of botanical medicines.

Sincerely,

Nick A. Fabes, DVM Phone (314) 344-1000 2000 Bryan Circle

Cardiology for
Dogs and Cats

Lori Siemens, DVM, DACVIM
P.O. Box 1898
Diamond Springs, CA 95619
Fax: (916) 735-2440
healinhearts@gmail.com
healinhearts.net

April 30, 2016

To: American Board of Veterinary Specialties (ABVS)
From: Dr. Lori Siemens

Dear ABVS Members,

As a veterinary cardiologist, I am always both grateful and relieved when I hear that a patient that I am treating for heart disease is also seeing an integrative veterinarian. The additional supportive care that they receive with herbal therapy, supplements, acupuncture, and diet clearly helps my patients' quality, and in my opinion, quantity of life. My clients benefit greatly too since they can see their pets thriving despite sometimes having advanced cardiac disease.

I am disheartened when I think about how little I know about herbal medicine; I received zero training in veterinary school and do not feel comfortable attempting to educate myself. Not only should veterinarians be given opportunities to attain a deeper understanding in this area of medicine but there should be specialists in botanical medicine to standardize and coordinate the dissemination of trustworthy educational material.

I would love to be able to become more familiar with the botanical therapy that my patients receive so I can feel more competent discussing it with my clients and their pets' other doctors, learn more about their benefits, and tailor my conventional therapy to better complement plant-derived treatments. I strongly support adding the American College of Veterinary Botanical Medicine to the list of specialties recognized by the AVMA.

Sincerely,

Lori M. Siemens, DVM
DACVIM (Cardiology)

May 22, 2016

Cynthia Lankenau, DVM
ACVBM

Dear Dr Lankenau,

I am a veterinary ophthalmologist in Jupiter, Florida. My patients include dogs, cats, horses, as well as many marine mammals including pinnipeds and cetaceans, as well as a few birds.

I have been using herbal extracts in the form of nutraceuticals to complement traditional therapy for over 15 years. I have found that these products have improved the healing of my patients, they have stabilized certain diseases including Progressive Retinal Atrophy and diabetic cataracts, when used consistently, and they have contributed to overall wellness in many other patients.

When I was part of the faculty of The Ohio State University's College of Veterinary Medicine, my laboratory did funded research on grapeseed extract and lutein. The grapeseed extract work has been published in the American Journal of Veterinary Research. We also studied resveratrol.

I am such a believer in these ingredients and products that I co-created a veterinary and a human vision supplement called OcuGLO™. In addition to OcuGLO™, my associates and we also co-created veterinary products for diabetes, dementia, and weight loss.

If I can be of more help or be a part of your College, please let me know! I would be honored to be a part of your group!

Warmly,

Carmen MH Colitz, DVM, PhD
Diplomate, American College of Veterinary Ophthalmologists

Medicine Wheel Veterinary Services, Inc.

Shauna Cantwell, DVM, MVSc, Diplomate ACVAA

www.shaunacantwell.com

February 16, 2016
American Board of Veterinary Specialties (ABVS)

Dear ABVS members;

I support the specialty of botanical medicine within the AVMA for a number of reasons.

After a number of years in general and emergency small animal medicine, I have been in a specialized field for 21 further years (anesthesia and analgesia) with a faculty position, scientific training, and clinical application of high quality medicine. I understand the scientific process and that herbal therapy can fit well into this process.

Client use of herbal therapy for themselves and their animals is commonplace and they are finding resources to self medicate. Laypeople are also starting to fill the gap of need by advising and providing herbal therapy to animal owners. This is inappropriate to good medicine and should be in the realm of veterinary oversight.

I now provide mostly holistic care and medicine for both small animals and equine. In this setting, herbal use is predominant, along with acupuncture and chiropractic. I see tremendous change clinically in patients that not only could be treated conventionally but mostly in those that cannot be such as in those with chronic degenerative or inflammatory diseases. These results are in the anecdotal realm but therapy would benefit and grow immensely with the guidance of specialty use and research. The AHVMA and its foundation has started some terrific research already as the basis for specialty medicine.

I have post-DVM training in all of these areas. I have taught many classes and workshops to veterinary students (and in post DVM certification programs), and see how readily the scientific training can be not only accepted but applied by burgeoning veterinarians in the basis of good scientific principles.

Just to emphasise, I daily use herbs, and successfully in my practice. I fully advocate more rigorous study and training in this already incredibly successful field for all of the above reasons.

Sincerely Yours,

● 2775 NW 49th Avenue, #205 – 359, Ocala, FL 34482 ● 352-538-3021 ● mwvetservice@gmail.com ●

Medicine Wheel Veterinary Services, Inc.

Shauna Cantwell, DVM, MVSc, Diplomate ACVAA

www.shaunacantwell.com

Shauna Cantwell, DVM, MVSc, DACVAA

● 2775 NW 49th Avenue, #205 – 359, Ocala, FL 34482 ● 352-538-3021 ● mwvetservice@gmail.com ●

November 25, 2015

Re: Letter of Support For The American College of Veterinary Botanical Medicine

Dear ABVS members,

Herbal medicine is a massive field that bridges ancient and modern times. The American Botanical Council reported in 2015 that sales of herbal supplements continue to rise.[1] At the same time research is growing exponentially.[2] Because veterinarians have a legal and ethical position responsible for the overall care of animals, it is vital that our profession embrace and recognize the importance of botanical medicine. Our profession faces rising difficulties in managing antibiotic resistant infections and a host of chronic diseases for which herbal medicine is being used daily to relieve suffering and even bring about resolution.[3] Research is booming in this area as it becomes a recognized gold mine of potential health solutions. The time has come for those using these agents to organize and associate in such ways that veterinary research is done and utilized to improve the quality of care available to animals in our country and the world.

I did not enter this field as a supporter. In fact, my first exposure to herbal medicine was seeing resolution in a case of biopsied, chronic, active hepatitis after conventional therapy failed. I contacted my veterinary school and was told that no evidence existed for herbal medicine in this case. It was embarrassing, to say the least, to advise clients that herbal medicine was "primitive" only to see the patient recover quickly once given milk thistle, a treatment we now recognize as effective. Since that time, I have developed an interest in botanical medicine and hold a graduate diploma in Chinese herbal medicine. Because of my training, many people travel long distances and lament that such professionals are not available in their area. I share their concern for this shortcoming in our profession.

The American College of Veterinary Botanical Medicine (ACVBM) formed to increase the competence and proficiency of veterinary health care professionals in the use of medicinal plants. This organization fully intends to develop into full-fledge diplomat status for the field. I support its efforts and see it filling a much needed function in the field of veterinary medicine. The college is dedicated in support of the American Veterinary Medical Association (AVMA) policy on complementary and alternative medicine, which states, "veterinarians should have requisite knowledge and skills in every treatment modality they consider using." An organization such as ACVBM is well equipped to offer quality, science-based continuing education, assist veterinarians in developing competence and understanding the use and abuse of botanicals in practice. Furthermore, ACVBM is serious about expanding properly done, high quality scientific research needed in the practice of modern veterinary medicine.

I am available to discuss this further as your group may need. Please support this application and encourage the group to fulfill its important mission in our profession.

Yours truly,

Richard Palmquist, DVM GDVCHM

References:

ABC: http://cms.herbalgram.org/press/2015/HerbalDietarySupplementSalesinUSRisein2014.html?ts=1448128594&signature=cadbf068182c04983449517442d9d17b. Accessed November 22, 2015

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PubMed 51 References on Antibiotic Resistance and Herbs. http://www.ncbi.nlm.nih.gov/pubmed/?term=Antibiotic+resistance+herb. Accessed November 21, 2015

DATE: December 9, 2015

TO: American Board of Veterinary Specialties (ABVS)

FROM: Veterinary Cancer Group

Dear ABVS members,

I am writing to you in support of the need for recognition of an additional subspecialty under the umbrella of the ABVS. As an oncologist, I am faced daily with the pet owning public expecting guidance from veterinarians regarding the use of botanicals and other complementary methodologies. When we are deficient in providing this expertise, pet owners will use every and any sources to guide them.

As a board certified doctor, this troubles me. Pet owners are more and more educated regarding the existence of specialists in veterinary medicine. Yet when it comes to the use of botanicals, there currently does not exist board certified specialists to guide them. Currently, any veterinarian can hang out a "shingle" announcing they are a holistic veterinarian, without any advanced training to guarantee a standardized proficiency.

The supplement industry is a multi-billion dollar industry and growing. We cannot lose sight of the fact these products are *drugs*. There is no regulation regarding the safety and appropriate use of these products. When pet owners are unable to get guidance, they purchase these botanicals and use them, sometimes to the detriment to their pet's health. Some pet owners may be reluctant to share they are self medicating, leading to the potential for dangerous interactions of supplements and pharmaceuticals. We know the use of high doses of antioxidants will negate the effects of chemotherapy and radiation, for instance. Specialists in botanical medicine would help the pet owning public to choose manufacturers that produce pure, high quality products and use them in the safest, most appropriate ways.

Our Veterinary Oath is based on safeguarding animal health and welfare. To this end, we need to advance our scientific knowledge regarding the use of botanicals for supportive care and treatment of animals. The best way to do this is to recognize the College of Veterinary Botanical Medicine.

Please do not hesitate to contact me if you have additional questions.

Sincerely,

Mona Rosenberg, DVM, DACVIM (Oncology)
Chief of Staff
Veterinary Cancer Group
www.vetcancergroup.com

DATE: November 23, 2015

TO: American Board of Veterinary Specialties (ABVS)

FROM: Chris Bessent, DVM, MSOM, Dipl.OM, L.Ac.
CEO HerbsmithRX

Dear ABVS members,

I support the petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Herbal medicine is of increasing interest to pet owners, pet professionals, and researchers. The veterinary or pet markets strongly follow the lead of the human supplement markets. Just as humans are seeking "natural - less side effect "options for themselves they are also seeking similar options for their pets. Many herbal formulas and herbs are sold in veterinary practices, over the counter and via the internet and veterinarians need to be versed in herbs to safely guide pet owners. As the ultimate authority in the care of animals, veterinarians must become proficient in the use of botanicals where clients request it, but more importantly, to help develop alternatives in an era of antibiotic resistance and other emerging medical challenges

I am a Veterinarian, have a Masters degree in Oriental Medicine, am a Diplomat of Oriental Medicine (Herbal medicine and Acupuncture) for use in humans by the NCCAOM, (National Certification Commission for Acupuncture and Oriental Medicine) and Licensed Acupuncturist. The NCCAOM - Herbal Medicine in humans recognizes the standard of care and the importance of training and the diplomat status. I recommend the ABVS do the same via the American College of Veterinary Botanical Medicine (ACVBM) diplomat program.

The American Veterinary Medical Association's policy on complementary, alternative, and integrative veterinary medicine (of which herbal medicine is considered a part) states that "veterinarians should have the requisite knowledge and skills for every treatment modality they consider using." The mission of the ACVBM is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomat status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill a much-needed role for our profession.

With the rise of herbal medicines used in veterinary practice worldwide, advanced training is now being offered. The College of Integrative Veterinary Medicine (online C.E. based in Australia) and the Chi Institute (Florida, USA) both offer Masters level training in specialty herb practice. Still, there is no recognized group of experts with such in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. These experts are consulted regularly by veterinarians and representatives of industry and government for information regarding appropriate use of botanicals in animals. I support their petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,

Chris Bessent, D.V.M, M.S.O.M., Dipl. O.M., L.A.C

DATE: December 21, 2015

TO: American Board of Veterinary Specialties (ABVS)

Dear ABVS members,

I am writing to support the need for quality, practitioner-level botanical medicine education for veterinarians. Herbs are increasingly being used by pet owners, trainers and breeders. Veterinarians must be educated to understand both the benefits and concerns of these practices.

As an owner of an herbal products company that makes its products for humans, we are constantly being asked questions by pet owners, professionals and vets. The need for high quality, clinically based information is critical.

Those involved with animals are already using herbs. They want to understand the alternatives; the veterinary practice must keep pace with these changes. Herbal medicine can offer excellent support in normalizing body systems, especially in the area of digestion, discomfort, stress, skin health, urinary tract & kidney health, liver health, cardiovascular system and Immune system.

Herbal medicine is critically important in treating feedstock animals raised organically. Livestock owners without access to appropriate herbal medicine put their investment and right livelihood at risk.

Many vets have been learning about Asian herbal formulas through their acupuncture education, but they need a deeper understanding of how the components of these formulas work. When they truly understand how these formulas work, they are able to know when to add in additional support to increase effectiveness for an individual's case.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine. As such, the ACVBM proposes to fulfill this much-needed role for veterinarians.

Sincerely,

Elizabeth K. Lambert
CEO – Herbalist & Alchemist, Inc.

March 15, 2016

TO: American Board of Veterinary Specialties (ABVS)
Dear ABVS members,

We write to you today to ask that you consider recognition of botanical medicine as a specialty organization of the AVMA.

Our personal experience with our 10 year old Briard who was diagnosed with osteosarcoma, in a very difficult anatomical site, led us to the creation of a "dream team" comprised of extraordinarily gifted and caring practitioners from both Western and Eastern disciplines. This team of oncology radiology and nutritional specialties along with a master herbalist and acupuncture practitioner forged a care plan that allowed for the best possible quality of life, reduction in pain, and no loss of hair for our beloved family member.

So strong is our hope that others can benefit from this individualized and integrative approach, that we have endowed both The DeeDee Arrison Holistic and Integrative Wellness Seminar Series at Cornell University and The May Arrison Fund for Acupuncture.

Our goal is to combine the best of both worlds of medicine, to truly allow for complementary, integrative, and individualized treatment options for all veterinary clients and to increase the size of the tool box from which practicing veterinarians can draw to create the best outcomes for each of their patients.

The mission of the American College of Veterinary Botanical Medicine (ACVBM) is to increase the proficiency and competence of veterinarians in the use of medicinal plants, ultimately leading to diplomate status in the specialty of veterinary botanical medicine. This would be a great step forward and a real help for animals now and in the future.

Sincerely yours,

35 Lincoln Parkway Buffalo, NY 14222 716-881-1761 karrison@mac.com

School of Nursing and Dental Hygiene
Department of Nursing

UNIVERSITY of HAWAI'I®
MĀNOA

December 13, 2015

Dear American Board of Veterinary Specialties Board:

I am writing to support the recognition of the American College of Veterinary Botanical Medicine (ACVBM) as a specialty organization of the American Board of Veterinary Specialties.

Professional recognition is imperative to maintain highest standards in today's climate for veterinarian services as so many unfounded claims for pet care exist on the Internet. Unsuspecting consumers often read the "testimonials" of these websites and fall prey to unsupported claims. This occurs at the expense of the pet owner, but more so, it ultimately costs the health and life of their pet family members.

Academic centers of excellence provide high quality, innovative academic-based care and advising that will enable vets and consumers to make sound decisions regarding educational, personal, and professional goals for the treatment of animals. The ACVBM is one organization diligently working towards making the goal of becoming an academic center for the advanced study of plant medicines a reality.

Recognition by the American Board of Veterinary Specialties will not only help veterinarians, but the animal feed and supplements industry by providing veterinarians who may be employed by such companies with better training and understanding how to utilize the safe use of herbs as feed supplements, herbal remedies to treat conditions, improve health and resistance to diseases.

I have been an internationally recognized advanced practice nurse and university professor nurse for over 35 years. Veterinary care is no different from human health care. The need for professional recognition of the ACVBM is similar to the credentialing and professional recognition required of health care professionals.

The bottom line is – do you want a quack taking care of you? The recognition of the ACVBM is no different from the professional recognition purposes for human health care. The ACVBM is seeking to assure the safety of veterinary practice and the public by:

- Standardizing training protocols and some of the treatment protocols, so that there is confidence that a veterinarian is well versed in veterinary botanical medicine, as a veterinarian treating animals and giving advice to the clients
- Assuring the referral of veterinarians and clients are made to a well-trained veterinarian in this area of medicine
- Assuring the lecturer giving a presentation is well trained in the subject being presented

2528 McCarthy Mall Honolulu, Hawai'i 96822-2233

Today's "do it yourself" trends are geometrically on the rise:

- The public demand for alternative therapies has caused many to seek Dr. Google for advice which can be faulty and even dangerous
- The public demand for herbal remedies has caused many to seek the internet for advice on how to use them, many times from non-veterinarian sources

Professional recognition of the ACVBM would provide the hallmark for educated speakers to help conventional veterinarians understand how alternative therapies work and be able to adjust their treatment regiments. In the era of evidence-based medicine, there is no excuse for the replication of traditional untested treatments which may compromise the health of animals, safety and quality of veterinary care.

Furthermore, we are now at a crossroad where public knowledge of alternative medicine is increasing and conventional veterinarians often ridicule pet owners causing them to seek information elsewhere or a worse case occurs – the client does not tell their veterinarian what they are doing. This may complicate treatment plans or the unthinkable – cause damage at the expense of the animal's life.

I strongly support the ACVBM's diligence in working towards creating an academic center for the advanced study of plant medicines. Safe veterinary practices are the cornerstone of high-quality animal health care. Much of the work in establishing the bases for reducing adverse animal health outcomes lay in the objectives of the ACVBM:

1. Establishing requirements for post-doctoral education and experience pre-requisite to certification in the specialty of veterinary botanical medicine
2. Providing programs of required study including: phytochemistry, phytopharmacology, pharmacognasy, ethnopharmacology, ethnoveterinary medicine, traditional and cultural uses of herbal medicines, traditional/oriental medicine & western medicine herbology
3. Supporting scientific research in phytochemistry, phytopharmacology, phytopharmacodynamics, and toxicology
4. Examining and certifying veterinarians as specialists in Botanical Veterinary Medicine.

With the rise of herbal medicines used in veterinary practice worldwide, advanced training is now a societal requirement. There is no recognized group of experts with such in-depth knowledge to whom the profession and industry can turn for help in advancing the practice and research base on herbal veterinary medicine, or for assisting pet owners in the responsible use of herbs for their pets.

The charter diplomats of the ACVBM represent veterinarians who are recognized specialists in herbal medicine. These experts are consulted regularly by veterinarians and representatives of industry and government for information regarding appropriate use of botanicals in animals.

I support the ACVBM petition to obtain recognition of botanical medicine as a specialty organization of the AVMA.

Sincerely,

Alice M. Tse, PhD, RN, APRN, FAAN
Associate Professor of Nursing

22. January. 2016

TO: American Board of Veterinary Specialties (ABVS)

FROM: Department of Veterinary Medicine, College of Bioresource Sciences,
NIHON UNIVERSITY

Dear ABVS members,

I am writing to support the use of botanical medicine within veterinary medicine. Botanical medicine can be used to treat most conditions recognized by conventional medicine and many that are not. Botanical medicines are often prescribed to treat conditions for which there is no diagnosis or treatment available or in cases where conventional medical treatment has failed or is contraindicated.

Botanical medicines are especially helpful in the treatment of organ failure, chronic and geriatric diseases and cancer. They are often used to relieve pain, help protect and restore internal organ function, strengthen and support the immune system and reduce the dosage and frequency of conventional medications and to reduce their side effects.

Often a botanical and conventional medical prescription will be used in an integrative approach for an enhanced therapeutic effect. It is important to have veterinarians properly educated in the use of botanical medicines to promote safety and efficacy, avoid side effects of herbal-drug combinations and promote better treatment outcomes.

Organic producers are requesting botanical medications in lieu of antibiotics due to public demand for antibiotic free meat, milk and eggs. Also, to prevent the development of antibiotic resistant organisms from antibiotic overuse.

According to surveys done by the National Center for Complementary and Alternative Medicine (NCCAM), the use of botanical medications is steadily rising in the human sector and by extension more people are requesting botanical medications to treat their pets in addition to themselves.

Due to these demands, it is incumbent upon the veterinary profession to educate veterinarians in the proper use of botanical medications, in order to safely prescribe them as a primary therapy or in combination with conventional drugs, reduce the incidence of adverse effects, and to educate clients and the veterinary profession as a whole on the safe use of botanical medicines.

Sincerely Yours,

Hiroshi Koie

Dr Hiroshi KOIE, DVM, Ph.D

DATE: December 19th, 2015

TO: American Board of Veterinary Specialties (ABVS)

FROM: Ryan Guldenpfennig, DVM

Dear ABVS members,

I am writing to support the use of botanical medicine within veterinary medicine. Botanical medicine can be used to treat most conditions recognized by conventional medicine and many that are not. Botanical medicines are often prescribed to treat conditions for which there is no diagnosis or treatment available or in cases where conventional medical treatment has failed or is contraindicated.

Botanical medicines may be helpful in the treatment of organ failure, chronic and geriatric diseases and cancer. They are often used to relieve pain, help protect and restore internal organ function, strengthen and support the immune system and reduce the dosage and frequency of conventional medications and to reduce their side effects.

Often a botanical and conventional medical prescription will be used in an integrative approach for an enhanced therapeutic effect. It is important to have veterinarians properly educated in the use of botanical medicines to promote safety and efficacy, avoid side effects of herbal-drug combinations and promote better treatment outcomes. Many veterinarians are asked about botanicals as therapeutic adjuncts but they are unfamiliar with their use. A reliable and consistent source would benefit inquiring veterinarians and their clients.

According to surveys done by the National Center for Complementary and Alternative Medicine (NCCAM), the use of botanical medications is steadily rising in the human sector and by extension more people are requesting botanical medications to treat their pets in addition to themselves.

Due to these demands, it is incumbent upon the veterinary profession to educate veterinarians in the proper use of botanical medications, in order to safely prescribe them as a primary therapy or in combination with conventional drugs, reduce the incidence of adverse effects, and to educate clients and the veterinary profession as a whole on the safe use of botanical medicines.

Sincerely Yours,

Dr. Ryan Guldenpfennig, DVM
Iowa State University, Class of 2007

25-January-2016

Bruce Ferguson, DVM, MS
www.naturalvet.org
naturalvet@earthlink.net
(904)-540-2795

To whom it may concern,

This is a letter of support for Botanical Medicine to be accepted as a valid and reliable practice in the Western Biomedical veterinary community today. This would mean recognition by the AVMA of the efficacy and usefulness of Botanical Medicine. I feel that this recognition should come soon.

Please let me share a little of my background to put this issue in perspective. I received both my Master of Science in Animal Behavior and my Doctorate of Veterinary Medicine degrees from the University of Florida. While attending the university, one of my older brothers was enrolled in the College of Pharmacy where he received his PharmD. I am a very curious person by nature, and found myself reading books from his courses quite often. I was very intrigued by one book, in particular, that was titled Pharmacognosy. For those of you unfamiliar with Pharmacognosy, it is the study medicinal drugs derived from the herbs. As many of you know, over 70% of our modern drugs are directly or indirectly derived from herbs. They are generally derived from what are known as secondary defensive compounds in plants (e.g. cardiac glycosides) which the plants have apparently evolved in their tissues to protect them against consumption by herbivores.

Why am I mentioning this now? Simply because plants MUST HAVE pharmacological activity for there to be the possibility of deriving drugs from them. And if plants DO have such pharmacological activity, then the plants in their natural or semi-processed states would seemingly be candidates for both further research and use as drug-like agents in veterinary practice. To me, at least, this seems like a completely logical conclusion from the previous brief discussion.

Are my comments just a Botanical Medicine fantasy? For the last decade I have been an adjunct senior lecturer at Murdoch University College of Veterinary and Biomedical Sciences, an AVMA-recognized school in Perth, Western Australia. I also had a clinical practice in Murdoch's Veterinary Teaching Hospital for 7 years, in which I practiced Traditional Chinese Veterinary Medicine (TCVM). The main treatment tools in my practice were Acupuncture and Herbal Medicine. My practice was busy, successful, and accepted by my Western Biomedical-practice colleagues because I had excellent results with some of the most complex cases that we see in Veterinary Teaching hospitals such as cancer, immune-mediated diseases, chronic infections, fever of unknown origin, chronic immune deficiency, chronic arthropathies, etc. Again, for emphasis, my tools were acupuncture and botanical medicines.

Did I use plants from my backyard to treat my patients? I would like to say that I am a "down-to-earth" herbalist with my hands in the soils, but I am not. What is true is that there are large, well-regulated industries which procure, test, process, and sell herbal products for both human and veterinary practice. And Australia, which has some of the strictest biosecurity laws on the planet, has approved many of these products for importation and use in Australia.

The manufacturers of the best products test the incoming raw herbs after having trained experts check the actual materials for their botanical identity. They then use mass spectography and gas

chromatography procedures to identity “fingerprints” or biomarkers of the most important pharmacologically active ingredients to assure themselves that the raw product will be useful when processed. The processing is a highly controlled procedure done in “clean rooms” under very closely observed conditions. Samples are saved and may be used for future investigation if there is any problem with the final manufactured product. The Botanical Medicines are, in fact, treated in a similar fashion as our most prized pharmacological agents used by Western Biomedical practitioners.

All of this is to say three rather simple things. First, it should be clear to everyone involved that many plants contain compounds that have pharmacological activity. And this pharmacological activity may be taken advantage of by utilizing plant products in Veterinary practice. This use, if by properly trained veterinarians in correctly diagnosed patients with quality products, can be at least as powerful and commonly more safe than the use of western biomedical drugs. Lastly, there already exists a well-regulated and highly respected industry of Botanical product manufacturers. The Botanical product industry makes the product, the animals can benefit from use of the product, and if trained veterinarians are available and supported by the AVMA, these Botanical products will be utilized and investigated by scientists such as ourselves. If we fail to grasp this opportunity, then such use will surely fall into the hands of the poorly-trained lay practitioner.

If you have any questions or comments, please feel free to contact me.

Sincerely,

Bruce Ferguson

Bruce Ferguson, DVM, MS

Appendix 3 Industry Support: Dairy

Northeast Organic Dairy Producers Alliance

January 21, 2016

Dr. Signe Beebe
AVMA

Dear Dr. Signe Beebe,

On behalf of the Northeast Organic Dairy Producers Alliance (NODPA) and the Midwest Organic Dairy Producers Alliance (MODPA) we are writing to you to express our enthusiastic support for the creation of the American College of Veterinary Botanical Medicine. There is a great need for this specialty within the organic farming community

NODPA is the largest organic dairy producer organization in the country with a membership of eight hundred and thirty six organic dairy producers in the Eastern US. NODPA's mission is to "**enable organic dairy family farmers, situated across an extensive area, to have informed discussion about matters critical to the well being of the organic dairy industry as a whole**." NODPA is not aligned with any one processor or cooperative and therefore is able to represent the views and needs of many different farmers.

MODPA represents organic dairy producers in WI, MN, ND, SD, IA, NE, KS, MO, IL, and MI with the mission to promote communication and networking for the betterment of all Midwest organic dairy producers and enhance a sustainable pay price.

In the experience of our organic dairy farmers learning to manage herd health issues within the confines of organic management is a very steep learning curve. Our farmers will always be grateful to the pioneering vets in this field who helped our farmers with our herd health problems and emergencies through internet lists, phone calls, and a few excellent books.

NODPA's Mission
To enable organic dairy family farmers, situated across an extensive area, to have informed discussion about matters critical to the well-being of the organic dairy industry as a whole.

30 Keets Rd, Deerfield, MA 01342 Tel: 413-772-0444 Fax: 866-554-94863
Email: ednodpa@comcast.net Website: www.nodpa.com

The truth is that most of our farmers live in areas that are simply not served by veterinarians with interest or skills in botanical/holistic medicine, especially in large animal practices. When there is an animal with a health issue, our farmers call their vets to diagnose the issue, then they explain to our farmers what they would normally prescribe or suggest on a conventional dairy. Our farmers are left to decide between the conventional treatment (most often this would be using drugs that are not allowed in organic production like antibiotics or steroids), or something else. But it is usually up to the farmer to research what that "something else" is to be.

One of the greatest possible outcomes would be that the newly formed College for Veterinary Botanical Medicine and its members and graduates could serve as a real source of information and experience. They could be the consultants for veterinarians not experienced in alternative forms of treatment --- so that there is better information used in determining the best treatment for an ailing animal.

Organic dairy is certainly a growing industry, with increases in sales of between 7-9 % per year and we believe this need will only continue to expend.

We strongly support the new American College of Veterinary Botanical Medicine as a board recognized specialty.

Thank you for your consideration. Please contact Ed Maltby if you require any more information.

Sincerely

Liz Bowden

NODPA Board Chair and New York organic dairy farmer

Darlene C Coehoorn

Darlene Coehoorn, MODPA President

Ed Maltby

NODPA Executive Director

Northeast Organic Dairy Producers Alliance

January 21, 2016

Dr. Signe Beebe
AVMA

Dear Dr. Signe Beebe,

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In the experience of our organic dairy farmers learning to manage herd health issues within the confines of organic management is a very steep learning curve. Our farmers will always be grateful to the pioneering vets in this field who helped our farmers with our herd health problems and emergencies through internet lists, phone calls, and a few excellent books.

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30 Keets Rd, Deerfield, MA 01342 Tel: 413-772-0444 Fax: 866-554-94863
Email: ednodpa@comcast.net Website: www.nodpa.com

The truth is that most of our farmers live in areas that are simply not served by veterinarians with interest or skills in botanical/holistic medicine, especially in large animal practices. When there is an animal with a health issue, our farmers call their vets to diagnose the issue, then they explain to our farmers what they would normally prescribe or suggest on a conventional dairy. Our farmers are left to decide between the conventional treatment (most often this would be using drugs that are not allowed in organic production like antibiotics or steroids), or something else. But it is usually up to the farmer to research what that "something else" is to be.

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Organic dairy is certainly a growing industry, with increases in sales of between 7-9 % per year and we believe this need will only continue to expend.

We strongly support the new American College of Veterinary Botanical Medicine as a board recognized specialty.

Thank you for your consideration. Please contact Ed Maltby if you require any more information.

Sincerely

Liz Bowden

NODPA Board Chair and New York organic dairy farmer

Darlene C Coehoorn

Darlene Coehoorn, MODPA President

Ed Maltby

NODPA Executive Director

Pam Moore <moohillfarm@verizon.net> Thu, Jun 18, 2015 at 11:02 PM
To: Signe Beebe <sebeebe@gmail.com>
Hello Dr. Beebe,

I am writing in support of the new American College of Veterinary Botanical Medicine as a board recognized specialty.

We own and operate a certified organic farm and over the last 20 years have used a variety of botanicals for the health and well-being of our livestock.* There is significant and growing need for this expertise by natural and organic-minded livestock producers, especially among conventional dairy farmers transitioning to organic certification who've long relied upon allopathic medicine and fast-acting pharmaceuticals. Complementary veterinary medicine needs to keep pace with the steadily rising demand for organic dairy and livestock products, as well as with increasing consumer awareness and expectations regarding animal welfare on all farms.

A continuing challenge that dates back to our certification in 1996 is the need for holistic veterinary care and expertise that is consistent with our natural approach to farming, and ideally is supported by evolving organic, grassfed and animal welfare standards. In the early years we were largely on our own for livestock health care, relying upon a limited selection of foreign books to help us manage our organic dairy and beef herd. Thankfully in the last decade additional veterinary texts have been published in this country and internet access has made online resources available, but none of this takes the place of veterinarians coming out to the farm to see and treat their patients. Many animals would likely have remained on our farm and in organic production if we were fortunate enough to have an "organic vet" in our region all these years.

The closest "alternative farm vets" are several hours away and don't serve our rural area. We are fortunate to have a veterinary center that is "open to alternatives" and has taken an interest in serving the organic community (due in part to the large number of certified organic dairies in their service area), but their expertise is limited. They've told us vets in their practice would attend training to broaden this understanding if education credits were available and they didn't have to travel halfway across the country to get them.

Our farm is located near Cornell's Veterinary School, where we understand there is growing interest among students in complementary approaches - but they are not taught. We're told that students determined to learn them must seek out alternative practitioners to intern with, but that there aren't enough such vets available. As the number of large animal veterinarians continues to decline we need to do more to nurture and meet the needs of those veterinary students whose interests extend to herbal and alternative therapies.

Botanicals are an excellent alternative to the pervasive dependance and overuse of antibiotics and other drugs in the dairy and livestock industries. A strong economic case for farmers can be made for the reduced withholding times on botanicals vs. pharmaceuticals. There will be significant public benefit if botanicals can replace more of the antibiotic use in the livestock sector. Cattle in particular respond well to herbal treatments.

Please let us know what more we can do to support the efforts of the new ACVBM.

Thank you,
Pam Moore
Moore Farms
Nichols NY

* including: St. Johnswort, Echinacea, Belladonna, Cedar, Peppermint, Cinnamon, Geranium, Thyme, Lemongrass, Rosemary, Calendula, Comfrey, Turmeric, Goldenseal, Hypericum, Aloe Vera, Garlic, Cayenne, White Willow, Chamomile, Fennel, Tea Tree, Eucalyptus, Apple, Kelp, Pulsatilla, Blue Cohosh, Wintergreen, Clove, Yucca, Black Walnut, Eye Bright

Dayna Locitzer <daynalocitzer@gmail.com>
8:41 AM (11 hours ago)
6-17-15

Hello,

I am writing to express my support for the American College of Veterinary Botanical Medicine. I am a herdsman at an an organic dairy farm in New York, Chaseholm Farm, where use of botanicals has been integral to our treatment protocols. Because we do not have access to antibiotics, steroids, and hormones, botanicals play an important role in the health of the herd. For example, my most favorite treatment I use is yarrow and goldenseal powder to heal the tissue when a cow has an abscess. Yarrow acts to heal the tissue while goldenseal is an antiseptic to prevent the infection from spreading. Another botanical we use often is oregano oil. This is used on top of their grain when they are fighting off infections like mastitis or pneumonia because it acts as an antibiotic. This past winter, we had a pneumonia outbreak and all the cows in the barn received herbal tinctures to help stave off the disease. They received tinctures to help with their immune system as well as to provide lung support. Most importantly, when a cow has mastitis we give them an oral garlic tincture treatment and that has been the most successful treatment for mastitis we have seen. The more veterinarians in support of this, the more successful organic dairy farming can be.
Organic Dairy Farms need the support of the veterinarians involved in the American College of Veterinary Medicine because we trust their advice and their experience and it is important for these veterinarian to have support.
Please consider this email in your decision making process

Thank You

APPENDIX IV Specialty Organising Committee Members

1. Signe Beebe Current Chair of the ACVBM
2. Ihor Basko current President-Elect of the ACVBM
3. Shauna Cantwell
4. Carmen Colitz
5. Connie DiNatale current President of the ACVBM
6. Barbara Fougere Vice-President of the ACVBM
7. Joyce Harman Board member of the ACVBM
8. Hubert Karreman
9. Cynthia Lankenau Secretary/Treasurer of the ACVBM
10. Steve Marsden Board member of the ACVBM
11. Richard Palmquist
12. Donna Raditic
13. Nancy Scanlan
14. Rob Silver
15. Susan Wynn advisor to the ACVBM Board
16. Huisheng Xie Board member of the ACVBM

APPENDIX V Curriculum Vitae of the ACVBM's Organizing committee

(Note, some of these CVs have been abridged to fit two pages each, full CVS are available on request)

Signe E Beebe DVM

Integrative Veterinary Center
5524 Elvas Avenue
Sacramento, CA 95819
DOB 4/25/57

Education

- 2007 Chi Institute of Chinese Veterinary Medicine, Reddick, FL, Chinese Veterinary Herbology
- 2006 University of Pretoria South Africa, Centre for Wildlife Management, South Africa, Ecosystems and Wildlife Management Course
- 2006 Chi Institute of Chinese Veterinary Medicine, Reddick, FL, Veterinary Tuina
- 2005 Chi Institute of Chinese Veterinary Medicine, Reddick, FL, 7 th Annual TCVM conference, Chinese Veterinary Food Therapy
- 2004 University Of Tennessee School of Veterinary medicine, Canine Rehabilitation and Physical Therapy course, Knoxville, TN
- 2002-2003 Chinese Veterinary Herbal Medicine Course (basic and advanced), Albuquerque NM
- 2000-2001 Chi Institute of Chinese Veterinary Medicine, Reddick, FL, Veterinary Acupuncture
- 1988 Purdue University School of Veterinary Medicine, DVM. W. Lafayette, IN
- 1981-1982 Kansas State University, Major Animal Science & Industry, Manhattan, KS
- 1981 University of Hawaii AA, Oahu, Hawaii

Professional Activities

Lecturer/ Instructor since 2000 for numerous organizations and institutions including most recently:

- 2016 10 th Annual China Medical Chinese Veterinary Medicine Seminars, Basel, Switzerland
- 2016 Qi Academy, Gangelt, Germany
- 2015 ACVBM and the AHVMA Augusta, Georgia
- 2015 Annual IVAS Congress on Herbal medicine Nova Scotia, Canada
- 2015 Lotus Institute of Integrative Medicine, City of Industry California
- 2015 Annual China Medical Chinese Veterinary Medicine Seminars, Basel, Switzerland
- 2014 Annual China Medical Chinese Veterinary Medicine Seminars, Basel, Switzerland
- 2014 Western Veterinary Conference, Las Vegas, Nevada
- 2013 AHVMA Kansas City, Kansas
- 2013 International Symposium on Veterinary Chinese Herbology, Bioethicus Institute and University Sao Paulo State, Botucatu, Brazil
- 2013 Annual China Medical Chinese Veterinary Medicine Seminar: China Medical, Basel, Switzerland

Scientific Organizations

American Veterinary Medical Association

Professional Organizations

- World Association of Traditional Chinese Medicine Board Member
- American Journal of Traditional Chinese Veterinary Medicine Associate editor
- LOTUS Institute of Integrative Medicine, faculty
- Veterinary Botanical Medical Association
- American Holistic Veterinary Medical Association
- Institute of Traditional Chinese Medicine

Offices held

- Current Chair, American College of Veterinary Botanical Medicine
- 2015 President, American College of Veterinary Botanical Medicine
- 2014-2015, President, American College of Veterinary Botanical Medicine

Bibilography (selected)

- 2014 Beebe S., Small Animal Chinese Endocrinology in: Practical Guide to Traditional
- Chinese Veterinary Medicine, Vol 2-Small Animals, Chi Institute Press, Reddick, Fla
  2011 Beebe S, Salewski M, Chen J, Chen T, Chinese Herbal Formulas for Veterinarians, City of Industry, California: Art of Medicine Press
- 2010 Beebe, S, Traditional Chinese Medicine Treatment of Anemia, American Journal of Traditional Chinese Medicine, Vol.5 No.1, February 2010
- 2009 Beebe, S, Treatment of Congestive Heart Failure with Conventional Pharmaceuticals plus Acupuncture, Chinese Herbal Medicine and Food Therapy in a Toy Poodle Dog, American Journal of Traditional Chinese Medicine, Vol.4 No.2, August
- 2008 Beebe, S, Chinese Veterinary Medicine Approach to Chronic Urinary Tract Infection, American Journal of Traditional Chinese Medicine, Vol.3 No.1, August 2008
- 2006 Beebe S, Salewski M, Monda L, Scott J. Clinical Handbook of Chinese Veterinary Herbal Medicine. Placitas, New Mexico: Herbal Medicine Press

Ihor John Basko DVM

6240 Helena Lane
Kapaa, HI 96746
DOB 2/13/1947

Education

- Wayne State University (Biology Major) 1965-1967
- Michigan State University Veterinary School 1968-1971 (DVM)
- University of California Los Angeles (Comparative Eastern Philosophy 1972-1973)
- Institute of Traditional Medicine Santa Cruz, CA (1984-1985) Completed course in Chinese Herbology
- The Tao Foundation San Francisco. (1981-1982) Completed course in Chinese Herbology
- Academy of Pain Control Research San Francisco, CA (1977-1978) Completed course in Pain Management

Professional Activities

- Small Animal and Equine Clinical Practices (1971-current)
    - All Creatures Great & Small Veterinary Services Kapaa, HI
    - Surf Paws Animal Hospital Honolulu, HI
- Herbal and Nutritional Consultant (Industry and Private Sector)
- Lecturer and Educator (Veterinarians, Pet Industry & Pet Parents)
- Research Consultant: Herbs and Supplements
- Heart Research Team / Bypass surgeries & Mitral Valve replacement
- Thoracic surgery training program/ MSU / Lansing General Hospital Lansing, MI (1970 – 1971**)**
- Author

Scientific Organizations

- American Veterinary Medical Association
- Hawaii State Veterinary Medical Association

Professional Organizations

- American Holistic Veterinary Medical Association
- Veterinary Botanical Medical Association

Honors

- Herbal Educator of the Year Award 2014 VBMA
- The Dr. Carvel Tiekert Life Achievement Award 2014 AHVMA
- Holistic Veterinarian of the Year 1998 AHVMA

Offices held

- Current President-Elect of the ACVBM
- Board of Directors: Veterinary Botanical Medical Association (2010- current)
- Council of Elders / American Holistic Veterinary Medical Association (1998- 2013)
- Research Advisory Board / National Animal Supplements Council (2003-2006)
- President of the Veterinary Botanical Medical Association (2001-2003)
- Board of Directors: American Holistic Veterinary Association (1999-2001)
- President / Hawaii State Veterinary Medical Association (1998-1999)
- Board of Directors / Hawaii State Veterinary Medical Association (1993 –1998)

Bibilography (Selected)

- Healing Your Horse: Alternative Therapies (co –author) 1993
- Veterinary Acupuncture: Ancient to Modern Medicine Vol I & II (co- author) 1994 & 2000
- Cooking For Da Hawaiian Kine Dog 1999
- Fresh Food & Ancient Wisdom: Preparing Healthy Meals For Your Dogs 2010

Shauna L Cantwell DVM MVSc Diplomate ACVAA

Medicine Wheel Veterinary Services, Inc
2775 NW 49th Ave, #205-359, Ocala FL 34482

Education

- Master of Veterinary Science Residency in Veterinary Anesthesiology 1995-1998 Western College of Veterinary Medicine University of Saskatchewan, Canada
- Doctor of Veterinary Medicine Western College of Veterinary Medicine 1985-1989 University of Saskatchewan, Saskatoon, SK, Canada
- Zoology (Honors Program - Cell Biology and Physiology) 1982-1985 (degree deferred for entrance to WCVM) University of Alberta, Edmonton, AB, Canada

Professional Activities

- Medicine Wheel Veterinary Services, Inc (SLCantwell, DVM) 2007-present
- Courtesy Appointment, Dept of Small Animal Clinical Sciences 2008-present College of Veterinary Medicine, University of Florida
- Courtesy Appointment in Dept of Anesthesia 2004-present College of Medicine, University of Florida
- Veterinary Orthopedics and Sports Medicine Hospital 2008-2009 Baltimore, MD. Consultant for Anesthesiology, Pain Management and Complementary Medicine
- Scientific publication review 1997-2013 J Acupunc and Meridian Studies, British J Anaesthesiology, Res Vet Sc, Vet Anesthesia and Analgesia, Am J TCVM, J Zoo Med and Wildlife, Can Vet J
- Lecturer 1996-2016 Midwest Veterinary Conference; Chi Institute; University of Florida CVM; St. George's University; Ross University School of Veterinary Medicine; Oregon State University; Western College of Veterinary Medicine and others
- Academic supervisor and student mentor 1999-2008 in anesthesia
- Always Helpful Veterinary Services (JMShoemaker, DVM) 2007-2009 American Holistic Veterinary Medical Conference; North American Veterinary Conference Complementary Medicine, Equine and Small animals (0.5 FTE) Nottingham, PA
- Clinical Assistant Professor, Anesthesiology and Pain Mgmt 2000-2008 (2007-2008 0.25 FTE) College of Veterinary Medicine, University of Florida
- Lecturer, Anesthesiology 1998-2000 College of Veterinary Medicine, University of Florida
- Various Small animal and mixed practices Manitoba 1989-1995

Scientific Organizations

- Diplomate of American College of Veterinary Anesthesiology and Analgesia
- American Veterinary Medical Association
- Florida Veterinary Medical Association and Licensure
- Florida Association of Equine Practitioners
- Pennsylvania Veterinary Medical Licensure
- Maryland Veterinary Medical Licensure
- South Carolina Veterinary Medical Licensure
- International Veterinary Academy of Pain Management

Professional Organizations

- American Veterinary Chiropractic Association
- American Holistic Veterinary Medical Association
- American College Veterinary Botanical Medicine

Honors

- ACVA/ANESCO Resident Research Award 1996

- NSERC Summer Studentship 1984
- Dean's Honor Role, University of Alberta 1983

Offices held (Selection)

- Current member of the ACVBM
- Current AHVMA Research Committee
- Current ACCAC Examination Committee
- Current Courtesy Appointment with College of Medicine, Dept Anesthesiology
- AAVA Organizing Committee for AVMA 2013
- AVCA Accreditation Commission

Bibilography (Selected)

Cantwell SL. Mechanism of Acupuncture Analgesia. In Egger, Love and Doherty (Eds), Pain Management in Veterinary Practice. Wiley-Blackwell. 2013.

Cantwell SL, Sapp HL, Kim MS, Xie H, Brooks DE,. Electro-acupuncture and cold laser acupuncture to decrease intraocular pressure in Rhesus monkeys with chronic glaucoma. Manuscript in preparation

Cantwell SL. Traditional Chinese Medicine: The Mechanism and Management of Acupuncture for Chronic Pain. Topics in Comp An Med. Feb:25(1): 53-58, 2010

CV Fiorello, M Cunningham, SL Cantwell, J Levy, E Neer, K Conley, P Rist. Diagnosis and treatment of presumptive post obstructive pulmonary edema in a Florida panther. JZooWildlMed. Jun;38(2):317-22 2007

Cantwell SL, Brooks DE, Xie H, Sapp HL. Electro-acupuncture to decrease intraocular pressure in Rhesus monkeys with chronic glaucoma. (abstract ARVO 2007, Fort Lauderdale, Fl) IOVS 2007;48:ARVO E-abstract 1281

Cantwell SL, Noble M. Investigation into the effect of acupuncture to prevent the development of low blood pressure after the administration of acepromazine and morphine to healthy surgical patients. Manuscript in preparation.

Cantwell SL. Pain Management III: Ancillary Therapies. In Seymore and Duke (Ed) BSAVA Manual of Canine and Feline Anaesthesia and Analgesia, 2nd Ed. 2007.

Cantwell SL, Robertson S. Equine Pain Management. In Auer JA and Stick JA (Ed) Equine Surgery. 3rd Ed, Saunders, 2005.

Valverde A, Cantwell S, Hernandez J, Brotherson C. Effects of Acepromazine on the Incidence of Vomiting Associated with Opioid Administration in Dogs. Veterinary Anaesthesia and Analgesia, 30:1-6, 2003

CARMEN MARIA HELENA COLITZ, DVM, PhD, Diplomate ACVO

Education

- The University of Paris, Paris, France, Summer 1985 Cours de Civilisation Française de la Sorbonne
- The University of Florida, Gainesville, FL, 1985 to 1988. Associate of Arts – 1986
- The University of Tennessee, Knoxville, TN, Spring 1989. Undergraduate
- The University of Tennessee, Knoxville, TN, 1989 to 1993. Doctor of Veterinary Medicine, May 1993
- The University of Tennessee, Knoxville, TN, 1993 to 1996. Doctor of Philosophy in Comparative and Experimental Medicine, 1996
- North Carolina State University, Raleigh, NC, 1996 to 1999. Residency, Comparative Ophthalmology and Post-Doctoral Research Associate

Professional Activities

- Co-owner: Jupiter Pet Emergency and Specialty Center November 2012 to present
- Owner: All Animal Eye Care, Inc January 2013 to present
- Principle, Animal Health Quest Solutions, LLC 2007 to present
- Sole Proprietor, Aquatic Animal Eye Care, LLC Consulting Veterinary Ophthalmologist 2009 to December 2012
- Consulting or Surgical Veterinary Ophthalmologist for z0os and aquariums
- Courtesy Faculty Appointment, Small Animal Clinical Sciences, Aquatic Animal Health
- Adjunct Associate Professor Raleigh, North Carolina 2007 to present
- Veterinary Ophthalmologist West Palm Beach, Florida, January 2007 to December 2009
- Assistant Professor, Comparative Ophthalmology Columbus, Ohio, October 2001 to November 2006
- Assistant Professor, Comparative Ophthalmology September 1999 to September 2001 Louisiana State University School of Veterinary Medicine
- Resident, Comparative Ophthalmology/ Post-Doctoral Research Associate July 1996-99 North Carolina State University College of Veterinary Medicine
- Post Doctoral Research Trainee Fellowship July 1993 to June 1996
- Small Animal Veterinarian August 1993 to June 1996

Scientific Organizations

- American Board of Veterinary Ophthalmologists, Member of the Board, 2012 to 2014.
- Association for Research in Vision and Ophthalmology, 1991- 2012.
- The American Veterinary Medical Association, 1993- present.
- American College of Veterinary Ophthalmology, Diplomate, 1999-present.
- American College of Veterinary Ophthalmology, Member of the Board of Regents, 2006 to 2012, President (2010-2011).
- International Society of Eye Research, 2002 – 2006.
- Veterinary Ophthalmology Editorial Board, 2004 – present.
- International Association for Aquatic Animal Medicine, 2006 - present.
- International Marine Animal Trainers' Association, 2009 – present.

Professional Organizations

- American Holistic Veterinary Medical Association

Honors

- Herbal Educator of the Year Award 2014 VBMA
- The Dr. Carvel Tiekert Life Achievement Award 2014 AHVMA

- Holistic Veterinarian of the Year 1998 AHVMA

Offices held

- OSU Search Committee for Small Animal Neurologist, 2006 to 2007.
- OSU Department of Veterinary Clinical Sciences Department Chair, 2006 to present, filled, Dr. Rustin Moore, start date November 2006.
- OSU Graduate Studies Committee, 2004-present.
- OSU Search Committee for Food Animal Faculty Position Summer 2003.
- OSU Search Committee for Small Animal Internal Medicine Faculty Position 2003-2004.
- Editorial Board Member or Ad Hoc Journal Review for:
    - Veterinary Ophthalmology, 1999 - present.
    - Comparative and Veterinary Oncology, 2006.
    - Experimental Eye Research, 2000 - present
    - Veterinary Pathology, 2002 - present.
    - American Journal of Veterinary Research, 2004 - present.
    - Veterinary Medicine, 2005 to present.
    - Journal of the American Veterinary Medical Association, 2005 to present.
    - Graefe's Archives of Clinical and Experimental Ophthalmology, 2005 to present.
    - Clinician's Briefs, 2007 to present.
    - The Veterinary Journal, 2007 to present.
    - Current Eye Research, 2009 to present.
    - Molecular Vision, 2009 to present.

Bibilography (selected)

- 78 Publications or Dissertations including:
    - D. Williams, A. Fitchie, C. Colitz. An oral antioxidant formulation delaying and potentially reversing canine diabetic cataract: A Placebo Controlled Masked Pilot Study. Int J Diabetes and Clin Res 2015 2:1-5.
    - D.L. Williams, C.M.H. Colitz. An Oral antioxidant formulation preventing and reversing canine diabetic cataract: a placebo controlled masked trial. In review PLOS One, 2014
    - J. Mejia-Fava, C.M. Colitz, Supplements for exotic pets. Vet Clin North Am Exot Anim Pract 2014 Sep; 17(3):503-525.
    - C.K. Hu, Y.J. Lee, C.M. Colitz, C.J. Chang, C.T. Lin. The protective effects of Lycium barbarum and Chysanthemum moifolum on diabetic retinopathies in rats. Veterinary Ophthalmology. 2012, Sep;15 Suppl 2:65-71.
    - C.M.H. Colitz, Terri McCalla. Antioxidants for Ocular Health. Integrative Veterinary Care. August, 2011.C.A. Barden, H.L. Chandler, P.Lu, J.A. Bomser, C.M.H. Colitz. The Effect of Grape Polyphenols on Oxidative Stress in Canine Lens Epithelial Cells. American Journal of Veterinary Research, 2008 Jan;69(1):94-100.
- 20 Book Chapters
- 134 Proceedings and Abstracts

CONNIE DINATALE DVM

742 Clay Street
Winter Park, FL 32789

Education

- Doctor of Veterinary Medicine 1992, University of Florida, Gainesville, FL
- Bachelor of Science, Microbiology 1987, University of Florida, Gainesville, FL
- Herbal Training Course 1999, Chi Institute

Professional Activities

- Practitioner, founder, owner Winter Park 1999- Present
- Associate Allen Shoen and Associates; Osceola Animal Clinic, Tony Weiratha 1993-1999
- Research
    - 1991 - 1992, Comparison of Colostrum-specific Gravity to IgG Content in Goat Milk Under Michelle LeBlanc, D.V.M., University of Florida1985 - 1987,
    - Small Colon Resection and Anastomosis Surgical Comparisons Under Alan Nixon, D.V.M. and Reid Hanson, D.V.M. University of Florida
    - 1985 - 1988, Research Assistant for Large Animal Surgical Sciences
- Lecturer/ Instructor
    - Chi Institute Clinical Applications of Herbal Medicine1999 – Present
    - Qi Academy (Germany) 2016
    - American Holistic Veterinary Medical Association 2013, 2012
    - North American Veterinary Conference 2004, 2007
    - AVMA Conference 2006

Scientific Organizations

- Central Florida Veterinary Medical Association
- American Veterinary Medicine Association

Professional Organizations

- American Holistic Veterinary Medical Association
- Veterinary Botanical Medical Association Central
- American College Veterinary Botanical Medicine

Offices held

- Current President of the ACVBM

Bibilography (Selected)

- Application of Chinese Herbal Medicine in Small Animal Practice, Xie's Veterinary Herbology Blackwell, 2010.
- Cancer treatment in geriatric animals, *Am J Trad Chi Vet Med* 7,1:55, 2012
- TCVM for Geriatric Medicine Practical Guide to TCVM-Small Animals, Chi Inst Press, 2014

BARBARA FOUGERE BSc BVMS (Hons)

College Integrative Veterinary Therapies Pty Lt trading as All Natural Vet Care
PO Box 474 Rozelle 2039 NSW 292 Lyons Rd Russell Lea 2046 NSW
DOB 10 September 1964

Education

- Masters Health Science (Herbal Medicine) (University New England 2005)
- Masters Organisational Development and Training (UNE/ Charles Sturt University 1995)
- Graduate Diploma Social Science (University New England 1994)
- Graduate Diploma of Business Management (University new England 1992)
- Graduate Diploma Phytotherapy (Australian College Phytotherapy/University new England 2004)
- Bachelor of Health Science Complementary Medicine (Charles Stuart University 2004)
- Bachelor of Veterinary Medicine and Surgery (Hons) (Murdoch University Veterinary School 1986)
- Bachelor of Science (Murdoch University Veterinary School 1984)

Professional Activities

- Current Vice President American College of Veterinary Botanical Medicine
- Current Board member Veterinary Botanical Medicine Association
- Past President International Veterinary Acupuncture Association
- Practitioner and Director Integrative Veterinary Services trading as All Natural Vet Care Veterinary Hospital which is an offsite training center for Sydney University Veterinary School for student rotations
- Principal and Director of the College of Integrative Veterinary Therapies – registered training organization
    - Teaching 2 year post graduate accredited courses in Veterinary Chinese Herbal Medicine and Veterinary Western Herbal Medicine
- Current external reviewer safety efficacy of complementary medicines Australian Pesticides and Veterinary Medicines Authority (Canberra)
- Lecturer WSAVA, New York Veterinary Conference, NAVC, AHVMA, VIN, AVA, IHS
- Adjunct Alabama Veterinary School Elective in Herbal Medicine

Scientific Organizations

- Chartered Member of the Australian Veterinary Association

Professional Organizations

- National Herbalists Association Australia (Registered Human Herbalist)
- American Holistic Veterinary Medicine Association
- Australian Integrative Veterinary Group
- Australian Holistic Veterinary Group
- Australian College of Nutritional and Environmental Medicine
- American Botanical Council
- College Integrative Veterinary Therapies
- Veterinary Botanical Medicine Association

Honors

- 2010 Practitioner of the Year Award 2010 (AHVMA) for services to Integrative Veterinary Medicine.
- 2011 Educator of the Year Award 2011 (AHVMA) for education in Integrative Medicine.

- Outstanding Leadership Award by the World Association of Traditional Chinese Veterinary Medicine, (2015)

Offices held

- Current Vice-President of the American College of Veterinary Botanical Medicine; Chair 2014-2015
- President Veterinary Botanical Medicine Association 2004, 2005, 2006 current International representative and board member.
- Board Member Australian Holistic Veterinarians 2000-2009.
- Board member Feline Health Research Council Australia 2003-2012
- Policy Council Member for the Australian Veterinary Association 1995-2003
- Therapeutic Advisory Committee veterinary medicines 2000-2004
- Reviewer for the Australian Veterinary Journal (current)

Bibilography (Selected)

- Fougere B, Wynn S Ed Text Book of Veterinary Herbal Medicine 2006
- Fougere B, in Goldstein's Integrating Alternative Medicine into Veterinary Practice 2008
- Fougere B, in Encyclopedia of Animal Behaviour 2010
- Child G, Foster DJ, Fougere BJ, Milan JM, Rozmanec M. Ataxia and paralysis in cats in Australia associated with exposure to an imported gamma-irradiated commercial dry food. Aust Vet J. 2009 Sep;87(9):349-51.
- McGreevy PD, Fougere B, Collins H, Bartimore KM, thoson PC, Effect of declining owned-cat population on veterinary practices in Sydney Aust Vet J 2002 Dec 80 (12) 740-5.

JOYCE HARMEN DVM MRCVS

1214 North Poes Rd.
Flint Hill, VA 22627
Date of Birth: June 22, 1955

Education

- BS 1980 University of Georgia, Animal and Dairy Science
- DVM 1984 Virginia Maryland Regional College of Veterinary Medicine
- MRCVS 1985 Member, Royal College of Veterinary Surgeons University of Bristol College of Veterinary Medicine, England

Professional Activities

- Instructor, lecturer Equine Herbal Medicine College of Integrative Veterinary Therapies 2013-Present
- Instructor, lecture Chi Institute 2002-2014 Reddick, FL
- Teaching/ Nutrition/Herb Company Veterinary Institute for Integrative Medicine
- 1995-present Boulder, Colorado
- CAVM Equine Practice Harmany Equine Clinic, Ltd. 1990 – Present Flint Hill, Virginia
- Equine Practice Dr. B. Furlong & Associates 1987 – 1989 Oldwick, New Jersey
- Research Veterinarian Bioengineering Isler 1986 – 1987 Zurich, Switzerland
- Equine Practice 1986 - 1986 Somerton Veterinary Hospital Curragh, Co. Kildare, Ireland
- Veterinary Research Assistan 1984 – 1986 Animal Health Trust, Suffolk, England

Scientific Organizations

- American Veterinary Medical Association
- American Association of Equine Practitioners

Professional Organizations

- American Holistic Veterinary Medical Association
- College Integrative Veterinary Therapies
- Veterinary Botanical Medicine Association

Offices held

- Current Board Member ACVBM
- 1992 - 1994: Chairman, American Association of Equine Practitioners, Subcommittee on Therapy Options
- 1996 - 1999 Board of Directors, Association of Equine Sports Medicine
- 1996 - 2000 Board of Directors, American Holistic Veterinary Medical Association
- 1996 - 2009 Veterinary Advisory Board, Animal Ambassadors and TTEAM
- 1998 - 1999 President, American Holistic Veterinary Medical Association
- 1999-2000 Member, American Veterinary Medical Association Task Force on CAVM
- 2006-present American Journal of Traditional Chinese Veterinary Medicine advisory board
- 1999-2010 Editorial Board, The Horse (magazine)
- 2004-present Veterinary Advisor to Board, The Holistic Horse (magazine)
- 2005-2007: Board of Directors, Veterinary Botanical Medical Association
- 2006-2007: President, Veterinary Botanical Medical Association

Bibilography (Selected)

- Glycogen repletion following different diets. RC. Harris, JC Harman, DJ Marlin, and DH Snow. Second International Conference on Equine Exercise Physiology, San Diego, 1986.
- Computerized Saddle Fitting. JC Harman. Pferdeheilkunde. 12(6):1996.
- Complementary (alternative) therapies for poor performance, back problems, and lameness. In Current Vet Therapy, ed Robinson, NE. WB Saunders Co, Philadelphia, PA. 1997: 131-137.
- Holistic Approach to Equine Practice. Harman, JC. In Complementary and Alternative Veterinary Medicine: Principles and Practice. Ed: Allen M. Schoen, DVM, Susan G. Wynn, DVM. Mosby, St. Louis, 1998 (601)
- Rehabilitation and Training. Harman JC and Ridgeway K. in Back Problems in Horses, ed K Haussler. Vet Clin N Am. 15:1, 263-280, 1999.
- The Role of Nutritional Therapy in the Treatment of Equine Cushing's Syndrome and Laminitis. JC Harman and M Ward. Alternative Medicine Review, 6:Suppl: 2001. pp S4-S16.
- The Toxicology of Herbs in Equine Practice. JC Harman. Clinical Techniques in Equine Practice. 1:2, 2002. (74-80).
- Herbs and Alternatives in Equine Practice. JC Harman. In Alternative Practices for Livestock., ed Morris TF, Keilty MT. Blackwell Publishing, Ltd, Ames, 2006.
- Herbal Medicine in Equine Practice. Harman, JC. Ed: Wynn S, Fougere B. Veterinary Herbal Medicine. Mosby Elsevier, St Louis, 2007.
- Long-Term Follow-up of Seizures in Three Horses Treated with Chinese Herbal Therapy. American Journal of Traditional Chinese Veterinary Medicine. V3:1. (48-53).
- Clinical Findings and TCVM Treatment of Equine Insulin Resistance Associated with Spleen Qi Deficieny with Damp Heat Accumulation. American Journal of Traditional Chinese Veterinary Medicine. V8:2, (67-71).
- Equine Endocrine and Metabolic Diseases. Wallis, C and Harman, JC. In Practical Guide to Traditional Chinese Veterinary Medicine-Equine. Ed Xie H, Crisman C. 2015.

HUBERT KARREMAN VMD

555 Red Hill Road
Narvon, PA 17555
DOB 9/25/62

Education

- 2008-2009 University of Pennsylvania School of Medicine, Certified Clinical Research
- 1991-1995 University of Pennsylvania School of Veterinary Medicine V.M.D
- 1980-1984 University of New Hampshire College of Life Sciences B.S.

Professional Activities

- 9/13-present The Rodale Institute
    - Conduct research studies with organic dairy farms in region
    - Conduct organic veterinary animal health classes
    - Partner with collaborators on pasture projects
    - Make presentations domestically and internationally
- 2010-present Bovinity Health, Lancaster PA & beyond
    - Natural treatment & products for non-antibiotic treatment of infectious disease
    - Part-time practice in medical treatment of certified organic cattle using non-antibiotic treatments against infectious disease and non-hormonal treatments against infertility via botanical derivatives, biological immuno-modulation, acupuncture, low dose therapeutics and hands-on physical therapy as needed.
    - Educational seminars for veterinary schools & organizations, farmer organizations, with optional in-barn instruction
- 6/95 – 12/09 Veterinary practitioner, full-time: Lancaster County, PA
    - Developed an effective non-antibiotic treatment of infectious disease in dairy cattle by utilizing botanicals and biologics
    - Integrative medicine techniques applied daily: botanical derivatives, biological immuno-modulation, acupuncture, low dose therapeutics and conventional approaches as each case requires.
    - Successful completion of 2005 SARE grant project ONE05-042 to analyze trends and associated management on organic farms using DHIA records in PA.

Scientific Organizations

- American Veterinary Medical Association
- American Association of Bovine Practitioners

Professional Organizations

- Veterinary Botanical Medicine Association

Honors

- 9/2007- 5/2009 Affiliate Assistant Professor, Dept of Animal and Nutritional Sciences, University of New Hampshire, Durham, NH

Offices held

- 1/2005- 1/2010 USDA National Organic Standards Board, Chair of Livestock Committee. Write recommendations for regulatory implementation by USDA.
- 5/99-3/01 AVMA Task Force on Alternative and Complementary Medicine, representing Food Animal sector to develop standards for CAVM within AVMA

Bibilography (Selected)

- Basics: Livestock, Industry Stats and Regulatory Aspects; Conventional and Alternative Treatments Allowable For Use With Organic Livestock; Utility of a Multi-Prong Approach to Treating Organic Livestock (Proceedings) Wisconsin Veterinary Medical Association (Madison, WI) (Oct. 2013)
- The Non-Antibiotic Treatment of Infectious Disease. Proceedings 1st Organic Congress of the East Black Sea (Kelkit, Turkey) (June 2013)
- P. Pinedo, H. Karreman, H. Bothe, J. Velez. Efficacy of a Botanical Preparation for the Intramammary Treatment of Clinical Mastitis on an Organic Dairy Farm. Canadian Veterinary Journal 2013 Mar; 54(5):479-484.
- Hubert J. Karreman. The Barn Guide to Treating Dairy Cows Naturally Acres USA, Austin, TX, 2011. (Soft cover, 191 pgs).
- Karreman, H J. (2010) Disease control on organic and natural cattle operations. Animal Health Research Reviews 10(2); 121–124. Proceedings: American Association of Bovine Practitioners (Albuquerque, NM) (Aug 2010)
- Hubert J. Karreman. Treating Dairy Cows Naturally: Thoughts and Strategies 2nd ed. Acres USA, Austin, TX, 2007 (Hardcover, 412 pgs).
- Current Veterinary Therapy, Food Animal Practice (Chapter 100: Therapeutic Options in Organic Livestock Medicine) (Anderson and Rings, eds.) Elsevier, 2008.
- Veterinary Herbal Medicine (Chapter 22: Phytotherapy for Dairy Cows) (Wynn and Fougere, eds.) Mosby, 2007.
- Alternative Livestock Health Practices (Chapter 9: Livestock Links From the Past to Holistic Alternatives of the Present) (Morrison and Kielty, eds.) Blackwell Scientific, 2005.

CYNTHIA JEAN LANKENAU DVM Grad Dip VCHM Grad Dip VWHM

9002 Sunset Drive,
Colden, NY 14033
DOB 11/30/1956

Education

- Cornell University; Agriculture and Life Sciences College; 1974-1977
- Cornell University, College of Veterinary Medicine: 1977-1981; Doctorate of Veterinary Medicine; (DVM, graduated with distinction)
- Herbal training in Chinese Medicine: 1995-1997 through the International Veterinary Acupuncture Society
- Chi Institute: Chinese Herbal Training Class: 2002-2003
- Sage Mountain Herbal Studies: 1994-2004: Herbal Practitioner
- College of Integrative Veterinary Therapies: Graduate Diploma Veterinary Chinese Herbal Medicine: 2009-2011
- College of Integrative Veterinary Therapies: 2012- 2015; Grad Dip Veterinary Western Herbal Medicine

Professional Activities

- 1981-1984: Private Practitioner: Dairy Practice in Boonville, NY
- 1984-1985: Government District Officer: Peace Corps, Malawi, Africa; District Veterinary Officer
- 1985-1993: Private Practitioner: Mixed Animal Practitioner in Middleport, NY
- 1993-now: Private Practitioner: 100 % alternative modality Practice: Holistic Center for Veterinary Care, Colden, NY
- 2015-now: lecturer for the College of Veterinary Integrative Therapies
- Registered Professional Herbalist by the American Herbal Guild RH(AHG)
- Lecturer:
    - Northeast Organic Farmer's Organization; 1995, 1996, 2014
    - Student Holistic Group at Cornell University College of Veterinary Medicine: 2013 and 2010.
    - Western NY Veterinary Medical Association: Herb-Drug Interactions; 2007.
    - International Herbal Symposium, Wheaton College, Norton, MA; 2013, 2011, 2013
    - IVAS and AHVMA: 2003, 2006, 2009, 2011, 2013; SUNY at Buffalo; Mini-vet school; 2008, 2010
    - World Association of Traditional Chinese Veterinary Medicine, 2016

Scientific Organizations

- American Veterinary Medical Association
- New York State Veterinary Medical Society

Professional Organizations

- Veterinary Botanical Medicine Association
- College Integrative Veterinary Therapies
- American Holistic Veterinary Medical Association

Honors

- Outstanding Leadership Award by the World Association of Traditional Chinese Veterinary Medicine, (2015)
- Excellent Speaker Award, by the World Association of Traditional Chinese Veterinary Medicine, Beijing, China, 2016

Offices held

- American College of Veterinary Botanical Medicine: secretary-treasurer (2014-now)
- Veterinary Botanical Medicine Association: board member (2008-2015) current past president
- American Holistic Veterinary Medical Association: on the Council of Elders (2007-now)
- New York State Veterinary Medical Society: committee head of Complementary and Alternative Medicine: (2012-now)
- New York Complementary and Alternative Medicine Association: founding Board Member: (2012-now)
- World Association of Traditional Chinese Veterinary Medicine (WATCVM): board member (2013-now)
- Peer reviewer of Herbal articles for RAIVE:2014

Bibilography (Selected)

- Lankenau, C.; Acute Tendinitis Treated with Chinese Medicine, Journal of the Veterinary Botanical Medical Association, Summer, 2014, PP13-17
- Lankenau, C.; Herbal Support in Ferrets with Cancer; Journal of AHVMA; Spring 2014, pp. 42-46.
- Lankenau, C: YooHoo: A Case of a Retained Pathogen: JVBMA, February, 2014; p.6-13
- Lankenau, C; The Treatment of an Equine with Osteomyelitis with a Chinese Herbal Formula, Xian Fang Huo Ming Yin; JAHVMA, 2012 pp. 47-50.
- Lankenau, C; Treatment of Mastitis in a Jersey Cow with Chinese Herbal Medicine, JVBMA; March, 2011 pp.57-62
- Lankenau, C; Idiopathic Hepatobiliary Disease in an Equine treated with Chinese Herbal Medicine, JVBMA; March 2011; pp. 39-48.
- Lankenau, C; Congenital Cardiac Defect in an Equine Supported with Chinese Herbal Medicine, JVBMA; March, 2011; pp. 11-21.
- Lankenau, C; Practice Pearls; from Clinical Cases; JVBMA; March 2011; pp. 11-14.
- Lankenau, C; Zoopharmacognosy at Work; Journal of Integrative Therapies, (4)2014; p.43.
- Lankenau, Cynthia: Herbal Wiki; Veterinary Botanical Medicine Association; Members Only web-site.

Steve Marsden DVM

8215 102 Street
Edmonton Canada
DOB 9/20/1963

Education

- Doctor of Veterinary Medicine, University of Saskatchewan, 1988
- Naturopathic Physician, National College of Natural Medicine, 1999
- Master of Science in Oriental Medicine, National College of Natural Medicine, 1999
- Licensed Acupuncturist, National Certification Commission for Acupuncture and Oriental Medicine (NCCAOM), USA, 1999
- Diplomate of Chinese Herbology, NCCAOM, 1999
- Graduate Diploma Veterinary Chinese Herbal Medicine 2012

Professional Activities

- Practitioner
- Teacher, lecturer internationally numerous conferences
- Faculty Position
    - College of Integrative Veterinary Therapies, Sydney, Australia
        - Director, Instructor
    - National College of Natural Medicine, Portland, Oregon
        - Director Emeritus
        - Adjunct Faculty Member, Department of Classical Chinese Medicine
- Board Member, American College of Veterinary Herbal Medicine

Scientific Organizations

- Canadian Veterinary Medical Association
- Alberta Veterinary Medical Association
- Canadian Naturopathic Doctors Association
- Alberta Association of Naturopathic Practitioners

Professional Organizations

- Veterinary Botanical Medicine Association
- College Integrative Veterinary Therapies
- American Holistic Veterinary Medical Association
- American Herbalist's Guild

Honors

- Small Animal Clinician of the Year, Canadian Veterinary Medical Association, 2009
- Teacher of the Year, American Holistic Veterinary Medical Association, 2010

Offices held

- Board Member ACVBM Current
- Board of Directors, National College of Natural Medicine, Portand OR, 2005-14
    - Treasurer, 2012-14
- Director of Continuing Education
    - Edmonton Association of Small Animal Veterinarians, 1989-1994
    - Alberta Veterinary Medical Assocation, 1989-1992

- Vice-President, Edmonton Humane Society
    - 1989-1994

Bibilography (selected)

- Foundations of Formula Design, AHVMA Press, Maryland, 1999
- New Understandings of the Pathophysiology of Pemphigus Foliaceous, JAHVMA, Jan. 2000
- Manual of Natural Veterinary Medicine (with Susan Wynn), Elsevier, New York, 2003
- Textbook of Veterinary Herbal Medicine, Elsevier, New York, 2006 (contributing author)
- Pain Management in Veterinary Practice, Wiley-Blackwell, in press (contributing author)
- Veterinary Clinics of North America: Cancer, in press (contributing author)

Richard Palmquist, DVM

721 Centinela Ave.
Inglewood, CA 90302

Education

- CSU 1983 DVM
- Grad Dip Veterinary Chinese Herbal Medicine (CIVT 2015)

Professional Activities

- Chief of Integrative Medicine, Centinela Animal Hospital, Inglewood, CA
- Teacher, lecturer internationally
- VIN, Alt Med Folder consultant www.vin.org
- CIVT, Faculty
- Nuova Biologics Research Associate: PVX drug development (canine distemper)
- AHVMF president, Past President (first)
- Western University School of Veterinary Medicine: Faculty as Clinical Preceptor rotation site in small animal integrative medicine as part of the 4th year professional degree program.
- Registry of Approved Integrative Veterinary Education (RAIVE) committee member
- Doctoral advisor. 2009-2010. Research in essential oils usage in canine veterinary medicine. Elizabeth Scott, PhD. South Dakota State University, Dept of Research.

Scientific Organizations

- AVMA
- CVMA
- SCVMA member and past program chairman for Northbay-Westside chapter

Professional Organizations

- Veterinary Botanical Medicine Association
- College Integrative Veterinary Therapies
- American Holistic Veterinary Medical Association
- AHVMA, Board of Directors, Research committee chairperson, Ethics committee member, member, and speaker
- ACVBM
- WATCVM

Honors

- Excellent speaker trophy 18th Annual TCVM Conference in Beijing, China
- COE Peacemaker Award 2012
- UpJohn Award for Proficiency in Small Animal Medicine

Offices held

- 2016 AHVMF, president and research chair
- Council of Elders and Editorial Committee, AHVMA
- AHVMF, Past President and research chair
- AHVMF president, Past President (first)
- World Association of TCVM (WATCVM) Board of Directors

Bibilography (selected)

- A preliminary clinical evaluation of Kibow Biotics®, a probiotic agent, on feline azotemia. JAHVMA. 2006 Jan-Mar; 2 (4):23-27.
- Ranganathan N, Patel BG, Ranganathan P, Dheer R, Palmquist R, Van Engelberg G. Effect of feeding Kibow Biotics to cats and dogs in kidney failure. American Society of Nephrology's 39th Annual Renal Week Meeting. Filename 551914. San Diego, CA, July 2006.
- Palmquist RE. 2006. Seeking reports of behavioral disorders (report of Standard Process Feline Whole Body Support for resolution of chronic plastic eating in a cat), letter to the editor. JAHVMA. July-Sept (25) 2; 42-43.
- Palmquist RE. 2007. The continuum of disease and health: A way to evaluate response to therapy. North American Veterinary Conference. January.
- Palmquist RE. 2007. Improving Evidence: a brief review of the use of clinical case reports in complementary and alternative veterinary medicine. *JAHVMA* , April-June, 26(1):15-21.
- Palmquist RE. Chronic cervical pain and an active scar relieved with essential oils and neuro auricular therapy: a canine case report. JAHVMA. 29(1):16-25.
- Modified Hospice for Canine Cutaneous Hemangiosarcoma. AHVMA 2011 Annual Meeting. San Diego, CA.
- NAVC 2013 - Approaching Integrative Veterinary Medicine for your Practice; Canine Cutaneous Hemangiosarcoma: A potentially new approach; and Translational medicine: A model for advancing medical care. Orlando, Florida.
- Palmquist, RE. 2013. Management of feline chronic gingivostomatitis of suspected viral origin with a thymic extract. JAHVMA 32(Spring):17-24.
- Perspective: Evidence Based Veterinary Medicine, Evidence Based Practice and Translational Medicine In Pioneering Areas. JAHVMA submitted Feb 2014.
- Coauthor, Goldstein R, editor. 2008. *Integrating Complementary Medicine into Veterinary Practice*, Wiley-Blackwell Publishing.

Donna Raditic, DVM DACVN

331 Confederate Drive
Knoxville, TN 37922

Education

- Cornell University Ithaca, New York Bachelor of Science with Honors and Distinction 1982
- Cornell University College of Veterinary Medicine, 1986
- American College of Veterinary Nutrition Alternative Residency Program, 2005-2010
- University of Tennessee College of Veterinary Medicine Residency in Nutrition, 2005-2010
- Bachelor of Science with Honors and Distinctions, 1982

Professional Activities

- Diplomat of America College of Veterinary Nutrition
- Adjunct Assistant Professor, Department of Small Animal Clinical Sciences, University of Tennessee College of Veterinary Medicine, 2008-Present
- Assistant Professor, Department of Small Animal Clinical Sciences, University of Tennessee College of Veterinary Medicine, November 2012-Present
- Medical Director, 2006-2008 VCA All Caring Animal Hospital Great Barrington, MA
- Veterinarian/Owner/Developer, 1997-2006 All Caring Animal Center, Inc. Great Barrington, MA
- Veterinarian, 1986-1997 Berkshire Veterinary Hospital Pittsfield, MA
  Research 2010 Evaluation of three herbal compounds used for management of lower urinary tract disease in cats. University of Tennessee COE grant
- Research The use of botanical therapy. NHV Inc. In progress

Scientific Organizations

- American Veterinary Medical Association
- Massachusetts Veterinary Medical Association
- New York State Veterinary Association
- American Academy of Veterinary Nutrition
- American Society of Parental and Enteral Nutrition
- Metabolomics Society

Professional Organizations

- Veterinary Botanical Medical Association
- CIVT (College of Integrative Veterinary Therapy)
- American Holistic Veterinary Medicine Association

Offices held

- AHVMA Foundation Professional Advisory Board – 2 years

Bibilography (Selected)

- Raditic DM, Nutrition and the food allergy trial for VetPartners.com. Vet Info Network VIN)
- Raditic DM, ELISA testing for common food antigens in four dry dog foods used in dietary elimination trails." *J Anim Phys Anim Nutr*; February 2010
- Raditic DM, Remillard RL. Refeeding Syndrome. In: *Small Animal Clinical Nutrition, 5th Edition.* Eds: Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novotny BJ. Mark Morris Institute:Topeka KS. 2010 pg 455.
- Raditic DM, Combined parenteral and enteral feeding in a cat. In: *Small Animal Clinical*

Nutrition, 5th Edition. Eds: Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novotny BJ. Mark Morris Institute:Topeka KS. 2010 pg 497-8.

- Raditic DM, Regulation of food intake. In: *Small Animal Clinical Nutrition, 5th Edition.* Eds: Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novotny BJ. Mark Morris Institute:Topeka KS. 2010 pg 440.
- Hamper B, Bartges JW, Kirk CA, Lusby A, Murphy M, Raditic DM. The unique nutritional requirements of the cat: a strict carnivore. In: *The Cat: Clinical Medicine and Management*. Little S, ed. Elsevier:St. Louis. 2011. 236-242.
- Lusby A, Bartges JW, Kirk CA, Hamper B, Murphy M, Raditic DM. Nutrition for the normal cat. In: *The Cat: Clinical Medicine and Management*. Little S, ed. Elsevier:St. Louis. 2011. 243-247.
- Bartges JW, Raditic DM, Kirk CA, Lusby A, Hamper B, Murphy M. Nutritional disorders. In: *The Cat: Clinical Medicine and Management*. Little S, ed. Elsevier:St. Louis. 2011. 248-254.
- Bartges JW, Raditic DM, Kirk CA, Lusby A, Hamper B, Murphy M. Nutritional management of diseases. In: *The Cat: Clinical Medicine and Management*. Little S, ed. Elsevier:St. Louis. 2011. 255-288.
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Nancy Scanlon DVM

404 N Mt Shasta Blvd, Rm B,
Mount Shasta CA 96067
DOB 10/21/1946

Education

- BS, University of California, Davis (on a National Scholarship Foundation 4-year scholarship) 1968
- Doctor of Veterinary Medicine, University of California, Davis, 1970
- Higher Education Administration Course, Carnegie Mellon (on a Bush Leadership Fellowship) 1982
- MS in Financial Planning (MSFP), Golden Gate University, 2004
- Currently enrolled in MS in Integrative Cancer Therapy program at University of South Florida medical school

Professional Activities

- Lecturer in CAVM
- RAIVE Committee member
- Practitioner
- Writer for numerous journals and magazines

Scientific Organizations

- California Veterinary Medical Association (delegate to their House of Delegates)
- American Animal Hospital Association
- Southern California Veterinary Medical Association

Professional Organizations

- Veterinary Botanical Medicine Association
- College Integrative Veterinary Therapies
- California Holistic Veterinary Medical Association (founder)
- American Holistic Veterinary Medical Association
- Veterinary Botanical Medical Association

Offices held

- American Holistic Veterinary Medical Association (past board member, past president, past executive director, current delegate to AVMA House of Delegates)
- Veterinary Botanical Medical Association (past board member, past president)
- California Veterinary Medical Association (delegate to their House of Delegates)
- California Holistic Veterinary Medical Association (founder)

Bibilography (Selected)

- Complementary Medicine for Veterinary Technicians and Nurses, Wiley, 2011
- Medical Terminology: Building a Vocabulary 1988
- Editor for: Chinese Herbal Formula for Veterinarians, Art of Medicine Press, 2012

Robert Silver DVM MS

7345 N 63rd St;
Longmont, CO 80503
DOB 03-22-1949

Education

- Colorado State University 1974 BS
- Colorado State University 1976 MS
- Colorado State University 1982 DVM

Professional Activities

- Small animal practice
- Instructor: Animal Health Technology program
- Consultant: Technical Writer (Pet food industry; Animal nutraceutical industry; Veterinary Holistic industry)
- Chief Medical Officer (Animal nutraceutical company)
- Post-graduate education presenter (RACE approved speaker)
-

Professional Organizations

- Veterinary Botanical Medicine Association
- American Holistic Veterinary Medical Association

Offices held

- President, Rocky Mountain Holistic Veterinary Medical Association
- Board of Directors, American Holistic Veterinary Medical Association
- Council of Elders, American Holistic Veterinary Medical Association
- President-elect, Veterinary Botanical Medical Association

Bibilography (Selected)

- "The Consumer Activist and the Pet Food Industry." in Supply Side Animal Nutrition Insights; July 2014. Virgo Health and Nutrition Publications, Phoenix AZ.
- "Emerging Trends in Pet Food Ingredients." in Supply Side Animal Nutrition Insights; September 2013. Virgo Health and Nutrition Publications, Phoenix AZ.
- Introduction to Veterinary Nutraceuticals (Atlantic Coast Veterinary Conference 2014)
- The Hyperpermeable Bowel and Its Relationship to IBD (The North American Veterinary Conference 2014)
- Quality of Life Instruments in the Evaluation of Multifactorial Integrative Veterinary Medical Protocols (IAAHPC 2013)
- Integrative Oncology (Pennsylvania Veterinary Medical Association: Hershey Conference 2013) Using Nutraceuticals to Maintain QoL in Pet Hospice (Atlantic Coast Veterinary Medical Conference 2014)
- Environmental Toxins and Animal Health (Atlantic Coast Veterinary Conference 2014)
- Genetically Modified Foods and Animal Health (Atlantic Coast Veterinary Conference 2014)
- Medicinal Cannabinoids and the Veterinary Patient (American Holistic Veterinary Medical Association 2014)

Susan Wynn DVM, DACVN

BluePearl Veterinary Specialists
455 Abernathy Rd
Sandy Springs, GA 30328
DOB 12/12/1960

Education

- Emory University, Atlanta GA 1978-1982 B.S., biology
- University of Georgia, College of Veterinary Medicine 1983-1987 DVM
- Emory University, Atlanta GA 1993-1996 (post-doctoral fellowship, viral immunology)
- University of Tennessee, College of Veterinary Medicine 2007-2010 Clinical Residency, Small Animal Nutrition
- Chinese Veterinary Herbal Medicine course, Chi Institute, Gainesville, FL 2000-2001
- Basic Medical Herbalism, Living with Herbs Institute, Atlanta, GA 2000-2001
- Professional herbalist (via peer review) by American Herbalist Guild 2002

Professional Activities

- Practitioner, Diplomat Clinical Nutrition Blue Pearl Veterinary Specialists
- 2005 to present, Adjunct Faculty, Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia
- Discussion Board Consultant (Alternative Medicine) Veterinary Information Network 1999 to present
- Faculty and teacher College Integrative Veterinary Therapies post graduate herbal medicine
- Lecturer and teacher numerous conferences

Scientific Organizations

- American College of Veterinary Nutrition
- American Veterinary Medical Association
- Georgia Veterinary Medical Association
- American Academy of Veterinary Nutrition

Professional Organizations

- Veterinary Botanical Medicine Association
- College Integrative Veterinary Therapies
- Veterinary Botanical Medicine Association (founder and lifetime member)
- American Herbalist Guild

Honors

- 2008, National Academy of Sciences, Ad hoc reviewer for *Safety of Dietary Supplements for Horses, Dogs and Cats*
- 1998, Office of Alternative Medicine, National Institutes of Health, Ad Hoc Review Group
- 1997, Behavioral and Neurosciences Special Emphasis Panel, National Eye Institute, Ad Hoc Review Group
- *Expert Panel Membership*
- 2004, A Model for Regulating Animal Medical Treatments by Non-veterinarians**,** Coalition on the Scope of Veterinary Practice, American Society of Veterinary Medical Association Executives, Lakewood, CO.
- 2000, Closed meeting for Advanced Research in Complementary and Alternative Medicine, Wellcome Trust, London

Offices held

- Current Advisor to the ACVBM Board
- 2012-2014: President, VetHeart of Georgia
- 2010 to 2016, District Director, Georgia Veterinary Medical Association
- 2006-2007, President, American Holistic Veterinary Medical Association
- 2000-2004 Secretary/Treasurer, American Academy of Veterinary Nutrition
- 2001 to 2006, founder and executive director, Veterinary Botanical Medicine Association
- 1995 to 2002, Founder, President, Executive Director, Georgia Holistic Veterinary Medical Association

Bibilography (Selected)

- Susan G. Wynn, Angela L. Witzel, Joseph W. Bartges, Tamberlyn S. Moyers, Claudia A. Kirk. Prevalence of asymptomatic urinary tract infections in morbidly obese dogs. PeerJ. 2016 Mar 14;4:e1711.
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Huisheng Xie DVM, MS PhD

Department of Small Animal Clinical Sciences
PO Box 100126 HSC
College of Veterinary Medicine
University of Florida
Gainesville, FL 32610
DOB 10/7/1963

Education

- 1983 Bachelor of Science Degree in Veterinary Medicine (equivalent to DVM), Sichuan College of Animal Sciences and Veterinary Medicine, Rongchang, Sichuan Province, PR China
- 1988 Masters Degree, Veterinary Acupuncture, at the College of Veterinary Medicine, Beijing Agricultural University, PR China
- PhD, University of Florida, Gainesville, FL "Acupuncture for pain control in horses and its mechanism"

Professional Activities

- 1983-1988 Lecturer and Staff Veterinarian: the Beijing Agricultural University, College of Veterinary Medicine. PR China
- 1988-1991 Assistant Professor and Staff Veterinarian: the Beijing Agricultural University, College of Veterinary Medicine. PR China
- 1992-1994 Associate Professor and Staff Veterinarian: the Beijing Agricultural University, College of Veterinary Medicine. PR China
- 1994-1998 Research Fellow Department of Animal Science, University of Florida
- 1999-2004 Lecturer, College of Veterinary Medicine, University of Florida
- 2005-2008 Clinical Assistant Professor, College of Veterinary Medicine, University of Florida
- 2009-now Clinical Associate Professor, College of Veterinary Medicine, University of Florida

Scientific Organizations

- American Association of Equine Practitioner (AAEP)
- American Veterinary Medical Association (AVMA)

Professional Organizations

- American Association of Traditional Chinese Veterinary Medicine (AATCVM)
- China Society of Traditional Chinese Veterinary Medicine, CHINA

Honors (A selection)

- 2014: Fu-xi Award (the highest honor) from the Chinese Association of Traditional Veterinary Science (CATVS), Taiwan
- 2014: Excellent Leadership Award for the World Association of Traditional Chinese Veterinary Medicine (WATCVM), Hebei, China
- 2013: Excellent Speaker Award for the 15th Annual International TCVM conference, World Association of Traditional Chinese Veterinary Medicine (WATCVM), September 11-15, 2013, El Escorial, Madrid, Spain
- 2013: Honorary professorship from South China Agricultural University, Guang-zhou, China
- 2012: Year of the Holistic Teacher from AHVMA, Birmingham, USA
- 2006 Honorary Professorship from Southwest University, Chong-qing, China

Offices held

- Advisor to the ACVBM Board
- American Association of Traditional Chinese Veterinary Medicine (AATCVM) Co-founder and Executive Director from 2006-now
- China Society of Traditional Chinese Veterinary Medicine, CHINAAdvisory Board Director from 2000-now
- Reviewer Journal of the American Veterinary Medical Association (JAVMA) from 2003-now
- Reviewer American Journal of Traditional Chinese Veterinary Medicine (also Executive Director) from 2006-2008

Bibilography (selected)

- Chrisman CL, Xie H. Conventional and Traditional Chinese Veterinary Medicine Perspectives of Estrus, Fertility and Infertility. American Journal of Traditional Chinese Veterinary Medicine. 9(1): 1-6, 2014
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- Xie H, Pasteur C, Smith L. TCVM for Equine Lameness in Horses-Diagnosis and Treatment. Integrative Veterinary Care. Summer issue: 54-58, 2013
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- Shmalberg, J, Xie H. Acupuncture and Chinese Herbal Medicines for Treating Horses. Compendium: Continuing Education for Veterinarians. May 2011 E1-E11
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- Atria S, Carson E, Xie H. Acupuncture and Chinese Herbal Medicine Treatment of Eighteen Florida Horses with Anhidrosis. American Journal of Traditional Chinese Veterinary Medicine. 5(2):25-36, 2010
- Xie H. How to Use TCVM for the Treatment of Thoracolumbar Intervertebral Disc Disease. In: Yang & Xie (Ed): Traditional Chinese Veterinary Medicine-Empirical Techniques to Scientific Validation. Jing-tang Publishing, Reddick:Florida. Page 183-190, 2010.

The written examination will consist of four (4) parts.

I. A general botanical medicine
II. Principles and practices
III. Clinical botanical medicine
IV. Botanical identification of 20 medicinal plants.

The examination shall consist of 100 or more questions for sections I-III in a multiple choice format with one correct answer and four distractors. Three (3) minutes will be allotted for each question. Depending on the number of questions, the total examination time may vary . The contents of the examination will be determined by the Credentials and Education Committee. Components of the examination will test Western or Chinese botanical medicine.

A proposed outline to be further developed by the Exam Committee is as follows:

Section I

- Pharmacognosy
- Phytochemistry
- Pharmacology
- Manufacture
- Dispensing
- Quality
- Safety
- Toxicology
- Pharmacy issues with herbs

Section II

- History and Philosophy
- Ethnoveterinary medicine
- Principles and Practices
- Contemporary herbal medicine issues (residues, conservation, safety)
- Herb Drug Interactions
- Botanical medicine actions
- Herbal Therapeutic strategies across all systems
- Materia medica knowledge

Section III

- Current research
- Case based botanical medicine across species:
    - Equine
    - Canine & Feline
    - Bovine & Production
- Dosing and Case management
- Herb Drug Interactions

Section IV

- Medicinal Plant Identification using organoleptic techniques

Appendix VII Training Programs currently Available

Current Training Programs available in Veterinary Chinese and Western Herbal Medicine range from short courses through to Post Graduate Degrees.

VBMA

- 16 basic to advanced webinar Topics in veterinary herbal medicine

VIN

- 16 hours Intermediate Herbal Medicine
- 12 hours Introduction to Herbal Medicine
- 10 hours Introduction to Veterinary Chinese Herbal Medicine
- 6 hours Using herbs for Liver and Kidney Disease

Chi

- 155 hour Chinese Veterinary Herbal Medicine Program approved by a majority of state boards, provides training Veterinary Chinese Herbalist and this contributes towards a Masters degree in Traditional Chinese Veterinary Medicine.

IVAS

- 160 hours Veterinary Chinese Herbal Medicine training
- 500 hours Advanced Veterinary Chinese Herbal Medicine training

CIVT

- 1305 hours Graduate Diploma Veterinary Chinese Herbal Medicine (Grad Dip VCHM) accredited post graduate degree competency based training
- 945 hours Graduate Diploma Veterinary Western Herbal Medicine (Grad Dip VWHM) accredited post graduate degree competency based training
- 500 hours Foundation Course in Western Veterinary Herbal Medicine
- 445 hours Advanced Western Veterinary Herbal Medicine
- 120 hours Foundation Course Veterinary Chinese Herbal Medicine
- 40 hours Getting Started Veterinary Chinese Herbal Medicine Introductory course
- 24 hours Essentials of Western Veterinary Herbal Medicine

Samples of Programs :

Chi Institute Veterinary Herbal Medicine Program

The Chinese Herbal Medicine Program is a 155 hour CE Program (Including the Intro to Herbal Medicine course) (approved by a majority of state boards) that trains students in Veterinary Chinese Herbal Medicine. It is presented in five modules, each covering different sets of organ systems and their affiliated health disorders. Each module offers five hours of wet lab, approved by many state boards. All five modules are offered each year, and can be taken either online or on-site. The modules are:

- Respiratory/Cardiovascular
- Gastrointestinal/Spleen
- Liver/Endocrinology
- Kidney/Urinary/Reproductive/Geriatric
- Dermatology/Oncology/Immune-mediated Diseases

The course is intended for small, mixed, and equine vets, and both small and large animals (mostly dogs and horses) are used during case studies and wet labs. General topics covered in each module include herbal medicine, a TCVM approach to Western diseases, and advanced TCVM theories and principles.

For further information:

http://www.tcvm.com/Programs/AdvancedPrograms/HerbalMedicine/Syllabus.aspx

IVAS Herbal Training Programs

IVAS Chinese Herbal Medicine Training

The IVAS Course in VCHM curriculum enables students to gain skills and knowledge in the principles of Veterinary Chinese herbal medicine, including materia medica, classic formula study, dispensary, herb drug interactions and herbal therapeutics with an emphasis on integrative approaches, biomedical understandings of formula and case based learning. Assessment includes case analysis assessment, submission of three case reports and multiple choice assessments. Support is provided by experienced veterinary Chinese herbal practitioners.

The IVAS Course curriculum covers a minimum of 100 herbs and a focused group of about 30 key classical formulas that can be modified, enabling a small pharmacy that meets the main needs of veterinary practice. Attention is paid to avoiding herbs and formulas composed of animal parts or endangered species and knowing relevant substitutions that are acceptable.

The 160 hour IVAS Course is RACE approved for 100 hours and trains graduates to be able to:

1. Explain the basis of how Chinese herbs work from a traditional medicine basis and from a biomedical and pharmacological perspective.
2. Explain how Chinese herbal products are made and therefore be able to ascertain quality issues
3. Take a history, conduct and interpret the necessary diagnostic procedures in order to make a TCM diagnosis by integrating patterns of disharmony with etiological factors and pathological processes and how these aspects interconnect - along with the Western diagnosis
4. Elucidate the treatment principles needed for patients and therefore be able to select an appropriate strategy based upon the actions of Chinese herbal medicine

5. Have the practical skills to dispense Chinese herbal medicine and integrate them with conventional medications
6. Monitor the patient's condition as a result of treatment, re-evaluate diagnostic information and differential diagnosis and modify treatment strategy as the patient's condition changes over time.
7. Be aware of safety issues including the potential for side effects (know how to interpret side effects)
8. Be able to integrate Chinese medicine principles with conventional veterinary medicine with emphasis on potential herb drug interactions and manage the risk.
9. Be aware of professional codes of ethics and the practice of veterinary Chinese herbal medicine
10. Be aware of significant research issues

For Further Information:

www.ivas.org

IVAS Advanced Chinese Herbal Medicine Training

The IVAS Advanced Course in VCHM curriculum enables students to gain advanced skills and knowledge in the principles of Veterinary Chinese herbal medicine, including materia medica, classic formula study, dispensary, herb drug interactions and herbal therapeutics with an emphasis on integrative approaches, biomedical understandings of formula and case based learning. Assessment includes case analysis assessment, submission of fifteen case reports and multiple choice assessments. Support is provided by experienced veterinary Chinese herbal practitioners.

The IVAS Course curriculum covers therapeutic strategies and some 80 key classical formulas. Attention is paid to avoiding herbs and formulas composed of animal parts or endangered species and knowing relevant substitutions that are acceptable.

The 500 hour IVAS Course trains graduates to be able to:

1. Explain the basis of how Chinese herbs work from a traditional medicine basis and from a biomedical and pharmacological perspective.
2. Explain how Chinese herbal products are made and therefore be able to ascertain quality issues
3. Take a history, conduct and interpret the necessary diagnostic procedures in order to make a TCM diagnosis by integrating patterns of disharmony with etiological factors and pathological processes and how these aspects interconnect - along with the Western diagnosis to an advanced level
4. Elucidate the treatment principles needed for your patients and therefore be able to select an appropriate strategy based upon the actions of Chinese herbal medicine to an advanced level
5. Have the practical skills to dispense Chinese herbal medicine and integrate them with conventional medications to an advanced level
6. Monitor the patient's condition as a result of treatment, re-evaluate diagnostic information and differential diagnosis and modify treatment strategy as the patient's condition changes over time.
7. Be aware of safety issues including the potential for side effects (know how to interpret side effects)
8. Deal with complex and critical case cases by integrating conventional and Chinese medicine

9. Be aware of professional codes of ethics and the practice of veterinary Chinese herbal medicine
10. Be current with recent relevant research that supports the evidence base

For Further Information:

www.ivas.org

CIVT Herbal Training Programs

Both of these courses are government accredited as post graduate degrees under the Australian Quality Skills Authority.

10014NAT Graduate Diploma of Veterinary Chinese Herbal Medicine

consists of 8 Modules which are the teaching components. The rest of the Graduate Diploma consists of keeping a reflective case log book of personal experience of the skills practiced; demonstrated professional ability and competence and preparing material and evidence for assessment of competencies.

There are 8 Units of Competency that are core units and are required before full qualification is granted. Students are assessed continuously throughout the training and in the final module submit their portfolio of evidence to CIVT for assessment. Qualified assessors of CIVT assess each final submission.

Units of Competency

VETCHM801A - Apply the veterinary Chinese herbal medicine diagnostic framework
VETCHM802A - Work within veterinary Chinese herbal medicine principles and practices
VETCHM803A- Plan and provide the veterinary Chinese herbal medicine treatment strategy
VETCHM804A - Perform the veterinary Chinese herbal medicine assessment
VETCHM805A - Provide specialised veterinary Chinese herbal medicine care
VETTCM802A - Operate a veterinary Chinese medicine practice
VETINT801A - Prepare veterinary case studies for publication
VETINT802A - Reflect upon integrated veterinary medicine practice

Part-time. 24 months. Expected volume of learning and study including clinical case time over the 24 months- 1310 hours. Participants are required to demonstrate evidence of competencies through assessment processes which comprise activities and assessments to demonstrate knowledge and skills largely based on actual and simulated cases in practice, over the two year period.
For further information:

http://www.civtedu.org/graduate-diploma-veterinary-chinese-herbal-medicin/

10049NAT Graduate Diploma of Veterinary Western Herbal Medicine

consists of 2 Parts, of which the programs - Year 1 Foundations Veterinary Western Herbal Medicine (Modules 1-4) and Year 2 Advanced Veterinary Herbal Therapeutics (Modules 5-8) are the teaching components (non-accredited when taken alone). The additional component of 10049NAT Graduate Diploma of Veterinary Western Herbal Medicine consists of keeping a reflective case log book of

personal experience of the skills practiced, demonstrated professional ability and competence and preparing material and evidence for assessment of competencies.

All 6 Units of Competency are core units and are required before full qualification is granted. Students are assessed continuously throughout the training and in the final module submit their portfolio of evidence to CIVT for assessment. Qualified assessors of CIVT assess each final submission.

UNITS OF COMPETENCE

UNIT
VETWHM801A - Work within a veterinary Western Herbal Medicine framework
VETWHM802A - Work within veterinary Western Herbal Medicine principles and practices
VETWHM803A - Operate a veterinary Western Herbal Medicine pharmacy
VETWHM804A - Perform the veterinary Western Herbal Medicine assessment
VETWHM805A - Plan and provide the veterinary Western Herbal Medicine treatment strategy
VETWHM806A - Prepare and present veterinary Western Herbal Medicine research findings

Part-time. 24 months. Expected volume of learning and study including clinical case time over the 24 months- 945 hours. Participants are required to demonstrate evidence of competencies through assessment processes which comprise activities and exercises based on actual and simulated cases in practice over the two year period.

For further information:

http://www.civtedu.org/graduate-diploma-veterinary-western-herb-medicine/

Appendix VIII Examples of recent journal articles

Effects of cranberry extract on prevention of urinary tract infection in dogs and on adhesion of *Escherichia coli* to Madin-Darby canine kidney cells

Hsin-I Chou DVM, MSc

Kuan-Sheng Chen DVM, PhD

Hsien-Chi Wang DVM, PhD

Wei-Ming Lee DVM, PhD

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From the Department of Veterinary Medicine (Chou, Chen, Wang, Lee) and the Veterinary Medical Teaching Hospital (Chen, Wang, Lee), College of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan. Dr. Chou's present address is International Diploma Program for Marketing & Project Management, University of California, Berkeley Extension, 1995 University Ave, Berkeley, CA 94704.

Address correspondence to Dr. Lee (wmlee@dragon.nchu.edu.tw).

OBJECTIVE
To determine effects of cranberry extract on development of urinary tract infection (UTI) in dogs and on adherence of *Escherichia coli* to Madin-Darby canine kidney (MDCK) cells.

ANIMALS
12 client-owned dogs (in vivo experiment) and 6 client-owned dogs (in vitro experiment).

PROCEDURES
12 dogs with a history of recurrent UTI received an antimicrobial (n = 6) or cranberry extract (6) orally for 6 months. Dogs were monitored for a UTI. For the in vitro experiment, cranberry extract was orally administered to 6 dogs for 60 days. Voided urine samples were collected from each dog before and 30 and 60 days after onset of extract administration. Urine was evaluated by use of a bacteriostasis assay. An antiadhesion assay and microscopic examination were used to determine inhibition of bacterial adherence to MDCK cells.

RESULTS
None of the 12 dogs developed a UTI. The bacteriostasis assay revealed no zone of inhibition for any urine samples. Bacterial adhesion was significantly reduced after culture with urine samples obtained at 30 and 60 days, compared with results for urine samples obtained before extract administration. Microscopic examination revealed that bacterial adherence to MDCK cells was significantly reduced after culture with urine samples obtained at 30 and 60 days, compared with results after culture with urine samples obtained before extract administration.

CONCLUSIONS AND CLINICAL RELEVANCE
Oral administration of cranberry extract prevented development of a UTI and prevented *E coli* adherence to MDCK cells, which may indicate it has benefit for preventing UTIs in dogs. (*Am J Vet Res* 2016;77:421–427)

Urinary tract infections are associated with a temporary or permanent breach in host defense mechanisms that allows virulent microbes to adhere, multiply, and persist within the urinary tract. Infections can be confined to a single site within the urogenital tract, such as the renal pelvis (pyelonephritis), ureter (ureteritis), bladder (cystitis), urethra (urethritis), prostate gland (prostatitis), or vagina (vaginitis), or can be found at multiple sites.[1] Although fungi and viruses also infect the urinary tract, UTIs are most commonly caused by bacteria such as *Escherichia coli*, which is the most common uropathogen.[2–4]

Cranberries consist primarily of water (88%), but they also contain organic acids (including salicylate), fructose, high amounts of vitamin C (200 mg/kg of fresh berries), flavonoids, proanthocyanidins, catechins, and triterpinoids.[4,5] The *E coli* strains that cause UTIs have proteinaceous macromolecules (fimbriae) that facilitate adhesion of bacteria to uroepithelial cells in the urinary tract. In vitro and in vivo studies[5,6] indicate that cranberry products prevent bacterial adhesion to cells in the wall of the urinary tract.

Studies[5,7] indicate that the consumption of cranberry extract can prevent UTIs in women. However, there is a paucity of studies on the benefits of cranberries for prevention of UTIs in dogs. Therefore, the objectives of the study reported here were to investigate the effect of cranberry extract on the development of UTIs in dogs with a history of recurrent UTIs and to evaluate effects of urine obtained from dogs provided cranberry extract on adhesion of *E coli* to MDCK cells.

ABBREVIATIONS

DMEM	Dulbecco modified Eagle medium
MDCK	Madin-Darby canine kidney
UTI	Urinary tract infection

Materials and Methods

Animals

The in vivo experiment involved 12 client-owned female dogs, each of which had at least 3 UTIs during

the preceding year. All dogs were confirmed to have recovered from the most recent UTI, as determined on the basis of results of urinalysis and bacterial culture of a urine sample.

The in vitro experiment involved 6 client-owned dogs (4 mixed-breed dogs, 1 Pug, and 1 Shih Tzu). There were 3 neutered males and 3 spayed females. Age of the dogs ranged from 7 to 11 years (mean, 8.9 years), and body weight ranged from 6.5 to 20.5 kg (mean, 13.6 kg). All 6 dogs were considered healthy at the time of enrollment, as determined on the basis of the medical history and results of a complete physical examination (no signs of urinary tract disease).

Owners provided consent for inclusion of the dogs in the study. All dogs received care in accordance with institutional animal care and use committee guidelines.

Experimental design

In vivo experiment—Dogs were allocated into 2 groups (6 dogs/group). One group comprised 4 Schnauzers and 2 Toy Poodles (3 spayed and 3 sexually intact; age of the dogs ranged from 7 to 14 years [mean, 9.8 years], and body weight ranged from 2.8 to 7.4 kg [mean, 5.6 kg]). These dogs received cephalexin[a] (20 mg/kg, PO, q 12 h for 14 days). The second group comprised 4 Schnauzers and 2 Chihuahuas (4 spayed and 2 sexually intact; age of the dogs ranged from 5 to 12 years [mean, 8.0 years], and body weight ranged from 2.5 to 7.3 kg [mean, 5.3 kg]). Dogs in the second group received powdered cranberry extract[b] daily for 6 months. The powder was mixed with food and administered to each dog at the morning meal. The amount of cranberry extract provided to each dog was the dose specified on the product (1 g for dogs < 25 kg and 2 g for dogs ≥ 25 kg). The first day of administration of cephalexin or cranberry extract was designated as day 1.

Dogs were monitored throughout the experiment. Blood samples and voided urine samples were collected from each dog immediately before onset of cephalexin or cranberry extract administration and then once per month for 6 months. Once each month, a complete physical examination, hematologic examination, biochemical analysis, urinalysis, and bacterial culture of a urine sample were performed.

In vitro experiment—Dogs received powdered cranberry extract[b] (1 g for dogs < 25 kg and 2 g for dogs ≥ 25 kg) daily for 60 days (1 day before administration of cranberry extract was designated as day 0). The powder was mixed with food and administered to each dog at the morning meal. Voided urine samples were collected from each dog immediately before onset of cranberry extract administration and on days 30 and 60.

Preparation of urine samples

Urine samples were collected in the morning and centrifuged at 1,000 × *g* for 5 minutes to precipitate particulate matter. Supernatant was removed with a sterile Pasteur pipette and vacuum-filtered by use of a commercial filtration unit with a 0.22-µm polyethersulfone filter[c]; filtered urine was collected in a sterile 50-mL conical tube and frozen at –20°C for use in a bacteriostasis assay.

Propagation of uropathogenic *E coli* strains and preparation of bacterial suspensions

Three uropathogenic *E coli* strains (C1-50, C2-48, and C3-48) were isolated from dogs with UTI examined at the Veterinary Medical Teaching Hospital of the National Chung Hsing University. The *E coli* strains were grown on blood agar plates at 35°C for 24 to 48 hours. After distinct bacterial colonies appeared, the plates were sealed and stored at 4°C until used for the bacteriostasis assay.

An *E coli* colony was selected; it was then streaked onto trypticase soy agar[d] and incubated overnight at 35°C. The next morning, *E coli* were suspended in 3 or 5 mL of saline (0.9% NaCl) solution. A standard bacterial concentration of 10^6 CFUs/mL, as determined by use of a 0.5-McFarland standard,[8] was used for the bacteriostasis assay.

Bacteriostasis assay

A swab specimen of the bacterial suspension was smeared onto plates containing Mueller-Hinton agar.[e] Seven holes were punched in each agar plate; 1 hole was filled with 100 µL of sterile saline solution (negative control sample), and the remaining 6 holes were each filled with 100 µL of the urine sample of 1 dog at 1 time point. A disk containing enrofloxacin[f] was used as the positive control sample. A positive result was considered to be an inhibition zone with a diameter > 21 mm. Plates were incubated at 35°C for 24 hours, and the inhibition zone around each hole was then assessed.

Preparation of MDCK cells

The MDCK cells were obtained from the Graduate Institute of Veterinary Pathobiology at National Chung Hsing University. They were maintained in DMEM[g] that contained 4.5 g of glucose/L, sodium pyruvate, and 4mM stable glutamine and 10% (vol/vol) heat-inactivated fetal bovine serum supplemented with 1mM sodium pyruvate and 1% (vol/vol) antimicrobial (penicillin, streptomycin, and amphotericin B) solution. Stock cultures of cells were propagated in 75-cm^2 plastic flasks at 37°C in a humidified 95% O_2–5% CO_2 atmosphere and passaged as needed.

Antiadhesion assay

The efficacy of cranberry extract for inhibiting bacterial adherence to MDCK cells was evaluated by use of an in vitro assay with modifications described elsewhere.[9] Antiadhesion assays were performed as follows.

The MDCK cells that had grown to confluence at 37°C were placed in 96-well plastic plates (10^4 cells/well) for the antiadhesion assay. Culture media were discarded, and each well was washed with PBS solution (100 µL). The wash solution was discarded, and plates were tapped dry on absorbent paper. Immediately before the assay, MDCK cells were fixed with 5% methanol. An aliquot (100 µL) of methanol was added to each well, and plates were allowed to sit undisturbed for 2 minutes. The methanol was then discarded, and plates were tapped dry on absorbent paper. Plates were then further dried in a laminar flow hood for 10 minutes.

A test sample of urine plus bacteria was created by mixing an aliquot of the bacterial suspension (a standard bacterial concentration of 10^6 CFUs/mL, as determined by use of a 0.5-McFarland standard[8]) with urine samples obtained before and 30 and 60 days after onset of cranberry extract administration. The ratio was 1:10 (1 part bacterial suspension to 9 parts urine sample). Each well of a 96-well plastic plate was prepared by adding 50 µL of the test sample (urine plus bacteria) and 150 µL of DMEM (final volume, 200 µL/well) to the methanol-fixed MDCK cells. Plates were then incubated at 25°C for 30 minutes.

After incubation was complete, the plates were incubated for an additional 60 minutes at 35°C to permit bacterial attachment. After this 60-minute incubation was complete, nonadhered bacteria and media were removed by aspiration, and the wells were rinsed 3 times with PBS solution (200 µL/rinse). Then, 200 µL of DMEM plus 5% heat-inactivated fetal bovine serum was added to each well. Plates were incubated at 35°C for 18 hours to allow growth of attached bacteria. After the 18-hour incubation was complete, absorbance for each well was determined at 650 nm by use of a microplate reader[h] and commercial software.[i]

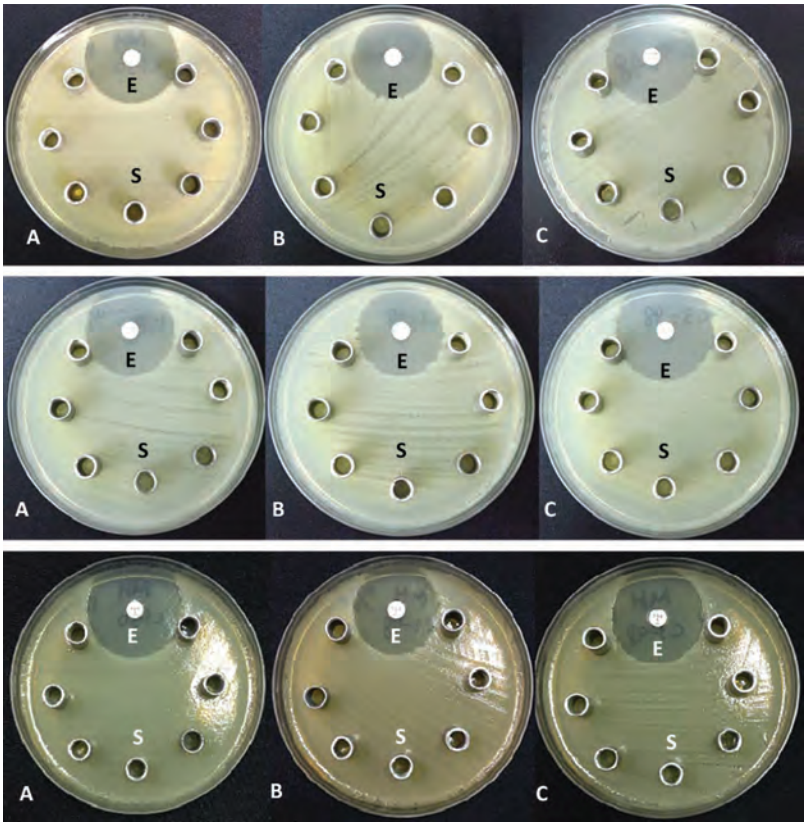

Figure 1—Photographs of a bacteriostasis assay against *Escherichia coli* strains C1-50(A), C2-48 (B), and C3-48 (C) cultured with urine samples obtained from a representative dog before (top row) and 30 (middle row) and 60 (bottom row) days after onset of cranberry extract administration. Notice that the inhibition zone for the positive control sample (enrofloxacin [E]) is > 30 mm, which indicates an inhibitory effect. Sterile saline (0.9% NaCl) solution (S) is the negative control sample.

Microscopic examination

The MDCK cells that had grown to confluence at 37°C were placed in 24-well plastic plates (5×10^4 cells/well) for the antiadhesion assay. Culture media were discarded, and each well was washed with PBS solution (100 µL). The wash solution was discarded, and plates were tapped dry on absorbent paper. Immediately before the assay, MDCK cells were fixed with 5% methanol. An aliquot (200 µL) of methanol was added to each well, and plates were allowed to sit undisturbed for 2 minutes. The methanol was then discarded, and plates were tapped dry on absorbent paper. Plates were then further dried in a laminar flow hood for 10 minutes.

A test sample of urine plus bacteria was created by mixing an aliquot of the bacterial suspension (a standard bacterial concentration of 10^6 CFUs/mL, as determined by use of a 0.5-McFarland standard[8]) with urine samples obtained before and 30 and 60 days after onset of cranberry extract administration. The ratio was 1:10 (1 part bacterial suspension to 9 parts urine sample). Each well of a 24-well plastic plate was prepared by adding 200 µL of the test sample (urine plus bacteria) and 300 µL of DMEM (final volume, 200 µL/well) to the methanol-fixed MDCK cells. Plates were then incubated at 25°C for 30 minutes.

After the initial incubation was complete, the plates were incubated for an additional 3 hours. Slides were stained with crystal violet and examined microscopically (1,000X magnification).

Statistical analysis

All data were expressed as mean ± SEM. Differences between groups were tested by use of the Student *t* test. Values of $P < 0.05$ were considered significant. Linear regression analysis was used to evaluate results for the antiadhesion assay and microscopic examination.

Results

In vivo experiment

None of the 12 dogs developed a UTI during the experimental period

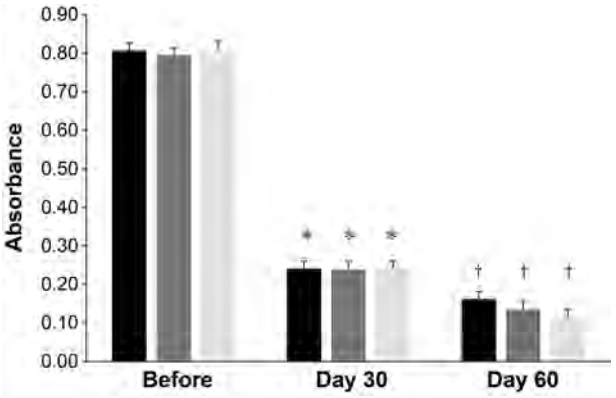

Figure 2—Mean ± SEM absorbance for *E coli* strain C1-50 (black bars), C2-48 (dark gray bars), and C3-48 (light gray bars) after culture in MDCK cells with urine samples obtained from 6 dogs before and 30 and 60 days after onset of cranberry extract administration. *Within a strain, value differs significantly ($P < 0.05$) from the value after culture with the urine samples obtained before the onset of cranberry extract administration. †Within a strain, value differs significantly ($P < 0.05$) from the values after culture with urine obtained before and 30 days after onset of cranberry extract administration.

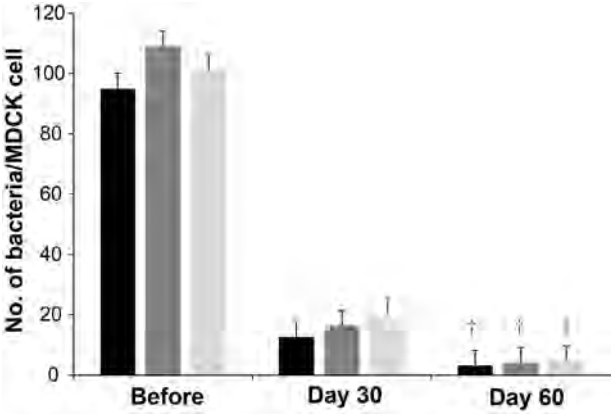

Figure 3—Mean ± SEM number of *E coli* (strain C1-50, C2-48, and C3-48) adhered to MDCK cells after culture with urine samples obtained from 6 dogs before and 30 and 60 days after onset of cranberry extract administration. ***See*** Figure 2 for key.

as determined on the basis of a lack of clinical signs and laboratory results (especially results for bacterial culture of a urine sample) that yielded no evidence of infection. On the basis of the medical history of these dogs, 12 cases of UTI (95% confidence interval, 10 to 14 cases of UTI) would have been expected during the 6-month experimental period. No adverse effects attributable to treatment were reported.

In vitro experiment

Results of the bacteriostasis assay were the same for urine samples obtained before and 30 and 60 days after the onset of cranberry extract administration to the 6 dogs. Enrofloxacin (positive control sample) yielded the only inhibition zone (diameter > 30 mm). No inhibition zone was observed around the sterile saline solution (negative control sample) or the urine samples of the 6 dogs **(Figure 1)**.

Bacterial adhesion was reduced for urine samples obtained at 30 and 60 days from each of the 6 dogs, compared with results for the urine sample obtained before administration of cranberry extract. Mean ± SEM absorbance for C1-50 *E coli* cultured in plates containing MDCK cells with urine samples obtained before and 30 and 60 days after onset of cranberry extract administration was 0.81 ± 0.03, 0.24 ± 0.01, and 0.16 ± 0.02, respectively **(Figure 2)**. Mean ± SEM absorbance for C2-48 *E coli* cultured in plates containing MDCK cells with urine samples obtained before and 30 and 60 days after onset of cranberry extract administration was 0.80 ± 0.03, 0.24 ± 0.01, and 0.14 ± 0.02, respectively. Mean ± SEM absorbance for C3-48 *E coli* cultured in plates containing MDCK cells with urine samples obtained before and 30 and 60 days after onset of cranberry extract administration was 0.81 ± 0.02, 0.24 ± 0.02, and 0.12 ± 0.01, respectively. Mean absorbance for the 3 *E coli* strains cultured with MDCK cells and urine samples obtained at 30 and 60 days was significantly lower than the absorbance for culture with the urine sample obtained before onset of cranberry extract administration. Moreover, mean absorbance of the 3 *E coli* strains cultured with MDCK cells and urine obtained at 60 days was also lower than that for urine samples obtained before and at 30 days after onset of administration of cranberry extract.

Adherence of the 3 *E coli* strains was decreased from a mean of 101.84 adherent bacteria/MDCK cell after incubation with urine samples obtained before cranberry extract administration to 16.44 and 4.00 adherent bacteria/MDCK cell after incubation with urine samples obtained at 30 and 60 days, respectively. Mean ± SEM number of C1-50 *E coli* adhering to MDCK cells was 95.17 ± 10.65, 12.67 ± 3.5, and 3.17 ± 2.04 for the urine samples obtained before and 30 and 60 days after onset of cranberry extract administration, respectively **(Figure 3)**. Mean ± SEM number of C2-48 *E coli* adhering to MDCK cells was 109.17 ± 10.61, 16.33 ± 3.5, and 4.17 ± 2.64 for the urine samples obtained before and 30 and 60 days after onset of cranberry extract administration, respectively. Mean ± SEM number of C3-48 *E coli* adhering to MDCK cells was 101.17 ± 9.52, 20.33 ± 3.56, and 4.67 ± 1.86 for the urine samples obtained before and 30 and 60 days after onset of cranberry extract administration, respectively. Compared with the mean adherence for the urine sample obtained before onset of cranberry extract administration, the mean *E coli* adherence to the MDCK cells for the urine samples obtained at 30 and 60 days was significantly lower. Moreover, the mean *E coli* adherence to the MDCK cells was significantly lower for the urine sample obtained at 60 days than for the urine sample obtained at 30 days.Adherence of

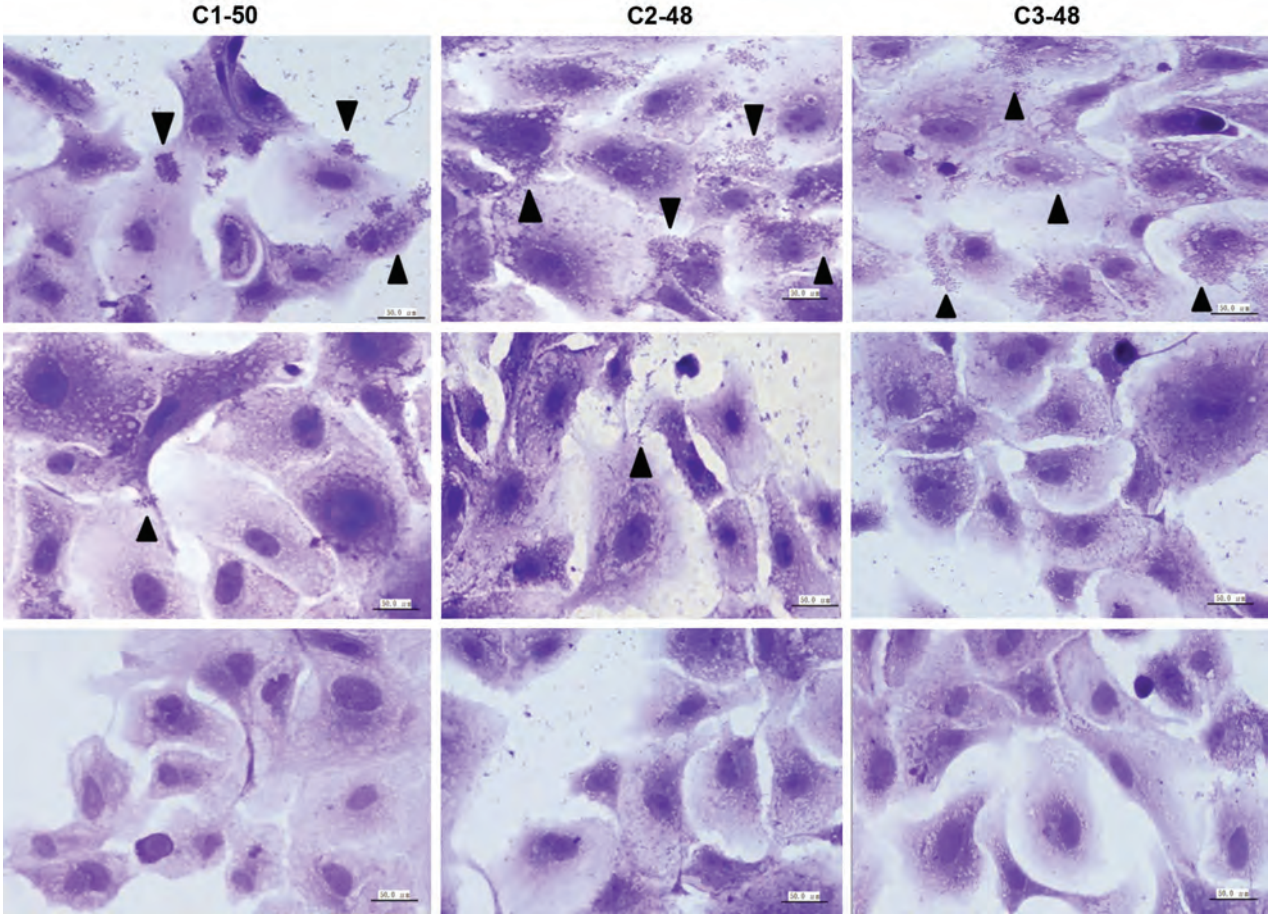

Figure 4—Photomicrographs of cultured MDCK cells indicating adherence of *E coli* strain C1-50, C2-48, and C3-48 to the cells after culture with urine samples obtained from a representative dog before (top row) and 30 (middle row) and 60 (bottom row) days after onset of cranberry extract administration. Notice the bacteria (arrowheads) that are adhered to the cells. Crystal violet stain; bar = 50 µm.

E coli to MDCK cells was evident during microscopic examination **(Figure 4)**.

Discussion

In the in vivo experiment reported here, an antimicrobial and powdered cranberry extract were administered to prevent UTIs in dogs. None of the dogs developed UTIs, as determined on the basis of clinical signs and laboratory results, which corresponded with results of another study.[5] Some studies[6,10,11] of humans indicate that the use of cranberries to prevent UTIs is better than the prophylactic use of low-dose antimicrobials because long-term use of antimicrobials increases the risk of antimicrobial resistance.

In the present study, the effect of cranberry extract on the prevention of bacterial adhesion was evaluated in vitro. Urine samples were collected from dogs receiving cranberry extract and used to determine antibacterial effects. Because *E coli* are the most common uropathogenic bacteria in dogs with UTIs,[2–4] those bacteria were used in the present study. Three *E coli* strains were prepared for use in bacteriostasis and antiadhesion assays and microscopic examination. The bacteriostasis assay revealed no inhibition zone around the urine samples and negative control sample, whereas the positive control sample (enrofloxacin) had an antibacterial effect (diameter of inhibition zone > 30 mm). This indicated 2 possibilities: the concentration of bacteria was too high for the cranberry extract to inhibit growth, or the urine samples from dogs receiving cranberry extract had no bacteriostatic activity.

Results of previous studies[12–15] as well as the present study suggest that cranberries do not have an effect on inhibition of bacterial growth. Instead, it is hypothesized that cranberries prevent UTIs by blocking adherence of bacteria to the uroepithelium.[16–18] Evidence to support this hypothesis was obtained in an in vitro study[11] of fimbriated *E coli* present in the urine 2 hours after ingestion of cranberry extract. In fact, the mean absorbance for the 3 *E coli* strains cultured with MDCK cells and urine samples obtained at 30 and 60 days was significantly lower than that after culture with the urine sample obtained before onset of cranberry extract administration, which indicated that the urine samples collected after administration of the cranberry extract had an antiadhesion effect. One possible mechanism of action may be that cran-

berry compounds act as receptor analogues and bind to the fimbriae of *E coli*, which thus competitively inhibits their adhesion. It has been confirmed that *E coli* isolated from dogs with UTIs most commonly express type-1 fimbriae.[19] Furthermore, the main mechanism of in vitro adherence to canine uroepithelial cells involves a mannose-sensitive mechanism.[20] Components of the cranberry extract also might have altered P-fimbriated uropathogenic bacteria in other ways, such as by reducing adhesion capabilities, reducing fimbrial length and density, or inducing other morphological changes.[18,21,22]

Mean *E coli* adherence to MDCK cells after incubation with urine samples obtained at 30 and 60 days was significantly lower, compared with adherence after incubation with the urine sample obtained before administration of the cranberry extract. Moreover, mean *E coli* adherence was significantly lower after incubation with the urine sample obtained at 60 days, compared with results after incubation with the urine sample obtained at 30 days.

In the present study, MDCK cells were used because they are a good in vitro method of screening to detect bacteria virulence[23] or determining the pathogenesis of various bacterial infections, including those attributable to uropathogenic *E coli*.[24,25] Adhesion of uropathogenic *E coli* to epithelial cells can lead to ascending UTIs, which range from nonclinical bacteriuria to cystitis and acute pyelonephritis to more severe acute lobar nephronia.[26] Bacterial adhesion to uroepithelial cells by fimbrial or nonfimbrial adhesins in bacterial renal infections is an important factor in the subsequent development of UTIs in the upper urinary tract (ie, calyx, renal pelvis, and ureter) via the ascending route.[27] The effect of cranberry extract on *E coli* adhesion to both kidney epithelial cells and uroepithelial cells derived from dogs has been described.[28] Results for the microscopic examination performed in the present study correlate with results of another study[28] that also revealed antiadhesion activity of cranberries or cranberry extract on *E coli* adherence to specific primary-cultured uroepithelial cells.[28] The antiadherence effect of cranberries is not restricted to a particular group of *E coli* strains, which might otherwise be caused by interference with specific receptor-ligand modes of bacterial adhesion or by inhibition of expression of the bacterial fimbriae.[21,29] The effect of cranberry intake might be synergistic, but the details remain unclear. In the present study, we minimized the possible bias associated with a noncontrolled trial by developing a bioassay to test adhesion of bacteria to MDCK cells that were cultured with urine obtained from dogs after they had received cranberry extract.

Antimicrobial resistance is an increasing concern. Therefore, alternative strategies such as consumption of cranberries or cranberry extract may be an option for prevention of UTIs in dogs. The present study revealed that the efficacy of cranberry extract for the prevention of UTIs was almost the same as that for an antimicrobial (cephalexin), with a lower risk of antimicrobial resistance or superinfection.[30] Analysis of the results of the study reported here indicated that cranberry extract decreased *E coli* adherence to MDCK cells but did not inhibit bacterial growth. This effect suggested that cranberry extract has a potential clinical benefit for the prevention of UTIs in dogs.

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Footnotes

a. Keflex, 250-mg capsule, Taiwan Biotech Co, Taoyuan City, Taiwan.
b. 120 g/pack, Cranimals, West Vancouver, BC, Canada.
c. Millex GV, Millipore Ireland BV, Carrigtwohill Co, Cork, Ireland.
d. Difco, Becton Dickinson and Co, Franklin Lakes, NJ.
e. BBL, Becton Dickinson and Co, Franklin Lakes, NJ.
f. Test disc, Oxoid, Basingstoke, Hampshire, England.
g. Mediatech Inc, Tewksbury, Mass.
h. Sunrise Infinite F200, Tecan, Männedorf, Switzerland.
i. Magellan, version 6.6, Tecan, Männedorf, Switzerland.

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Review Article

Topical Herbal Application in the Management of Atopic Dermatitis: A Review of Animal Studies

Younghee Yun,[1,2] Kyuseok Kim,[3] Inhwa Choi,[2,3] and Seong-Gyu Ko[4]

[1] *Department of Dermatology of Korean Medicine, College of Korean Medicine, Graduate School of Kyung Hee University, Seoul 130-701, Republic of Korea*
[2] *Department of Dermatology of Korean Medicine, Kyung Hee University Hospital at Gangdong, No. 149 Sangil-dong, Gangdong-gu, Seoul 134-090, Republic of Korea*
[3] *Department of Ophthalmology & Otorhinolaryngology & Dermatology of Korean Medicine, College of Korean Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea*
[4] *Department of Preventive Medicine, College of Korean Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea*

Correspondence should be addressed to Inhwa Choi; inhwajun@hanmail.net

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Herbs are widely used in the treatment of atopic dermatitis (AD) in Eastern Asian countries, and certain herbs regarded have anti-inflammatory properties that can help with AD. With the goal of developing a topical herbal agent for AD, we conducted a systematic review of *in vivo* studies of AD-like skin models for screening potential herbs. Searches were conducted from PubMed and EMBASE. After all, 22 studies were included for this review. We judged most of the domains of all studies to be at unclear risk of bias. Among 22 included studies, 21 herbs have been reported to reduce AD-like skin lesions in mouse models by suppressing Th2 cell response. Our findings may offer potential herbs for the topical application treatment of AD.

1. Introduction

There are many chemical substances that have been derived from plants for use as drugs, and these include some of the most utilized drugs such as aspirin, atropine, digoxin, ephedrine, morphine, quinine, and taxol. The latest version of the Dictionary of Natural Products (DNP; http://dnp.chemnetbase.com/) has just over 260,000 entries. Over the past decades, natural sauces have only taken a secondary role in drug discovery and development after the advent of molecular biology and combinatorial chemistry. However, as a basis for drug development, a new interest in the role of natural sauces has been concentrated, because various "-omics" technologies now allow scientists to detail the exact biological effects of natural sauces [1].

Atopic dermatitis (AD) is a chronic inflammatory skin disease with an increasing prevalence in industrialized countries. AD is characterized by pruritus; eczematous lesions accompanied by excessive infiltration of inflammatory cells such as lymphocytes, macrophages, and granulated mast cells in the skin lesions; eosinophilia in the peripheral blood; and high levels of serum immunoglobulin IgE. Although the pathogenesis of AD has not yet been fully understood, genetic, environmental, pharmacological, psychological, immunological, and skin barrier dysfunction factors are believed to contribute to the underlying pathogenic mechanisms [2–4].

Topical steroids are commonly used to treat moderate-to-severe AD, but long-term use of steroids at high concentrations is associated with a number of side effects [5]. Among various natural sources such as plants, animals, or microorganisms, herbs are widely used in the treatment of atopic dermatitis (AD) in Eastern Asian countries, and certain herbs regarded have anti-inflammatory properties that can help with AD. Our interest is to develop a safe and curative herb derived agent for AD using medical knowledge and clinical experience of herbal medicine combining with molecular biology and combinatorial chemistry technologies.

Table 1: Search strategy for PubMed.

Intervention/exposure	"Herbal Medicine" [MeSH term] or "Herbals" [MeSH terms] or "Herbals as Topic" [MeSH terms] or "Plant Extracts" [MeSH terms] or "Drugs, Chinese Herbal" [MeSH term] or "Plants, Medicinal" [MeSH terms] or "Ethnobotany" [MeSH term] or "Medicine, Traditional" [MeSH Terms] or "Phytotherapy" [MeSH terms] or "Herb" [tiab] or "Extract" [tiab] or "Extracts" [tiab] or "Chinese medicine" [tiab] or "Korean medicine" [tiab] or "Kampo" [tiab]
Disease of interest	"dermatitis, atopic" [MeSH Terms] or "Atopic dermatitis" [title/abstract] or "Eczema, Atopic" [title/abstract]
Animal species	Using "Animal search filter" [8]
Outcome measures	Not included in the search strategy
Filter	Full text available AND English AND Published in the last 5 years

Recently, since Sandercock and Roberts drew attention to the need for more animal studies before beginning studies in human patients [6], there has been an increasing interest in the systematic reviews of research involving animals.

Systematic reviews can aid in the development of more effective therapeutic agents for AD by extrapolating the results of animal studies to humans [7]. We performed a systematic review with this goal in mind and our objectives were (i) to screen topically applicable herbs for AD, (ii) to suggest potential mechanisms of action of topical herbal application in animal models of AD, and (iii) to ascertain the conditions of animal experiments used in the studies.

2. Methods

2.1. Criteria for Considering Studies for This Review

2.1.1. Inclusion Criteria

(i) Studies on the use of topical herbs for AD in animal models
(ii) Published between 2009 and 2013
(iii) Full text available
(iv) Article in English.

2.1.2. Exclusion Criteria

(i) Not related to AD or allergic dermatitis
(ii) Not an animal study
(iii) Animal cell studies
(iv) Not an investigational study of herbs
(v) Not an investigational study of herbs alone
(vi) Studies investigating compounds isolated from herbs
(vii) Use of fermented herbs by *Lactobacillus plantarum* and so forth
(viii) Studies investigating oils from herbs
(ix) Pharmacoacupuncture
(x) Preexisting herbal drugs
(xi) Anal, intraperitoneal, or oral administration of herbs
(xii) Herbal mixtures
(xiii) Biomarkers not used as outcome measurements.

Table 2: Search strategy for EMBASE.

Intervention/exposure	Medical plant [EMTREE]
Disease of interest	Atopic dermatitis [EMTREE]
Outcome measures	Not included in the search strategy
Filter	English AND Published from 2009 to 2013 AND (animal experiment OR animal tissue OR animal model)

2.2. Search Methods for Identification of Studies

2.2.1. Data Sources and Searches. Literature searches were performed using PubMed and EMBASE databases. Search terms contained three components: (A) intervention/exposure, (B) disease of interest, and (C) animal species, with adjustments made for the different databases. Herbs were defined as plants, part of plants, or plant extracts that are used for medical purposes. Since the administration method of the herbs, as well as the outcome measures, was commonly described in the main article and rarely indexed in many papers, we excluded the administration method and outcome measures in the search strategy. For identification of MeSH terms, we used the PubMed thesaurus and the MeSH database, while we used EMTREE terms for searches using EMBASE. To identify all animal studies in PubMed, we used the "Animal search filter" that Hooijmans et al. [8] designed, while we used the filters in EMBASE. The full lists of search terms are presented in Tables 1 and 2.

2.2.2. Selection of Studies. Two authors (Yun and Kim) independently conducted the database searches. Duplicate articles were removed by the first author (Yun). Moreover, the references lists of review articles on relevant topics were manually searched by the two authors. For identifying eligibility of each study, the two authors read all potentially relevant articles. Disagreements were resolved by discussions with the corresponding author (Choi and Ko).

3. Results

3.1. Identification of Studies. After adding the search results from PubMed ($n = 165$) and EMBASE ($n = 33$), duplicate articles ($n = 24$) were removed. References lists in review articles ($n = 8$) were searched but did not result in any

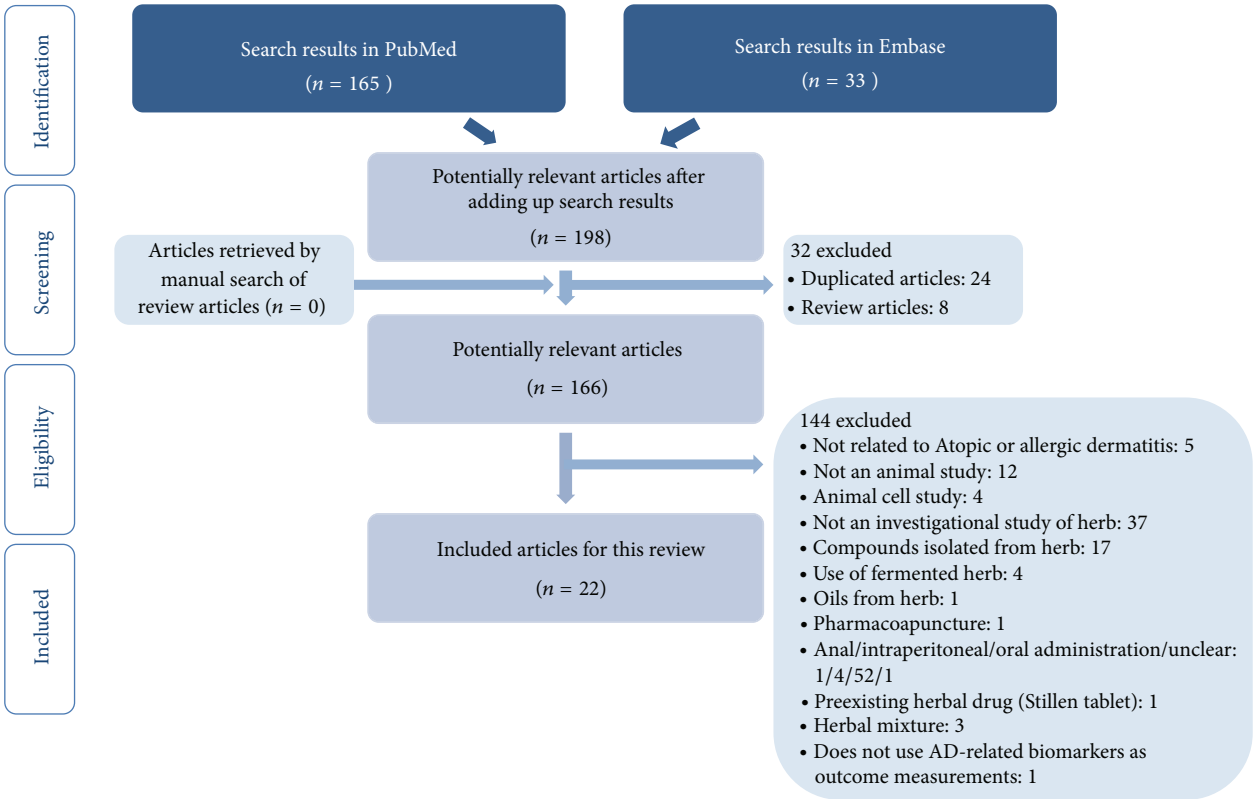

Figure 1: Flowchart of the study selection process.

articles being retrieved. From the potentially relevant articles (n = 166), we excluded 144 articles based on the predefined exclusion criteria, resulting in a total of 22 studies being included in this review (Figure 1).

3.2. Risk of Bias. Figure 2 shows the study quality checklist items reported for each included study, including random allocation to treatment groups (n = 8, 36.4%), compliance with animal welfare regulations (n = 22, 100%), and statements of a potential conflict of interest (n = 16, 72.7%). None of the studies reported allocation concealment, examiner blinding, sample size calculation, and if results were based on analysis of the intent-to-treat population.

3.3. Basic Characteristics and Investigated Herbs in the Included Studies. Twenty-one studies were conducted in Korea and one was conducted in Japan. In two studies, herbs of the genus *Chrysanthemum* were investigated. Otherwise, there were no studies investigating the same herb (Table 4). Herbal extracts were prepared using ethanol, water, methanol, butanol, chloroform, 1,3-butylene glycol, or indirect heat.

3.4. Animal Models Used in the Included Studies. All studies used mice to investigate topical herbal application in an *in vivo* setting. The NC/Nga mouse (n = 16) was the most frequently used mouse model in these studies, followed by BALB/c (n = 4), C57BL/6 (n = 1), and hairless mice (n = 1).

The methods used for induction of AD-like skin lesions varied depending on the study. Repeated cutaneous application of chemical allergens and house dust mite allergens was used in 15 and 10 studies, respectively. Skin injury by stripping using surgical tape was used in 2 studies. For barrier disruption, sodium dodecyl sulfate (SDS) was applied to the lesions in 8 studies. Dorsal skin, ears, or a combination of dorsal skin and ears was used in most of the studies (Table 3).

3.5. Main Outcomes Investigated, Results, and Suggested Mechanisms of Action. In most of the studies, clinical symptoms, serum IgE levels, and Th1- and/or Th2-related cytokines and/or chemokines were assessed as outcome measurements (Table 4). The clinical severity of dermatitis was scored, and severity was found to have decreased after topical herbal application in 15 studies. Epidermal, dermal, or ear thickness was measured and was found to be decreased in 13 studies, indicating a decrease in the severity of the inflammatory process. Frequency of scratching was measured by counting scratching episodes in 5 studies, either directly or by reviewing videos of the animal. After topical herbal application, the frequency of scratching was decreased in all 5 studies.

Elevated serum IgE levels are important characteristics of AD. Serum or plasma IgE levels were measured in 21 studies, and, in 20 of these, serum or plasma IgE levels were decreased after the topical herbal application. However, in one study [9], neither topical *Rehmannia glutinosa* extract nor tacrolimus reduced the increased serum IgE levels after

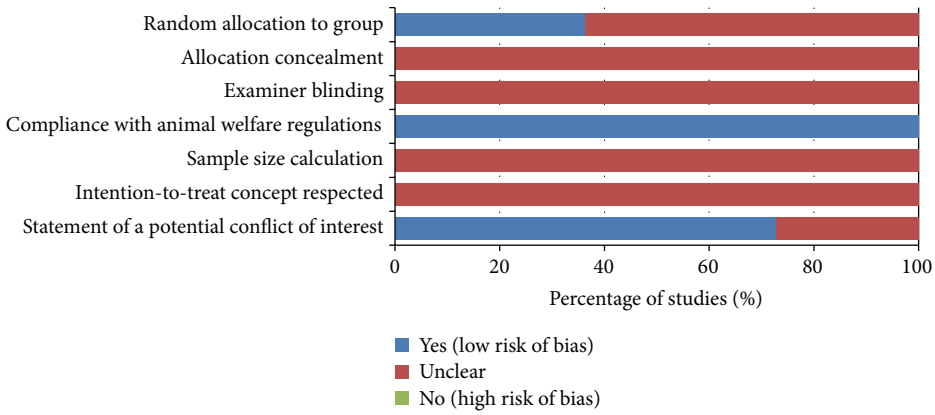

Figure 2: Risk of bias in the studies.

Table 3: Basic characteristics of the animal models used in the included studies.

Study	Sex/model species	Induction of AD-like skin	Barrier disruption	Investigation site
Qi et al. [27]	F/hairless mouse	DNCB	N/A	Dorsal skin
Lee et al. [14]	M/NC/Nga mouse	DfE	SDS	Dorsal skin, ears
Choi et al. [15]	F/NC/Nga mouse	DfE	N/A	Dorsal skin
Sohn et al. [28]	M/BALB/c mouse	DNCB	N/A	Dorsal skin
Wu et al. [29]	M/NC/Nga mouse	DNFB	N/A	Dorsal skin
Yang et al. [16]	F/NC/Nga mouse	DNCB	N/A	Dorsal skin, ears
Sung et al. [30]	M/NC/Nga mouse	DfE	SDS	Dorsal skin, ears
Nam et al. [31]	M/C57BL/6 mouse	DNFB	N/A	Abdominal skin, ears
Choi et al. [24]	F/BALB/c mouse	DNCB, DfE, and skin injury	N/A	Ears
Sung et al. [9]	M/NC/Nga mouse	DfE	SDS	Dorsal skin, ears
Lee et al. [22]	M/NC/Nga mouse	DfE	SDS	Dorsal skin, ears
Ngatu et al. [17]	M/NC/Nga mouse	TNCB	N/A	Abdominal skin, ears
Yang et al. [18]	F/NC/Nga mouse	DNCB	SDS	Dorsal skin, ears
Hwang et al. [25]	F/BALB/c mouse	DNCB, DfE, and skin injury	N/A	Ears
Choi et al. [32]	M/NC/Nga mouse	DNCB	N/A	Dorsal skin, ears
Sung et al. [33]	M/NC/Nga mouse	DNCB and DfE	SDS	Dorsal skin, ears
Kang and Shin [34]	M/NC/Nga mouse	DNCB	N/A	Dorsal skin
Sung et al. [35]	M/NC/Nga mouse	DfE	SDS	Dorsal skin, ears
Park et al. [10]	F/NC/Nga mouse	DNCB	N/A	Dorsal skin, right ear
Yang et al. [36]	M/NC/Nga mouse	DfE	N/A	Dorsal skin, ears
Kim et al. [37]	F/BALB/c mouse	DNCB	SDS	Dorsal skin
Choi et al. [38]	M/NC/Nga mouse	DNCB	N/A	Dorsal skin, ears

DNCB: 1-chloro-2,4-dinitrobenzene, DfE: D. farinae extract, DNFB: 2,4-dinitrofluorobenzene, TNCB: 2,4,6-trinitrochlorobenzene, N/A: not applicable, SDS: sodium dodecyl sulfate, M: male, and F: female.

allergen sensitization, although they both suppressed the expression of interleukin- (IL-) 4 mRNA in the ear lesions. Antigen-specific IgE levels were measured in two studies, both of which used house dust mite allergen and DNCB to induce AD-like skin lesions.

Most of the included studies investigated the Th2-response suppressing effects and/or Th1-response modulating effects upon topical herbal application in the AD-like mouse models. In 14 studies, only Th2-related biomarkers were measured, while both Th1- and Th2-related biomarkers were measured in nine studies. In all 21 studies that measured Th2 responses, topical herbal application resulted in decrease of Th2-related cytokines, chemokines, proinflammatory factors, and adhesion molecules. Conversely, among the eight studies that measured interferon- (IFN-)γ, topical herbal application resulted in increased IFN-γ levels in two studies, decreased levels in five studies, and no induced changes in one study. Interestingly, in one study [10], *Chrysanthemum indicum* L. decreased both Th1 (IFN-γ) and Th2 cytokines (IL-4 and IL-13); however, the ratio of Th1 to Th2 cytokines was increased by herbal application.

4. Discussion

Herbal medicine is the use of medicinal plants for prevention and treatment of disease. Herbs and their derivatives have been, and continue to be, rich sources for drug discovery. Recently, results from several studies have indicated that

Table 4: Investigated herbs, results, and suggested mechanisms of action in the included studies.

Ref. number	Herb	Outcomes and results	Suggested mechanisms
[14]	*Bambusae caulis*	TEWL↓ serum IgE↓ eosinophil↓ spleen IFN-γ ↑ TNF-α ↓ IL-4↓ IL-13↓	Suppression of Th2 response and promotion of Th1 response
[15]	*Broussonetia kazinoki*	Plasma IgE↓ IL-4↓ skin mast cell↓	Suppression of Th2 response
[28]	*Alnus japonica*	Clinical score↓ serum IgE↓ eosinophil↓ skin IL-4↓ IL-5↓ IL-13↓ iNOS↓ COX-2↓	Suppression of Th2 response
[29]	Korean red ginseng	Scratching↓ serum IgE↓ IL-4↓ IL-10↓	Suppression of Th2 response
[16]	*Cordyceps bassiana*	Clinical score↓ epidermal thickness↓ serum IgE↓ histamine↓ skin IFN-γ ↓ IL-4↓ mast cell↓	Suppression of both Th1 and Th2 responses
[30]	*Chelidonium majus*	Clinical score↓ ear thickness↓ scratching↓ serum IgE↓ IL-4↓ TNF-α ↓	Suppression of Th2 response
[31]	*Cinnamomum cassia*	Serum IgE↓ histamine↓ TNF-α ↓ skin IL-4↓ TNF-α ↓ TARC↓	Suppression of Th2 response
[24]	*Terminalia chebula* Retzius	Ear thickness↓ skin inflammatory cells↓ eosinophils↓ ear IL-31↓ T-bet positive cell↑ MMP-9↓	Suppression of Th2 response and promotion of Th1 response
[9]	*Lindera obtusiloba*	Ear thickness↓ serum IgE↓ DfE specific IgE↓ histamine↓ ear mast cell↓ IL-4↓ IL-13↓ IL-31↓ TNF-α ↓	Suppression of Th2 response
[22]	*Rehmannia glutinosa*	Clinical score↓ ear thickness↓ serum IgE↑ histamine↓ ear IL-4↓ TNF-α ↓ TARC↓ MDC↓ RANTES↓ ICAM-1↓ VCAM-1↓	Suppression of Th2 response
[17]	*Angelicae Dahuricae Radix*	Clinical score↓ plasma IgE↓ histamine↓	Suppression of Th2 response
[18]	*Vernonia amygdalina*	Clinical score↓ ear thickness↓ scratching serum IgE↓ IL-4↓ IL-5↓ MCP-1↓ eotaxin↓	Suppression of Th2 response
[25]	*Chrysanthemum boreale* Makino	Clinical score↓ ear thickness↓ scratching↓ serum IgE↓ TNF-α ↓ IL-4↓	Suppression of Th2 response
[32]	Mycelium of *Phellinus linteus*	Clinical score↓ ear thickness↓ serum IgE↓ DfE specific IgE↓ total IgG— ear IL-12↓ IFN-γ ↓ IL-4↓ IL-5↓ IL-10↓ IL-13↓ TNF-α ↓ CCL4↓ CCL22↓ CCL17— CCL20— eotaxin— IL-2↓	Suppression of both Th1 and Th2 responses
[33]	*Psidium guajava*	Clinical score↓ serum IgE↓ TARC↓ IL-10↑ ear IFN-γ ↓ TNF-α ↓ IL-4↓ IL-5↓ IL-13↓	Suppression of both Th1 and Th2 responses and upregulation of IL-10
[34]	*Drynaria fortunei*	Clinical score↓ ear thickness↓ serum IgE↓ IgG1↓ IgG2a— IL-4↓ IL-6↓ TNF-α ↓ ear IFN-γ— IL-4↓ TNF-α ↓ IL-6↓ ICAM-1— VCAM-1	Suppression of Th2 response
[35]	*Schisandra chinensis*	Clinical score↓ scratching↓ serum IgE↓ IgM↓ histamine↓ skin histamine receptors↓ spleen IL-4↓ IL-5↓ FcεRIβ ↓	Suppression of Th2 response
[10]	*Illicium verum*	Clinical score↓ ear thickness↓ serum IgE↓ histamine↓ IL-6↓ ICAM-1↓ ear IFN-γ ↑ IL-4↓ IL-6↓ TNF-α ↓ TARC↓ RANTES↓ ICAM-1↓ VCAM-1↓	Suppression of Th2 response and promotion of Th1 response
[36]	*Chrysanthemum indicum* L.	Clinical score↓ ear thickness↓ serum IgE↓ IgG1↓ IFN-γ ↓ IL-4↓ skin eosinophil↓ mast cell↓ IFN-γ ↓ IL-4↓ IL-13↓ IL-4 : IFN-γ ratio↓	Suppression of both Th1 and Th2 responses and balancing of Th1/Th2 cell responses

Table 4: Continued.

Ref. number	Herb	Outcomes and results	Suggested mechanisms
[37]	*Catalpa ovata*	Clinical score↓ serum IgE↓ skin mast cell↓ IL-4↓ IL-5↓ IL-6↓ IL-13↓ IL-1b↓ TNF-α ↓	Suppression of Th2 response
[38]	*Astragalus membranaceus*	Epidermal thickness↓ dermal thickness↓ serum IgE↓ IL-4↓ IL-5↓ IL-6↓ IL-13↓ TNF-α ↓ skin NF-κB↓	Suppression of Th2 response
[11]	*Pleurotus eryngii*	Clinical score↓ ear thickness↓ serum IgE↓ TARC↓ skin mast cell↓ ear IFN-γ ↓ TNF-α ↓ IL-4↓ IL-5↓ IL-13↓	Suppression of both Th1 and Th2 responses

"—": no change, TEWL: transepidermal water loss, Ig: immunoglobulin, IFN: interferon, IL: interleukin, TNF: tumor necrosis factor, iNOS: inducible nitric oxide synthase, COX: cyclooxygenase, TARC: thymus and activation-regulated chemokine, MMP: matrix metalloproteinase, MDC: macrophage-derived chemokine, RANTES: regulated on activation normal T-cell expressed and secreted, ICAM: intercellular adhesion molecule, VCAM: vascular adhesion molecule, MCP: monocyte chemoattractant protein, CCLCC: chemokine ligand, FcεRI: high-affinity IgE receptor, and NF-κB: nuclear factor-κB.

patients with AD may benefit from herbal medicines [11–13]. Certain herbs are regarded to have anti-inflammatory properties that can reduce the symptoms of AD. In Asian herbal medicine, herbs are categorized according to their functions. One such group of categorized herbs is named the clear heat drug group (清熱藥), and these herbs can be used for treating fever, infectious disease, and inflammatory conditions. Among the included 22 studies, seven studies [9, 10, 14–18] investigated herbs belonging to the clear heat drug group. Among these seven studies, two studies investigated herbs of the genus *Chrysanthemum*.

Since multiple genetic and environmental factors may underlie AD, the notion of developing a single comprehensive animal model is unrealistic [19]. Since the description of the Nc/Nga mouse as the first spontaneously occurring model of AD in 1997 [20], a number of mouse models have been developed. They can be classified into three groups: (1) models induced by epicutaneous application of sensitizers, (2) mice that spontaneously develop AD-like skin lesions, and (3) transgenic mice that either overexpress or lack selective molecules [21].

NC/Nga mice were used in 16 of the analyzed studies. These mice are free of dermatitis in pathogen-free conditions but develop a spontaneous AD-like eruption when conventionally housed, and they have historically been viewed as one of the best animal models for assessing this condition [19]. In other studies, models induced by epicutaneous application of sensitizers were used. However, none of the included studies used genetically engineered mouse models.

In most of the included studies, clinical symptoms, serum IgE levels, and Th1- and/or Th2-related cytokines and/or chemokines were measured as outcome measurements. Based on the decreased clinical scores, ear or epidermal thickness, scratching behaviors, and histological inflammations after herbal application, it can be hypothesized that topical herbal application has anti-inflammatory effects. However, we could not conduct a meta-analysis to integrate quantitative analyses, since the studies included in our study all investigated different types of herbs.

Elevated serum IgE levels are an important feature of AD. Several studies have demonstrated that serum IgE levels are elevated in patients with AD; furthermore, serum IgE levels have been shown to be elevated in NC/Nga mice with AD-like skin lesions [20]. Among the 22 included studies, serum or plasma IgE levels were measured in 21 studies, and, in 20 studies, serum or plasma IgE levels were decreased after herbal treatment. However, in one study [9], neither topical application of *Rehmannia glutinosa* extract nor tacrolimus reduced the increased serum IgE levels after allergen sensitization, although they both suppressed the expression of IL-4 mRNA in the ear lesions and serum. The authors discussed two possible reasons for this observation: (i) that locally expressed IL-4 in the ear lesions did not contribute to systemic IgE production or (ii) that, in a short-term study with topical herbal application, effects on serum IgE may not be observed [22]. However, we noted that the authors did not measure the levels of allergen-specific IgE, and, in general, total IgE concentrations are a relatively crude method of detecting allergic disorders, since normal values do not exclude the presence of allergic disease, particularly to a single allergen, and since elevated levels of total IgE can be found in many patients with no evidence of allergy [23].

Conversely, both total and allergen-specific IgE levels were measured and found to be decreased after topical application of water-soluble extract of *Lindera obtusiloba* and *Phellinus linteus* in two studies, which used both house dust mite allergens and DNCB to induce AD-like skin lesions [24, 25]. Furthermore, water-soluble extract of *P. linteus* did not affect the total IgG levels, and it was found to be more potent than ceramide in reducing mite-specific IgE levels. These data suggest that certain herbs can suppress allergic responses in an allergen-specific manner. Nevertheless, immunological and clinical parameters for the assessment of antigen-specific immune responses were not measured in most of the studies.

Both Th1- and Th2-type cytokines contribute to the pathogenesis of AD, and their expression patterns are not mutually exclusive [2]. Th2 cytokines such as IL-4, IL-5, and IL-13 play key roles in the hyperproduction of IgE, whereas Th1 cytokines, especially IFN-γ, are strong inhibitors of IgE synthesis, Th2 cell proliferation, and IL-4 receptor expression on T-cells [26]. Development of AD is induced by Th2-type responses, while the chronic inflammatory responses are dominantly mediated by Th1-type reactions.

Among the 22 included studies, 21 herbs were reported to reduce AD-like skin lesions in mouse models by suppressing Th2 cell response with or without balancing of the Th1/Th2 cell response. In eight studies, Th1 cytokines were measured and showed different results. Based on this review, it seems that investigators mainly assess Th1- and Th2-related mechanisms to explain the anti-inflammatory effects of herbs.

In the present study, out of 166 potential studies, we identified 22 studies that met all the selection criteria. It showed that there is room for methodological improvement in the studies. Most studies were at an unclear risk of bias; therefore, it was not possible to accurately determine the degree of bias of the described treatment effects. Further research should be conducted with well-designed methodological research protocols using random allocation, allocation concealment, assessor blindness, sample sizes calculation, and intention-to-treat-respected analyses.

Twenty-one studies were conducted in Korea and one was conducted in Japan. For identifying all potentially relevant researches, the search strategy in the present study included American (PubMed) and European (EMBASE) databases. No attempts were made to retrieve articles from Chinese, Korean, or Japanese databases. Also, non-English articles from PubMed and EMBASE were not included. Because of these, studies may have been excluded. However, when we made our search strategy, we did not expect different results among the countries (Korea, Japan, and China) which conduct a relatively large number of studies on herbs. We assume that the great interest in topical use of herbs and the large number of research and development (R&D) projects on herbs in Korea have been important factors of the results.

In summary, we have reviewed studies investigating topical herbal application in AD-like animal models. For all studies, we judged most domains to be at unclear risk of bias. Herbs of the genus *Chrysanthemum* were used in two studies, and seven studies investigated herbs of the clear heat drug group. Among the AD-like animal models, NC/Nga and BALB/c mice treated with chemical haptens, DNCB, DNFB, or TNCB were used in most of the studies. Clinical symptoms, serum IgE levels, and Th1- and/or Th2-related cytokines and/or chemokines were assessed as outcome measurements. Among the 22 included studies, 21 herbs were reported to reduce AD-like skin lesions in mouse models by suppressing Th2 cell responses. By summarizing the results from the published literature, we hope that this study might aid in finding a potential herbal therapeutic agent for the treatment of AD. The limitation of this study was that a meta-analysis was not conducted because of the variety of investigated herbs included in the studies. Nevertheless, this review may assist in identifying directions for further researches endeavors.

Conflict of Interests

The authors state that there is no conflict of interests. No financial support or benefits were received by the authors. The authors have no commercial associations or financial relationships to disclose.

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Dietary plant extracts modulate gene expression profiles in ileal mucosa of weaned pigs after an *Escherichia coli* infection[1]

Y. Liu,* M. Song,*[2] T. M. Che,*[3] J. J. Lee,* D. Bravo,† C. W. Maddox,‡ and J. E. Pettigrew*[4]

*Department of Animal Sciences and ‡Department of Pathobiology,
University of Illinois, Urbana 61801; and †Pancosma SA, Geneva, Switzerland

ABSTRACT: This study was conducted to characterize the effects of infection with a pathogenic F-18 *Escherichia coli* and 3 different plant extracts on gene expression of ileal mucosa in weaned pigs. Weaned pigs (total = 64, 6.3 ± 0.2 kg BW, and 21-d old) were housed in individual pens for 15 d, 4 d before and 11 d after the first inoculation (d 0). Treatments were in a 2 × 4 factorial arrangement: with or without an F-18 *E. coli* challenge and 4 diets (a nursery basal, control diet [CON], 10 ppm of capsicum oleoresin [CAP], garlic botanical [GAR], or turmeric oleoresin [TUR]). Results reported elsewhere showed that the plant extracts reduced diarrhea in challenged pigs. Total RNA (4 pigs/treatment) was extracted from ileal mucosa of pigs at d 5 post inoculation. Double-stranded cDNA was amplified, labeled, and further hybridized to the microarray, and data were analyzed in R. Differential gene expression was tested by fitting a mixed linear model in a 2 × 4 factorial ANOVA. Bioinformatics analysis was conducted by DAVID Bioinformatics Resources 6.7 (**DAVID;** National Institute of Allergy and Infectious Diseases **[**NIAID, NIH**],** http://david.abcc.ncifcrf.gov). The *E. coli* infection altered ($P < 0.05$) the expression of 240 genes in pigs fed the CON (148 up- and 92 down-regulated). Compared with the infected CON, feeding CAP, GAR, or TUR altered ($P < 0.05$) the expression of 52 genes (18 up, 34 down), 117 genes (34 up- and 83 down-regulated), or 84 genes (16 up- and 68 down-regulated), respectively, often counteracting the effects of *E. coli*. The *E. coli* infection up-regulated ($P < 0.05$) the expression of genes related to the activation of immune response and complement and coagulation cascades, but down-regulated ($P < 0.05$) the expression of genes involved in protein synthesis and accumulation. Compared with the CON, feeding CAP and GAR increased ($P < 0.05$) the expression of genes related to integrity of membranes in infected pigs, indicating enhanced gut mucosa health. Moreover, feeding all 3 plant extracts reduced ($P < 0.05$) the expression of genes associated with antigen presentation or other biological processes of immune responses, indicating they attenuated overstimulation of immune responses caused by *E. coli*. These findings may explain why diarrhea was reduced and clinical immune responses were ameliorated in infected pigs fed plant extracts. In conclusion, plant extracts altered the expression of genes in ileal mucosa of *E. coli*-infected pigs, perhaps leading to the reduction in diarrhea reported previously.

Key words: *Escherichia coli*, gene expression, immunomodulation, pigs, plant extracts

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INTRODUCTION

Escherichia coli postweaning diarrhea is one of the major causes of mortality in weaned pigs (NAHMS, USDA, 2008). The *E. coli* infection affects not only gut physiology but also immune responses of pigs. The toxins secreted by *E. coli* can stimulate the small intestine to increase water and electrolyte secretion and decrease fluid absorption, resulting in hypersecretory diarrhea (Gyles, 1993; Nataro and Kaper, 1998). In addition, lipopolysaccharide (**LPS**), the major component

[1]Financial support from Pancosma SA, Geneva, Switzerland is greatly appreciated.

[2]Current address: Department of Animal Science and Biotechnology, Chungnam National University, Daejeon, South Korea.

[3]Current address: Department of Animal Production, Nong Lam University, Ho Chi Minh, Vietnam.

[4]Corresponding author: jepettig@illinois.edu

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of the outer membrane of *E. coli*, can activate the innate immune system of pigs, triggering increased production of proinflammatory cytokines from antigen-presenting cells (Webel et al., 1997; Wright et al., 2000).

Feeding low levels of capsicum oleoresin (**CAP**), garlic botanical (**GAR**), or turmeric oleoresin (**TUR**) reduced diarrhea and mitigated disruption of intestinal morphology of weaned pigs caused by *E. coli* infection. Feeding these plants extracts also reduced serum TNF-α and haptoglobin concentrations and the number of white blood cells and lymphocytes in *E. coli*-infected pigs (Liu et al., 2013). These results indicated benefits of plant extracts in alleviating the negative effects of pathogenic infection and maintenance of normal intestinal integrity and function. The 3 plant extracts tested showed different effects, indicating they may work through different mechanisms.

The mucosal layer of the intestine is in direct contact with luminal contents, and contributes to the immune defense against pathogens (Schenk and Mueller, 2008). Therefore, investigating the alteration of ileal mucosa function is important to help understand the underlying mechanisms, by which the plant extracts protect against the consequences of the *E. coli* infection. The objective of this study was to characterize gene expression of ileal mucosa of pigs as affected by experimental infection with a pathogenic *E. coli* and by plant extracts using the porcine genome array followed by quantitative real-time PCR (**qRT-PCR**) validation.

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The experiment was conducted in the disease containment chambers of the Edward R. Madigan Laboratory at the University of Illinois at Urbana-Champaign.

Animals, Housing, Experimental Design, and Diet

A total of 64 weaned piglets with the same number of gilts and barrows (G-Performer boars × Fertilium 25 sows; Genetiporc Inc., Alexandria, MN) averaging 6.3 kg initial BW were selected from 12 sows at the Swine Research Center of the University of Illinois at Urbana-Champaign. The sows and piglets used in this experiment did not receive vaccines against *E. coli*, antibiotic injections or antibiotics in creep feed. After weaning, all pigs were transferred to the disease containment chambers. They were blocked by weight within sex and randomly assigned to treatments with the restriction that litters were balanced across treatments to the extent possible. The pig was the experimental unit. Pigs were housed in individual pens for 9 d (4 d before and 5 d after the first *E. coli* challenge [d 0]). There were 2 suites of 8 chambers, and each suite was used for either *E. coli*-challenged or unchallenged pigs. There were a total of 64 individual pens, 4 in each of 16 chambers, and each diet was represented in each chamber. The piglets had ad libitum access to feed and water.

The treatments were in a 2 × 4 factorial arrangement (with or without *E. coli* challenge and 4 different dietary treatments) with 8 replicates per treatment. In the *E. coli* challenge group, all pigs were inoculated orally with 3 mL F-18 *E. coli*/d for 3 consecutive days from d 0 post infection (**PI**). The *E. coli* strain was derived from a field disease outbreak by University of Illinois Veterinary Diagnostic Lab (isolate number U.IL-VDL # 05–27242). The isolate expressed heat-labile (**LT**), heat-stable (**STb**) and Shiga-like (**SLT-2**) toxins. The inoculum provided at 10^{10} cfu per 3 mL dose in PBS, a dose that previously caused mild diarrhea (Song et al., 2012). In the unchallenged group, pigs were inoculated with 3 mL PBS per day as the sham control (**Sham**) for the 3 consecutive days. The 4 dietary treatments were the complex nursery basal diet (**CON**), and the addition of 10 ppm CAP, 10 ppm GAR, or 10 ppm TUR (Pancosma S. A., Geneva, Switzerland) to the CON, respectively. Capsicum and turmeric are extracted oleoresins, which were standardized to 6% capsaicin and dihydrocapsaicin, and 98% curcuminoides, respectively. Garlic botanical is an extract from garlic, standardized to 40% propyl thiosulfonates. The CON diet was formulated to meet or exceed the NRC (1998) estimates of nutrient requirements of weaned pigs (Table 1). The same batches of ingredients were used for the preparation of all the diets. For mixing the 3 plant-extract diets, 1 of plant extracts was first premixed with 0.8 kg of soybean oil, and then this mixture was remixed with a small amount of corn (10 kg). Finally, the premix of plant extract, soybean oil, and corn was added to and mixed with the remaining ingredients. The same diets were fed throughout the experiment.

Sample Collection

After inoculation, all pigs in the *E. coli* challenged groups were verified to be successfully infected by F18 *E. coli* by analyzing β-hemolytic coliforms in feces (Song et al., 2012). As shown previously (Song et al., 2012) and duplicated in the current study in which the clinical signs of pigs used in this experiment were reported (Liu et al., 2013), d 5 PI is the peak of *E. coli* disease with the greatest diarrhea score and greatest proportion of β-hemolytic coliforms in the feces. Therefore, one-half of the pigs (4 pigs from each treatment, 2 males and 2 females) were euthanized on d 5 PI. Before being euthanized, pigs were anesthetized by intramuscular injection of a 1-mL combination of telazol, ketamine, and xylazine (2:1:1) per 23 kg BW. The final mixture contained

100 mg telazol, 50 mg ketamine, and 50 mg xylazine in 1 mL (Fort Dodge Animal Health, Fort Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway, Vortech Pharmaceuticals, Ltd., Dearborn, MI) per kilogram of BW. The 5-cm ileal samples (collected within 10 cm of the ileocecal junction) were cut longitudinally and washed with ice-cold PBS. The specific location for ileal sample collection were based on the presence of a continuous aggregate of lymphoid tissue as a bulky area of granular appearance, which is involved in the gastrointestinal mucosal immunity (Solano-Aguilar et al., 2000). The mucosal layer of the entire ileal sample was carefully removed by scraping with a surgical scalpel and immediately stored in liquid N for further analysis.

Total RNA Extraction and Gene Expression by Microarrays

Total RNA (4 pigs/treatment) from ileal mucosa isolated at d 5 PI was extracted using a kit according to the manufacturer's instructions (PureLink RNA Mini Kit; Invitrogen, Carlsbad, CA). The RNA quality and quantity were assessed using an analyzer (Agilent 2100 Bioanalyzer; Agilent Technologies, Inc., Santa Clara, CA) and a spectrophotometer (ND-1000 Nanodrop; Thermo Scientific, Wilmington, DE), respectively. All samples used for further analysis had a ratio of optical density read at 260 and 280 nm from 1.9 to 2.1, a ratio of optical density read at 260 and 230 nm of >1.8, and an RNA integrity number of ≥7.7. Double-stranded cDNA was first synthesized and employed as a template for in vitro amplification and labeling (GeneChip Expression 3'-Amplification IVT Labelling Kit; Affymetrix Inc., Santa Clara, CA). Then, cDNA was used to synthesize cRNA, which was hydrolyzed to produce fragmented cRNA in the 35 to 200 nucleotide size range for proper hybridization. The fragmented cRNA was labeled and further hybridized to the porcine genome array (Affymetrix GeneChip Porcine Genome Array; Affymetrix Inc.). Each array consisted of 23,937 probe sets to interrogate 23,256 transcripts in the pig genome, which represents 20,201 genes. Thirty-two chips in total were used in this experiment.

Analysis of Microarray Data

All quality-control assessments, data processing, and statistical analyses were done in R using packages (Bioconductor Project; Gentleman et al., 2004). Quality-control assessment (MacDonald, 2005) showed that all arrays were of acceptable quality. The arrays were processed with the guanine cytosine robust multi-array analysis algorithm, which performs a guanine-cytosine-based background-correction, does a quantile normalization between arrays, and summarizes the multiple probes into a single probe set value using a median polish algorithm (Wu and Irizarry, 2005). Testing for differential gene expression was done by fitting a mixed linear model equivalent to a 2 × 4 factorial ANOVA using the limma package (Che et al., 2011), which uses an empirical Bayes correction that helps to improve detection power by borrowing information across genes (Smyth, 2004). The statistical model included effects of *E. coli* challenge, diet, and their interaction as fixed effects. Block was a random effect. The appropriate pairwise comparisons were fit as contrasts from the model. The following 4 comparisons were of interest: infected control (**ICON**) vs.CON, infected CAP (**ICAP**) vs. ICON, infected GAR (**IGAR**) vs. ICON, and infected TUR (**ITUR**) vs. ICON. A total of 23,937 gene probe sets were included in the porcine array, but only 16,363 probe sets were detected in the ileal mucosa samples. The limma model was fit and *P*-values were calculated using all 16,363 probe sets on the array. The modulated genes were defined by 1.5-fold difference and a cutoff of $P < 0.05$ by parameter tests.

Table 1. Ingredient composition of basal diet (as-fed basis)

Item	Amount, %
Corn, ground	41.54
Whey, dried	15.00
Soybean meal, dehulled	10.82
Fishmeal	10.00
Lactose	10.00
Soy protein concentrate	5.00
Poultry byproduct meal	4.27
Soybean oil	2.67
Mineral premix[1]	0.35
Vitamin premix[2]	0.20
L-Lysine·HCl	0.05
DL-Met	0.05
L-Thr	0.03
L-Trp	0.02
Total	100.00
Calculated energy and nutrients	
ME, kcal/kg	3,480
CP, %	22.67
Fat, %	6.34
Ca, %	0.80
P, %	0.72
Available P, %	0.49
Lys, %	1.50
Lactose, %	21.00

[1]Provided per kg of diet: 3,000 mg of NaCl; 100 mg of Zn from zinc oxide; 90 mg of Fe from ferrous sulfate; 20 mg of Mn from manganese oxide; 8 mg of Cu from copper sulfate; 0.35 mg of I from calcium iodide and 0.30 mg of Se from sodium selenite.

[2]Provided per kg of diet: 2,273 μg of retinyl acetate; 17 μg of cholecalciferol; 88 mg of DL-α-tocopheryl acetate; 4 mg of menadione from menadione sodium bisulfite complex; 33 mg of niacin; 24 mg of D-Ca-pantothenate; 9 mg of riboflavin; 35 μg of vitamin B_{12}; 324 mg of choline chloride.

Bioinformatics Analysis

A bioinformatics resources (DAVID Bioinformatics Resources 6.7; National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD; http://david.abcc.ncifcrf.gov) consists of an integrated biological knowledge base with analytic tools used to systematically extract biological meaning from large gene lists (Huang et al., 2009). In brief, the analysis of selected genes in DAVID was as follows. First, all 16,363 probe sets in the porcine genome array (Affymetrix GeneChip Porcine Genome Array, Affymetrix Inc.) were submitted to DAVID and 5,168 of them were mapped with identified functions for 3,715 porcine genes. These 5,168 genes' Entrez [National Center for Biotechnology Information (NCBI), USHHS, Washington, DC] identifications were uploaded as background for the analysis. Second, the modulated genes with 1.5-fold difference and a cutoff of $P < 0.05$ in each comparison were uploaded as the tested gene list. Third, the parameters and subparameters of interest in this experiment were established. In the present analysis, the main parameters in DAVID included gene ontology and pathways. The sub-parameter under gene ontology was gene ontology for biological process, and the sub-parameter under pathways was Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathways. Finally, the functional annotation chart, which provided typical gene-term enrichment analysis, was run. The expression analysis systematic explorer (**EASE***)* score, a modified Fisher Exact *P*-value, was used to examine the significance of gene-term enrichment with a modified Fisher's exact test. The EASE score <0.05 was considered as significantly affected.

Quantitative Real-Time PCR

The same total RNA (4 pigs/treatment) from ileal mucosa used to run the microarray was also employed for qRT-PCR. First-strand cDNA was produced from 1 μg of total RNA per sample(SuperScript III First-Strand Synthesis SuperMix for qRT-PCR; Invitrogen) in a total volume of 20 μL. Total RNA was denatured at 65°C for 5 min and immediately annealed on ice for at least 1 min. Then, the reverse transcription reaction was performed at 50°C for 50 min, followed by heat inactivation at 85°C for 5 min.

To verify the results from the microarray, quantitative analysis of IL-1 β (***IL1b)***, tumor necrosis factor α ***(TNFa)***, toll-like receptor 4 (***TLR4)***, lipopolysaccharide binding protein ***(LBP)***, and myeloid differentiation primary response gene 88 (***MyD88)*** were assayed by qRT-PCR. In addition, the expression levels of mucin 2 (***MUC2)***, p65 nuclear factor kappa-light-chain-enhancer of activated B cells ***(p65 NFkB)***, p38 mitogen-activated protein kinases ***(p38 MAPK)***, cystic fibrosis transmembrane conductance regulator ***(CFTR)***, and cyclooxygenase 2 (***COX2)*** in ileal mucosa were analyzed by qRT-PCR. Data normalization was accomplished using β-actin as a housekeeping gene, which has been validated as an effective internal control for studying gene expression in porcine ileal mucosa (data are not shown). Primers (Supplementary Table 1) were designed based on published sequences in pigs using the NCBI (USHHS) online primer design tool and published literature, and synthesized commercially (Applied Biosystems, Foster, CA). One hundred nanograms of total RNA were assayed for each sample in triplicate. Each PCR reaction consisted of 5μL of mixture (SYBR Green PCR Master Mix; Applied Biosystems, Foster, CA), 0.4 μL of 10 μM forward primer, 0.4 μL of 10 μM reverse primer, 0.2 μL of DNase/RNase free water, and 4 μL of diluted cDNA. The qRT-PCR analysis was done (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Foster, CA). Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles with 15 sec at 95°C and 1 min at 60°C. The dissociation cycle was 95°C for 15 s plus 65°C for 15 s. Standard curves were generated using serial dilutions of pooled cDNA from all samples. The arbitrary values were calculated based on the standard curve and normalized using the housekeeping genes.

Table 2. Gene expression profiles induced by *Escherichia coli* infection and dietary supplementation of plant extracts to *E. coli*-infected pigs[1]

Dietary supplement	Comparison[2]	Up-regulated	Down-regulated	Total
Escherichia coli	ICON vs. CON	252	166	418
Capsicum oleoresin, 10 mg/kg	ICAP vs. ICON	35	39	74
Garlic botanical, 10 mg/kg	IGAR vs. ICON	53	150	203
Turmeric oleoresin 10 mg/kg	ITUR vs. ICON	37	146	183

[1]The gene expression changed by fold-change cutoff of 1.5 and a *P*-value cutoff of 0.05. All data were analyzed by DAVID Bioinformatics Resources 6.7 (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD).

[2]CON = uninfected control diet–fed pigs; ICAP = infected capsicum oleoresin–fed pids; ICON = infected control diet–fed pigs; IGAR = infected garlic botanical–fed pigs; ITUR = infected turmeric oleoresin–fed pigs.

RESULTS

Gene Expression Profiles Induced by E. coli *and Plant Extracts*

The *E. coli* infection of pigs fed the CON diet altered ($P < 0.05$) the expression of 418 genes in ileal mucosa of pigs compared with the uninfected CON (Table 2). The supplementation of the 3 plant extracts displayed different effects on the gene expression in ileal mucosa of *E. coli*-infected pigs. Compared with

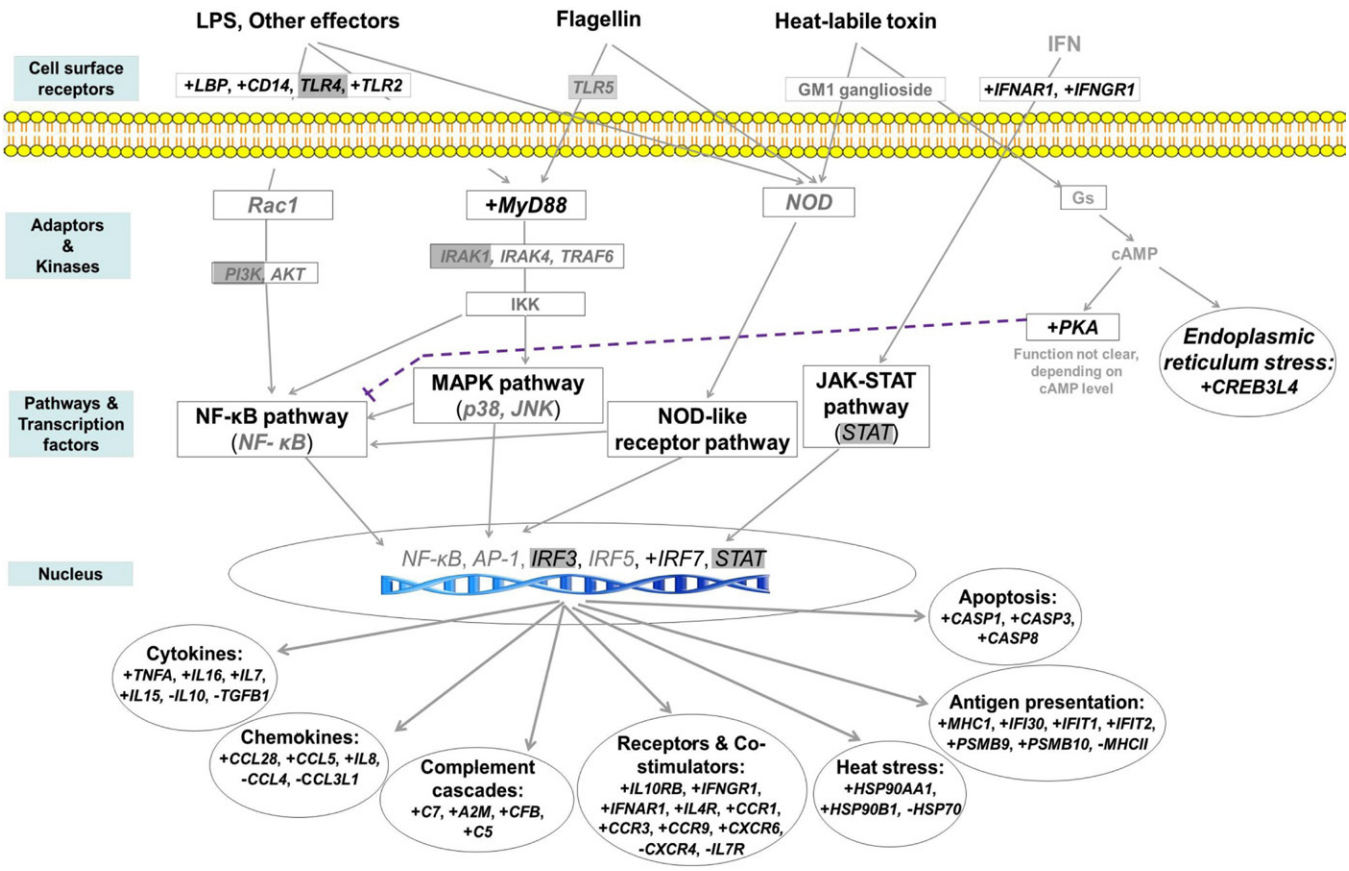

Figure 1. *Escherichia coli* infection induces gut immune responses of pigs. The bolded + sign and bolded − sign represent genes up-regulated and down-regulated, respectively. Genes with gray-shaded backgrounds were not affected by *E. coli* at d 5 postinoculation, whereas genes with gray font did not have information from the gene chip (Affymetrix Inc., Santa Clara, CA). The arrow symbol indicates induction or interaction with other molecules; the blocking symbol with dashed line indicates inhibition. Receptor-mediated pathways: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway; mitogen-activated protein kinases (MAPK) pathway; nucleotide-binding oligomerization domain (NOD)-like receptor pathway; Janus kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway. A2M = α-2-macroglubin; Akt = serine/threonine protein kinase; AP = activator protein; C = complement component; CASP = caspase, apoptosis-related cysteine peptidase; CCL = C–C motif ligand; CCR = chemokine (C–C motif) receptor; CFB = complement factor B; CREB3L4 = cAMP responsive element binding protein 3-like 4; CXCR = chemokine (C–X–C motif) receptor; Gs = stimulatory guaninie nucleotide-binding protein; HSP70 = heat shock protein 70; HSP90AA1 = heat shock protein 90, α; HSP90B1 = heat shock protein 90, β; IFI30 = interferon, γ-inducible protein 30; IFIT = interferon-induced protein with tetratricopeptide; IFN = interferon; IFNAR = interferon (α and β) receptor 1; IFNGR = interferon γ receptor 1; IKK = IkB kinase; IL4R = interleukin 4 receptor; IL7R = interleukin 7 receptor; IL 10RB = interleukin 10 receptor, β; IRAK = interleukin-1 receptor-associated kinase; IRF = IFN-regulatory factor; LBP = lipopolysaccharide binding protein; LPS = lipopolysaccharide; LT = heat-labile toxin; MAPK = motigen activated kinase-like protein; MHC = major histocompatibility complex; MyD88 = myeloid differentiation factor 88; NOD = nucleotide-binding oligomerization domain; PI3K = phosphoinositide-3-kinase; PKA = cAMP dependent protein kinase; PSMB = proteasome subunit β; Rac1 = ras-related C3 botulinum toxin substrate 1; STAT = signal transducers and activators of transcription; TGFB1 = transforming growth factor, β 1; TLR = toll-like receptor; TNF = tumor necrosis factor; TRAF = TNF receptor-associated factor. Feeding plant extracts had several effects counter ($P < 0.05$) to those of *E. coli*. Capsicum oleoresin: *C7, CCL5, CCR3, IFIT1, IFIT2, IFNGR1, IRF7, MHCII,* and *TNFA*; Garlic botanical: *C7, CASP3, CASP8, CCL3L1, CCL4, CFB, HSP90AA1, IFIT1, IFIT2, IL7, IRF7, LBP, TGFB1,* and *TNFA*; Turmeric oleoresin: *C5, C7, CASP3, CASP8, CCL5, CCL28, CCR9, CXCR4, CFB, HSP90AA1, HSP90B1, IFNAR1, IFNGR1, IFIT1, IFIT2, IL8, IL10, IL16, IL7R, IRF7, LBP, MHCII, TGFB1,* and *TNFA*. See online version for figure in color.

the infected CON, CAP-fed pigs had altered ($P < 0.05$) expression of 74 genes, GAR-fed pigs exhibited ($P < 0.05$) 203 changed genes, and TUR-fed pigs showed ($P < 0.05$) 183 altered genes. All these altered genes were mapped in DAVID Bioinformatics Resources.

Differential Immune Gene Expression in Ileal Mucosa of Pigs

The modulated immune gene expression indicated that *E. coli* infection induced gut immune responses of pigs fed the CON diet (Fig. 1). The *E. coli* infection altered the expression level of genes related to LPS activation (increase in *LBP* and *TLR2*), and *MyD88*, cytokines (increase in *IL16, IL7, IL15,* and *TNFA*, decrease in *IL10* and *TGFB1*), chemokines (increase in *CCL28, CCL5* and *IL8*; decrease in *CCL4* and *CCL3L1*), complement cascades (increased *C7, A2M, CFB,* and *C5*), receptors and costimulators (increase in *IL10RB, IFNGR1, IFNAR1, IL4R, CCR1, CCR3, CCR9,* and *CXCR6*; decrease in *IL7R* and *CXCR4*), heat stress (increase in *HSP90AA1* and *HSP90B1*; decrease in *HSP70*), antigen presentation (increase in *MHCI, IFI30, IFIT1, IFIT2, PSMB9,* and *PSMB10*, decrease in *MHCII*), apoptosis (increase in

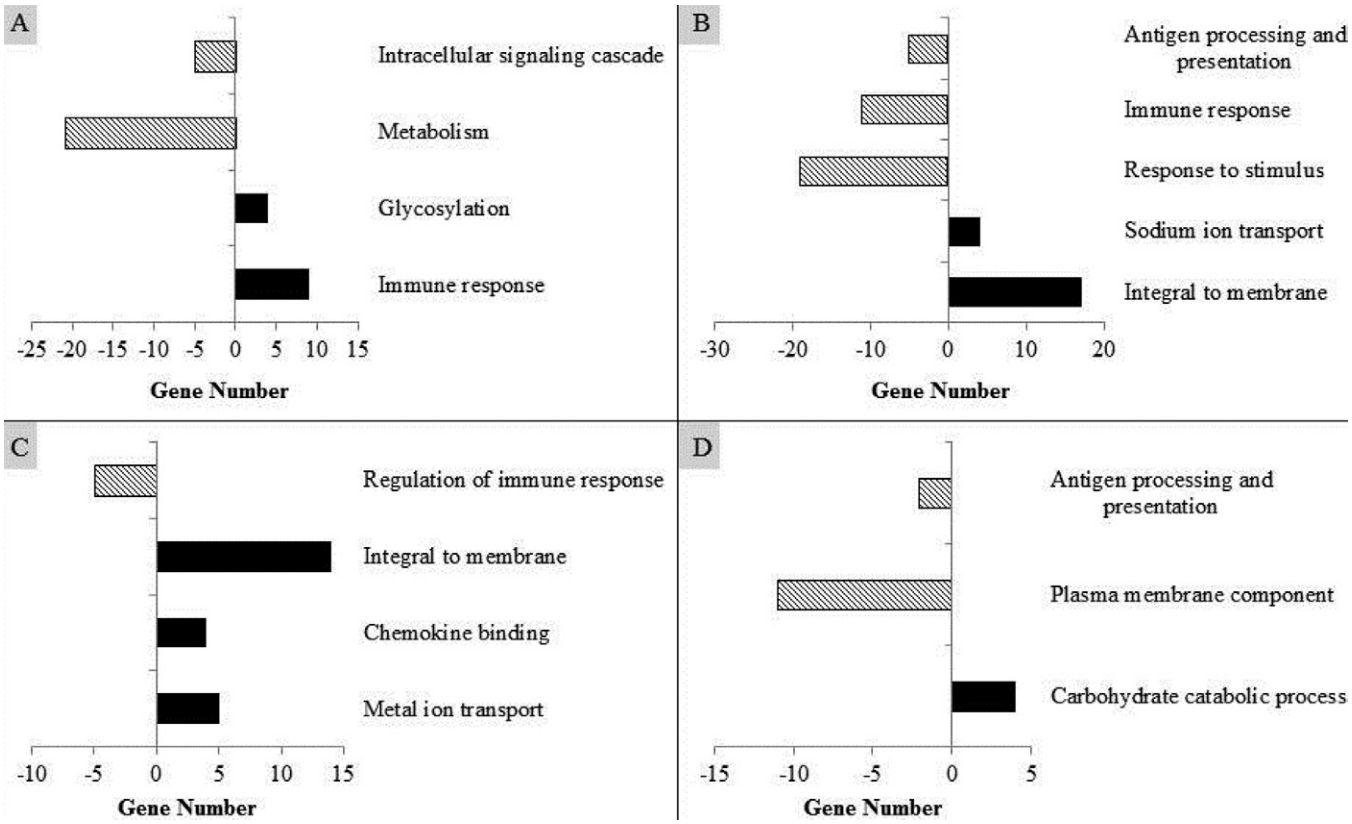

Figure 2. Modulation of biological process of ileal mucosa of pigs by E. coli infection (A) and supplementation of capsicum oleoresin (GAP; B), garlic botanical (GAR; C), or turmeric oleoresin (TUR; D).

CASP1, CASP3, and *CASP8*), and endoplasmic reticulum stress (increased *CREB3L4*).

Feeding each of the plant extracts had effects on expression of several genes that were counter to the effects of *E. coli*. Feeding CAP counteracted ($P < 0.05$) effects of *E. coli* on *C7, CCL5, CCR3, IFIT1, IFIT2, IFNGR1, IRF7, MHCII,* and *TNFA*. Feeding GAR counteracted ($P < 0.05$) the disease effects on *C7, CASP3, CASP8, CCL3L1, CCL4, CFB, HSP90AA1, IFIT1, IFIT2, IL7, IRF7, LBP, TGFB1,* and *TNFA*. Finally, feeding TUR reversed ($P < 0.05$) the effects of *E. coli* on expression of 24 genes, including *C5, C7, CASP3, CASP8, CCL5, CCL28, CCR9, CXCR4, CFB, HSP90AA1, HSP90B1, IFNAR1, IFNGR1, IFIT1, IFIT2, IL8, IL10, IL16, IL7R, IRF7, LBP, MHCII, TGFB1,* and *TNFA*, compared with *E. coli*-infected control.

Biological Process Analysis

The altered genes were analyzed by the gene ontology biological process using DAVID and presented in Fig. 2 and Supplementary Tables 2 to 5. The *E. coli* infection up-regulated (EASE score < 0.05) the expression of genes related to the biological processes of immune response and glycosylation, but down-regulated (EASE score < 0.05) metabolism and intracellular signaling cascade biological processes (Fig. 2A). Compared with the infected CON, the supplementation of CAP up-regulated (EASE score < 0.05) gene expression integral to membranes and Na ion transport, but down-regulated (EASE score < 0.05) the expression of genes associated with 3 different biological processes: response to stimulus, immune response, and antigen processing and presentation (Fig. 2B). Feeding GAR up-regulated (EASE score < 0.05) the expression of genes related to metal ion transport, chemokine binding, and integral to membrane biological processes, but down-regulated (EASE score < 0.05) regulation of immune response in the ileal mucosa of *E. coli*-infected pigs (Fig. 2C). In addition, the addition of TUR up-regulated (EASE score < 0.05) gene expression related to carbohydrate catabolic process, but down-regulated (EASE score < 0.05) the expression levels of genes associated with plasma membrane component and antigen processing and presentation (Fig. 2D).

The Kegg Pathway Analysis

The altered genes were analyzed by the Kegg Pathway using DAVID and presented in Fig. 3 and Supplementary Tables 6 to 9. Compared with the sham CON, *E. coli* infection up-regulated (EASE score < 0.05) genes involved in the following pathways, complement and coagulation cascades, amino sugar and nucleotide sugar metabolism, and metabolism of xenobiotics by cytochrome p450, but down-regulated (EASE score < 0.05) the expression of genes involved in the pathway of spliceosome (Fig. 3A). Compared with the infected CON, CAP up-regulated

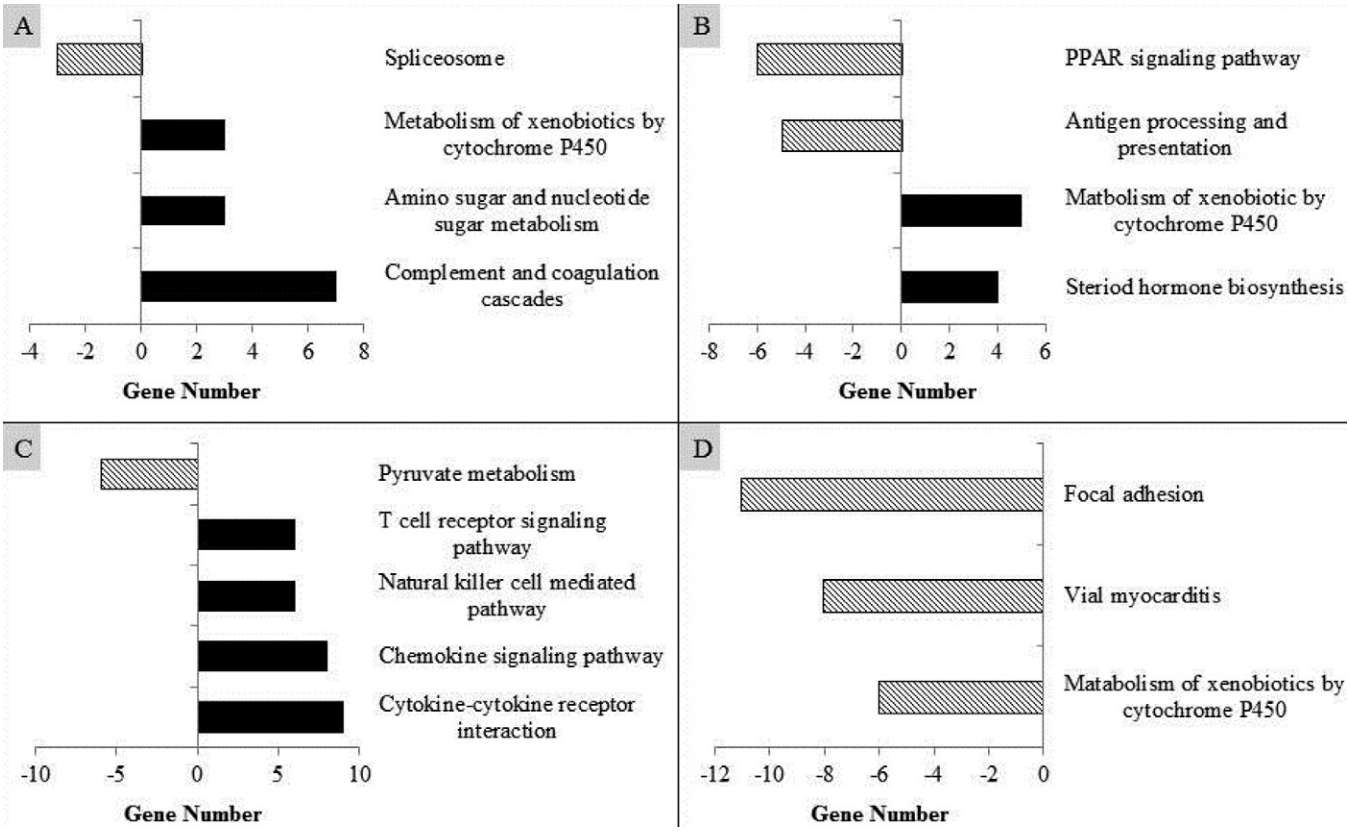

Figure 3. Modulation of Kyoto Encyclopedia of Genes and Genomes Pathway in ileal mucosa of pigs by *E. coli* infection (A) and supplementation of capsicum oleoresin (GAP; B), garlic botanical (GAR; C), or turmeric oleoresin (TUR; D). PPAR = peroxisome proliferator-activated receptor.

(EASE score < 0.05) the expression of genes related to steroid hormone biosynthesis and metabolism of xenobiotics by the cytochrome p450 pathway, but down-regulated (EASE score < 0.05) the gene expression associated with the antigen processing and presentation pathway and peroxisome proliferator-activated receptor (**PPAR**) signaling pathway (Fig. 3B). The supplementation of GAR up-regulated (EASE score < 0.05) gene expression related to 4 different pathways: cytokine–cytokine receptor interaction, chemokine signaling pathway, natural killer cell mediated pathway, and T cell receptor signaling pathway; but down-regulated (EASE score < 0.05) gene expression related to the expression of genes involved in pyruvate metabolism (Fig. 3C). Moreover, the supplementation of TUR down-regulated (EASE score < 0.05) the expression of genes related to metabolism of xenobiotics by cytochrome p450, vial myocarditis, and focal adhesion pathways (Fig. 3D).

Quantitative Real-Time PCR

Five genes, *IL1b, LBP, MyD88, TLR4,* and *TNFa* were tested by qRT-PCR to verify the expression of genes detected by microarray and as shown in Table 3, the transcriptional changes in these genes as assessed by qRT-PCR showed similar patterns when compared with the original microarray data, although the magnitude of the responses of those genes varied from one method to another with the exception to the *MyD88* responses.

Another 5 genes not included in the microarray, *CFTR, COX2, MUC2, p38 MAPK,* and *p65 NFkB*, were tested by qRT-PCR. The *E. coli* infection increased ($P < 0.05$) the expression of *COX2* and *p38 MAPK* in the ileal mucosa of pigs, but feeding the plant extracts counteracted ($P < 0.05$) this effect. In addition, *E. coli* infection reduced ($P < 0.05$) the expression of *p65 NFkB* in the ileal mucosa of pigs. No effect of plant extracts on *p65 NFkB* was detected. Otherwise, feeding CAP increased ($P < 0.05$) the expression of *CFTR* and *MUC2*, whereas feeding TUR enhanced ($P < 0.05$) *MUC2* gene expression in the ileal mucosa compared with the infected control.

DISCUSSION

General

The present results describe the impacts of infection with a pathogenic *E. coli* on gene expression in the ileal mucosa of the weaned pigs and indicate that feeding specific plant extracts to pigs challenged with *E. coli* changed the expression levels of genes related to the biological processes of membrane integrity and immune responses. Several of the changes in expression

Table 3. Verification of gene expression in ileal mucosa by quantitative real-time PCR (qRT-PCR)[1,2]

	Fold Change							
	ICON vs. CON		ICAP vs. ICON		IGAR vs. ICON		ITUR vs. ICON	
Gene[3]	Microarray	qRT-PCR	Microarray	qRT-PCR	Microarray	qRT-PCR	Microarray	qRT-PCR
IL1B	−1.13	−3.64*	1.03	2.69*	1.75	3.69*	1.56	6.78*
LBP	4.39	1.68*	−1.24	−1.63*	−2.41	−1.18	−3.77	−1.34
MyD88	1.54	1.02	−1.97	−1.28*	1.13	−1.48*	−1.39	−1.13
TLR4	1.23	1.21	−1.62	−1.52*	−1.44	−1.08	−1.72	−1.10
TNFa	2.21	1.04	−1.89	-−1.34	−1.58	−1.32	−1.62	−1.37
CFTR	–	1.31	–	1.72*	–	1.21	–	1.05
COX2	–	5.37*	–	−1.73*	–	−1.62*	–	−2.65*
MUC2	–	1.19	–	1.36*	–	1.17	–	1.43*
p38 MAPK	–	1.47*	–	−1.55*	–	−1.78*	–	−1.40*
p65 NFkB	–	−1.19*	–	1.07	–	1.07	–	1.02

*Different ($P < 0.05$) in qRT-PCR studies.

[1]The total RNA samples (4 pigs/treatment) that were used to run the porcine microarray (Affymetrix Inc., Santa Clara, CA) were employed for qRT-PCR.

[2]Negative value indicates reduction in gene expression. CON = uninfected control diet–fed pigs; ICAP = infected capsicum oleoresin–fed pigs; ICON = infected control diet–fed pigs; IGAR = infected garlic botanical–fed pigs; ITUR = infected turmeric oleoresin–fed pigs.

[3]The average threshold cycle values for *IL-1B*, lipopolysaccharide binding protein (*LBP*), myeloid differentiation primary response gene 88 (*MyD88*), toll-like receptor 4 (*TLR4*), tumor necrosis factor alpha (*TNFa*), cystic fibrosis transmembrane conductance regulator (*CFTR*), cyclooxygenase 2 (*COX2*), mucin 2 (*MUC2*), p38 mitogen-activated protein kinases (*p38 MAPK*), p65 nuclear factor kappa-light-chain-enhancer of activated B cells (*p65 NFkB*) were 28.7, 30.2, 22.6, 24.6, 27.3, 25.1, 23.2, 23.2, 17.4, 22.0, respectively; β-*actin* (ACTB) was used as endogenous control for qRT-PCR.

of key genes caused by plant extracts were counter to the changes caused by *E. coli*, and may help explain the benefits of reduced diarrhea and improved gut morphology previously observed (Liu et al., 2013).

Host Gene Response to E. coli *Infection*

The mucosal surface in the intestine has dual functions. The surface allows exchange of nutrients and ions across the intestinal epithelium and supports an immune defense against potentially harmful luminal antigens and microbes (Schenk and Mueller, 2008). Therefore, the delicate balance of intestinal immunity is important for animal health and growth. Pathogenic *E. coli* and other pathogenic bacterial infections can induce a defect of intestinal mucosal immunity and enhance inflammation in animals and humans (Savkovic et al., 2003; Sansonetti, 2006; Srinivasan and McSorley, 2006). In the present study, the increased expression of immune genes, such as *LBP, MyD88, C7, CCL28, CFB, TLR2, C5, SLA-1, AMCF-II, B2M, IRF7, IL8,* and *TNFA* indicates that the F18 *E. coli* used here induced gut inflammation of weaned pigs (Fig. 1). The increased expression of *LBP* and *MyD88* indicates that LPS, the major component of the outer membrane of *E. coli*, may be involved in the activation of immune responses. These results are consistent with other studies that found activation of the innate immune system of pigs by LPS (Webel et al., 1997; Wright et al., 2000). Several pathways, such as the NF-κB pathway (Savkovic et al., 1997), MAPK pathway (Czerucka et al., 2001; Savkovic et al., 2001), nucleotide-binding oligomerization domain–like receptor pathway (Chen et al., 2009), and Janus kinase-Signal Transducer and Activator of Transcription pathway (Ceponis et al., 2003), may be involved in the process of *E. coli* infection, but it was previously not clear which pathways were of primary importance. The qRT-PCR results showed *E. coli* infection up-regulated p38 MAPK but down-regulated p65 NFkB, indicating both of these 2 pathways may be involved in the *E. coli* infection. Such changes result in downstream upregulation of production of proinflammatory cytokines and secretion of chemokines, followed by the enhancement of the complement system (Parkin and Cohen, 2001). In the present study, *E. coli* infection increased the expression level of some genes related to the Kegg Pathway of complement and coagulation cascades, indicating increased immune responses in the intestinal mucosa of infected pigs. Otherwise, the increased IL-8 expression in the ileal mucosa indicated that another potent pro-inflammatory mediator from the flagella of *E. coli*, flagellin, might be involved in the activation of intestinal immune responses. Zhou et al. (2003) and Im et al. (2009) have reported flagellin induced the production of IL-8 in the epithelial cells. Interleukin-8 can facilitate the recruitment of inflammatory associated immune cells, such as polymorphonuclear leucocytes (Savkovic et al., 1996; 2003; Zhou et al., 2003). Cyclooxygenase-2 is the inducible form of the prostaglandin synthetase enzymes that catalyzes the committed step in the prostaglandin production pathway (Dubois et al., 1998). The expression of COX-2 is increased by several different stimuli under inflammatory conditions, including proinflammatory cytokines such as TNFα (Akarasereenont et al., 1995; Abdalla et al., 2005). The qRT-PCR revealed upregulation

of *COX-2* genes, which is directly related to the intensity of organ inflammation (Poljakovic et al., 2001; Jana et al., 2009), providing further evidence of increased gut mucosa inflammation caused by *E. coli*. Therefore, the present results support the clinical responses of *E. coli*-infected pigs in our previous study, indicated by increased white blood cells and serum proinflammatory cytokines and acute phase proteins (Liu et al., 2013).

The present study indicated that *E. coli* infection reduced the expression levels of genes linked to several metabolic processes of pigs, including protein synthesis (*Rps23, rps20,* and *rpl14*), unwanted protein degradation (*Uchl1* and *UBC1*), fatty acid metabolism (*Cpt1b* and *PLCD4*), carbohydrate metabolism (*PGAM2* and *GAPDH*), DNA repair (*RAP80*), and intracellular transcription factors (*NOR-1* and *OCT2*). However, the infection increased the expression level of several genes related to amino sugar and nucleotide sugar metabolism. In addition, previous studies reported that the reduced growth caused by *E. coli* infection was related to suppressed protein synthesis and accumulation in the muscle tissues (Jepson et al., 1986; Tian and Baracos, 1989). Similarly, our clinical data showed *E. coli* infection reduced growth rate and feed efficiency (Liu et al., 2013), which may be related to the increased immune responses of pigs, decreased protein synthesis, or both.

Capsicum Oleoresin on E. coli *Infection*

Capsicum oleoresin, obtained from peppers, contains capsaicin as the major active component involved in maintenance of mucosal integrity and protection against gastric mucosal injury induced by noxious agents (Abdel Salam et al., 1997, 1999, 2005). In the present study, the supplementation of CAP up-regulated the expression level of genes related to the integrity of membranes, such as *Ocln, SLC5A1, ST6GALNAC-V, SLC5A4, SLC4A4, Slc5a2, Abcg2, LPAR2, SLC7A7, Slc15a1,* and *ITGB8*. Among these genes, *Ocln* encodes an integral membrane protein, occludin, that is required for formation and maintenance of tight junctions (McCarthy et al., 1996). The *ITGB8* encodes integrin β 8, a receptor, which plays an important role in mediating cell–cell and cell–matrix interaction and communication (Benoit et al., 2009). The genes *SLC5A1, SLC5A4, SLC4A4, Slc5a2, Abcg2, LPAR2, SLC7A7,* and *Slc15a1* are related to the cell membrane transporters and channels, which facilitate the transport of glucose and different ions, and help to maintain ion homoeostasis in the small intestine (Sterling and Casey, 2002; Robey et al., 2009; Choi et al., 2010). Diarrhea may occur when tight junctions of epithelial cells that cover the small intestine become disordered (Hecht, 1995; El Asmar et al., 2002; Sawada et al., 2003). The microarray results indicate that feeding dietary CAP enhances the integrity of membranes, especially several proteins involved in the tight junctions, which may support the reduced diarrhea of pigs infected with *E. coli* compared with the control diet in our clinical data. Mucins play a central role in maintenance of the gut barrier function through interfacing with food, water, and luminal microoragnisms. Mucin 2 is the major core polypeptide of membrane-associated and secretory gel-forming mucins in the intestine, and it plays an important role in defense against inflammation (Hollingsworth and Swanson, 2004; Nishida et al., 2009; Hansson, 2012). Therefore, qRT-PCR results show that feeding CAP up-regulated the expression of MUC2 and supported a beneficial effect of CAP on the gut barrier.

Capsicum oleoresin fed to pigs affected the gut mucosal immune responses by downregulating the expression of genes in the categories of responses to stimulus and antigen processing and presentation. These genes include *DDX58, C7, SLA-DRB1, Hsp27, IL1RN, IRG6, SLA-2, C2, mx1, TNFRSF1A, SLA-1, CD163,* and *B2M*. The downregulation of *B2M* and *DDX58* genes and genes of the major histocompatibility complex, including both *MHCI* (*SLA-1* and *SLA-2*) and *MHCII* (*SLA-DRB1*), may indicate moderation of the increase of antigen processing and presentation caused by *E. coli* infection. The primary function of *MHCI* is to continuously present intracellular antigen-derived peptides to cytotoxic T lymphocytes, ensuring a rapid immune response against infectious pathogens and initiating the adaptive immune response (Yang, 2003). Similarly, *MHCII* presents processed peptides from exogenous antigen to helper T lymphocytes, driving both the humoral and cell-mediated immune responses (Radosevich and Ono, 2003). The DDX58 encodes a pattern-recognition receptor, called retinoic-inducible gene-1 protein that has antiviral responses and regulation of immune response (Takeuchi and Akira, 2008). Perhaps the reduced antigen presentation contributes to the attenuation of the expression of components in the complement system, such as *C2* and *C7* (Sarma and Ward, 2011), as well as the reduced mRNA expression of other inflammatory mediators, including *IRG6, TNFα* and *TNFRSF1A, IL1RN, mx1,* and *Hsp27*. Among these genes, *IRG6* encodes an interferon-induced antiviral protein, viperin, which is induced in lymphoid cells and dendritic cells during acute infection and is highly induced in neutrophils and macrophages (Hinson et al., 2010). The *TNFRSF1A* expresses a major receptor for TNF-α, activates the transcription factor NF-κB, and regulates inflammation (Micheau and Tschopp, 2003). The *Hsp27* encodes an ATP-independent chaperone that can regulate cell apoptosis and enhance NF-κB activity (Parcellier et al., 2003). Moreover, the qRT-PCR results showed that feeding CAP down-regulated *COX-2* gene expression in the ileal mucosa. Consistent with our clinical data, these gene expression results support the observation that feeding CAP attenuated the increased

total white blood cell numbers and serum inflammatory mediators, TNF-α and haptoglobin, caused by *E. coli* infection (Liu et al., 2013). Thus, CAP may reduce the immune responses of pigs infected with *E. coli*.

Garlic Botanical on E. coli *Infection*

Garlic botanical is extracted from garlic, standardized to 40% propyl thiosulfonates. Feeding GAR to *E. coli*-infected pigs down-regulated the mRNA expression of several immune genes, such as *C7, CASP3, CASP8, CFB, HSP90AA1, IFIT1, IFIT2, IL7, IL10RB, IRF7, LBP, MHCI*, and *TNFA*, related to the regulation of immune responses. The reduced *LBP* indicated that GAR might decrease the impact of LPS, a cell-wall component of *E. coli*. The IRF7 is a transcription factor and potentially induced by TNF-α and LPS. It is involved in regulation of a variety of cellular functions, such as IFN-mediated immune responses, cell growth, and apoptosis (Ning et al., 2011). Therefore, at the transcriptional level, the *IRF7* expression was consistent with the reduced *LBP*, indicating that the failure of LPS activation may block the transcriptional signaling pathway mediated by *IRF7*. The *CASP3* and *CASP8*, encoding death proteases involved in cell apoptosis, were also in agreement with the possibly reduced LPS activation by GAR (Porter and Jänicke, 1999). The reduced mRNA expression of *CFB, IFIT1, IFIT2,* and *MHCI* indicated feeding GAR may decrease antigen presentation (de Veer et al., 1998; Jensen, 2007; Pichlmair et al., 2011).

On the other hand, the supplementation of GAR up-regulated other genes related to cytokine–cytokine receptor interaction, chemokine signaling pathway, natural killer cell mediated pathway, and T cell receptor signaling pathway. Among these pathways, several immune genes, including *CXCR6, Ccr3, IFNG, Cxcl9, CCL4, CCL3L1, CCR9, CCR5, FYN, FCGR3B, CD8B, Cd3 g, Cd8a*, and others were involved. The *cxcl9, CCL4, CCL3L1, CXCR6, Ccr3, CCR9,* and *CCR5* encode several chemokines and chemokine receptors, which play an important role in host defense because of their abilities to trigger leukocyte mobilization to sites of injury (Bennett et al., 2011). The *CD8B, Cd3 g*, *cd8a* and *IFNG* code the molecules involved in the cytotoxic T cell signaling pathway, which recognizes peptides bound to the MHCI and helps to remove these pathogenic peptides from the host (Frankenberger et al., 2005). However, the CD8-positive T cells require various stimuli, such as cytokines, costimulatory molecules, and other immune cells, to become fully activated and to induce differentiation and proliferation (Ito and Seishima, 2010), and these other stimuli were not increased by GAR. Therefore, the increase in molecules related to cytotoxic T cells does not indicate increased antigen presentation and immune responses.

In the present study, GAR increased the expression of some genes of the immune system and reduced expression of others. Overall, the reduction in the transcriptional signaling pathway and antigen presentation indicates the net effect of GAR was to attenuate the increased immune responses caused by *E. coli* infection. These results were also in agreement with the qRT-PCR result, indicated by the reduced COX-2 gene expression, and also by our clinical data, indicated by the reduced serum haptoglobin and total white blood cell numbers when GAR was fed to *E. coli*-infected pigs (Liu et al., 2013). Further evidence for the modulation of immune responses in response to GAR comes from the study of Kim et al. (2013) showing that feeding GAR to *Escherichia acevulina*-infected chickens inhibited the expression of *TLR3, TLR5, TNFRSFB*, and other genes involved in the inflammatory response.

Turmeric Oleoresin on E. coli *Infection*

Turmeric oleoresin is specifically known for wound-healing and anti-inflammatory properties (Aggarwal et al., 2007; Krishnaswamy, 2008). In the present study, feeding TUR reduced the mRNA expression of several immune genes in the ileal mucosa of *E. coli*-infected pigs. These genes were involved in LPS recognition and subsequently transcriptional signaling (*LBP* and *MyD88*), interferon effects (*IFNAR1* and *IFNGR1*), antigen presentation (*IFIT1, IFIT2, MHCI,* and *MHCII*), cytokines and receptors (*IL16, IL10RB,* and *TNFA*), chemokines (*CCL5, CCL28, CCR9,* and *IL8*), complement cascades (*C5, C7,* and *CFB*), heat stress (*HSP90AA1* and *HSP90B1*), and cell apoptosis (*CASP3* and *CASP8*; Porter and Jänicke, 1999; Jensen, 2007; Ning et al., 2011). Curcuminoids are the vital constituents of turmeric that were beneficial in treatment of various chronic inflammatory conditions such as cancer and arthritis (Shishodia et al., 2005; Aggarwal et al., 2007; Goel et al., 2008). Ahmed and Gilani (2011) reported that a curcuminoid mixture effectively decreased caspase-3 (encoded by *CASP3* gene) level in the hippocampus after 20 d of treatment. The current microarray results indicated that feeding TUR reduced the gut inflammation of *E. coli*-infected pigs, especially the impact of LPS released by *E. coli*. In addition, the qRT-PCR results showed that feeding TUR down-regulated the expression of COX-2, which suggests a reduction of gut inflammation by TUR.

Feeding TUR down-regulated the mRNA expression of plasma membrane components, including the genes involved in the tight junctions (*CLDN3, cdh5, CLDN4,* and *Ocln*). The *CLDN3* and *CLDN4* encode proteins claudin 3 and claudin 4, which are considered as major proteins in the structural backbone of tight junctions (Van Itallie et al., 2001; Elkouby-Naor and

Ben-Yosef, 2010). The *Ocln* codes another transmembrane protein, occludin, associated with tight junctions. Occludin interacts with claudin and is involved in the regulation of intermembrane diffusion and paracellular diffusion of small molecules (Balda et al., 1996: Tsukita and Furuse, 1999). The *cdh5* encodes cadherin, a transmembrane protein that function as adherens junctions (Oda and Takeichi, 2011). Previous studies reported that dysfunctional tight junctions may induce diarrhea of humans and animals during a pathogenic bacterial infection (Hecht, 1995; Sears and Kaper, 1996; Sawada et al., 2003). However, the qRT-PCR measurement of the *MUC-2* gene indicates that feeding TUR increases the secretion of mucins, which may enhance the gut barrier function and contribute to the reduced diarrhea of *E. coli*-infected pigs. Therefore, the information about the delicate relationships between tight junction proteins and diarrhea of pigs is still fragmentary, and more research may need to be conducted.

Conclusions

Overall, the analysis of gene expression patterns shows that dietary plant extracts affected the expression of genes in ileal mucosa of pigs at d 5 post infection with *E. coli*. The increased expression of genes related to membrane structure and function in the ileal mucosa of *E. coli*-infected pigs consuming CAP and GAR may enhance the gut mucosa health. Feeding CAP and TUR increased the *MUC2* gene expression. These findings support earlier observations that feeding these plant extracts are associated with reduced diarrhea of pigs infected with *E. coli* (Liu et al., 2013). Moreover, feeding the 3 plant extracts each reduced the mRNA expression of several immune genes involved in antigen presentation and other immune responses–related pathways, indicating that these plant extracts may attenuate the increased immune responses caused by *E. coli* infection. These results also support observations that feeding each of the 3 plant extracts reduce total white blood cell numbers and serum inflammatory mediators compared with the control diet (Liu et al., 2013). In conclusion, the current findings provide new insights into the immunomodulatory and physiolomodulatory properties of plant extracts. Feeding plant extracts provide benefits by reducing the over stimulation of the immune system by *E. coli* and enhancing gut physiological defenses.

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Phytotherapy as an alternative for treating fish disease

G. M. R. VALLADÃO
S. U. GALLANI &
F. PILARSKI

Laboratory of Aquatic Organisms Pathology, Aquaculture Center of São Paulo State University (CAUNESP), Jaboticabal, Brazil

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Intensification of livestock rearing often promotes an increase in inappropriate practices that disregard care for the environment, animal health, and workers' health. Intensive fish farming systems are often associated with higher stocking density and massive use of artificial feed. Currently, outbreaks of parasitic, bacterial, and fungal diseases act as major limiting factors for fish farming, meaning that producers have to make use of massive amounts of antibiotics, disinfectants, and pesticides in order to control mortality and avoid huge economic losses. Because of adverse effects on the aquatic environment, terrestrial organisms, and human health (both fish handlers and consumers), this therapy has been criticized. Use of herbal medicines within animal production has shown promise, in that it is natural and biodegradable and has antimicrobial activity against various pathogens, including those relating to fish. Recently, researchers have reported promising effects from many herbal medicines for treating parasitic diseases caused by protozoa and metazoa, and broad activity against bacteria and fungi. This review addresses the current issues regarding indiscriminate use of chemicals and antibiotics in aquaculture and discusses the main findings and methodologies of the latest research on herbal medicines to stimulate and accelerate research in this field, especially in developing countries.

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Gustavo Moraes Ramos Valladão, LAPOA – Laboratory of Aquatic Organisms Pathology, Aquaculture Center of São Paulo State University (CAUNESP), Via Prof. Paulo Donato Castellane, s/n, CEP: 14884-900 Jaboticabal, SP, Brazil. E-mail: gmrvalladao@gmail.com

INTRODUCTION

Over recent years, farming of aquatic organisms has been encouraged throughout the world. It received special emphasis in 2012, when this form of farming overtook beef production (Earth Policy Institute, 2013). In the production chain, the demand for maximum productivity over a short period of time and at lower cost leads to some barriers that directly or indirectly affect the health status of the product. High stocking densities (Garcia *et al.*, 2013), poor nutritional quality, poor quality of the aquatic environment (Longo *et al.*, 2013), and lack of biosafety measures are among the major problems in aquaculture, especially in rearing fish, because these conditions favor the spread of pathogen and/or immunosuppression of host.

Researchers have been stimulated to find solutions to health problems in aquaculture. The solutions for controlling and preventing fish diseases include development of prophylactic agents as vaccines (Pridgeon & Klesius, 2013) and development of immunostimulant diets (Skalli *et al.*, 2013). In this regard, several natural products have shown promising prophylactic results and improvement of immune response in fish (Samad *et al.*, 2014; Vaseeharan & Thaya, 2014). Nonetheless, to control outbreaks of mortality caused by pathogens, it is essential to use a therapeutic product at some stage of production, with the aim of at least reducing the pressure of pathogens on the host.

There is a big gap in discussing treatments used to control fish diseases. The lack of veterinary products registered for use in aquaculture is a current problem in most countries worldwide. Until the present day, much of the information about treatments has been extrapolated from cold water fish (especially salmonids) to freshwater fish (Athanassopoulou *et al.*, 2004). The drugs used in aquaculture are antimicrobials, disinfectants, pesticides, and other chemicals, which have an uncertain future because of the various negative effects that they present toward the aquatic environment (Rico & Van den Brink, 2014), animals, and humans (Sapkota *et al.*, 2008). In a review on the use of chemotherapy in salmon, Burridge *et al.* (2010) reported that there was a great need for research to develop appropriate interventions with lower risks.

Phytotherapeutics with activity for treating bacterial infections and parasitic infestations of humans and animals have been described for centuries (Silva & Fernandes-Júnior, 2010). Thus, such agents can be considered to have promise with regard to therapeutic control over pathogens of fish. *In vitro* tests have recently been conducted to search for parasiticides (Ji *et al.*, 2012; Yi *et al.*, 2012), bactericides (Ostrand *et al.*, 2012; Albert & Ransangan, 2013), and fungicides (Xue-Gang *et al.*, 2013) against major pathogens in aquaculture. Moreover, some *in vivo* tests have shown positive effects relating to treatment of and recovery from various fish diseases (Harikrishnan *et al.*, 2005; Muniruzzaman & Chowdhury, 2008; Abd El-Galil & Aboelhadid, 2012; Schelkle *et al.*, 2013).

The present review clarifies the prospects in relation to current and future treatments of diseases in fish farming and discusses the main herbal medicines surveyed with a view to use as parasitic, bacterial, and fungal agents.

REVIEW OF THE LITERATURE

Intensification of aquaculture and pathogens

For decades, one of the main health barriers caused by intensification of fish farming has been occurrences of parasitic infestations (Kugel *et al.*, 1990). Several parasitic agents affect fish production, with effects ranging from reduced productivity (Evans *et al.*, 2007) to outbreaks of mortality (Khan, 2009), which consequently generate significant economic losses. Among the major parasitic diseases in aquaculture, those caused by parasites that are visible to the naked eye, that is, lernaeids (Raissy *et al.*, 2013) and branchiurans (Pekmezci *et al.*, 2011), can be highlighted, along with microscopic parasites such as myxosporeans (Müller *et al.*, 2013), trichodinids (Valladão *et al.*, 2014b), monogeneans (Akoll *et al.*, 2012), and *Ichthyophthirius multifiliis* (Wei *et al.*, 2013). Each of these types is responsible for causing huge losses in fish production.

Opportunistic infections caused by bacterial agents are common in aquaculture and may have primary or secondary origin. However, this usually occurs when the host is exposed to adverse environmental or farming conditions that lead diminished effectiveness of the immune system, which may be due to poor nutrition, stress, or recurrent challenges by parasite infestations (Xu *et al.*, 2012a,b).

Among the main bacterial diseases in fish from continental waters are those notoriously caused by bacteria of the genera *Streptococcus* (Zhang *et al.*, 2013a), *Aeromonas* (Griffin *et al.*, 2013), *Flavobacterium* (Sebastião *et al.*, 2013; Evenhuis *et al.*, 2014), *Edwardsiella* (Park *et al.*, 2012; Hawke *et al.*, 2013), and *Francisella* (Soto *et al.*, 2013).

Treatment in aquaculture

Conventional treatments for fish diseases. The treatment of diseases in farm animals is necessary regardless of the species farmed or type of rearing. The pathogen pressure on hosts is high in intensive farming systems, and sometimes it is essential to use therapeutic drugs. The situation is no different in aquaculture. The most common substances used in aquaculture are disinfectants, pesticides (Rico & Van den Brink, 2014), and antibiotics (Cabello, 2006).

The main criticisms surrounding the use of these drugs is the presence of residues in water, sediment, and fish; toxicity in nontarget organisms such as plants, crustaceans, and even wild fish; carcinogenic potential for handlers and consumers; and bacterial resistance (Sapkota *et al.*, 2008; Tavechio *et al.*, 2009; Burridge *et al.*, 2010; Rico *et al.*, 2013; Rico & Van den Brink, 2014). Nonetheless, producers have made use of highly toxic substances, including molecules that have not been registered for treating fish diseases, which is worrying.

In aquaculture, disinfectants are used to reduce the load of pathogenic micro-organisms on the biological surface of aquatic organisms, and their use on fish is associated with broad-spectrum, fast-acting, and low-cost characteristics (Burka *et al.*, 1997). The best-known substances are hydrogen peroxide, quaternary ammonia, formalin, and malachite green. These compounds are mainly used for treating parasitic (Picón-Camacho *et al.*, 2012) and fungal diseases (Sudova *et al.*, 2007) in fish.

Among the disinfectants, formalin is one of the main products used in treating diseases caused by *Saprolegnia parasitica* (Gieseker *et al.*, 2006), monogeneans (Pahor-Filho *et al.*, 2012), trichodinids (Noga, 2010), and *I. multifiliis* (Heinecke & Buchmann, 2009). However, fish that survive the treatment may present compromised health (Tieman & Goodwin, 2001). Although disinfectants have low potential for bioaccumulation in the environment and in aquatic organisms, their deleterious effects on fish and on workers through direct contact with this product present risks, thus making it uncertain whether there is a future for their use in aquaculture (Picón-Camacho *et al.*, 2012).

Malachite green is widely used for controlling various fish parasites and especially for combating *I. multifiliis*. It has been shown to be effective against this important pathogen of fish (Srivastava *et al.*, 2004). However, this product has been banned in several countries because of its mutagenic and carcinogenic effects (Picón-Camacho *et al.*, 2012) and its high persistence in the environment, given that it bioaccumulates in the ecosystem and in fish tissue (Henderson *et al.*, 1997).

Use of antibiotics and veterinary drugs in aquaculture can pose risks to food safety and consumers (Heuer *et al.*, 2009; Love *et al.*, 2011), as well as causing toxic injuries to fish and deleterious effects on the aquatic ecosystem (Tavechio *et al.*, 2009). Most parasiticides and disinfectants currently used in aquaculture are highly toxic and are able to affect nontarget organisms. These factors contribute to environmental degradation (Rico *et al.*, 2012). In addition to affecting human health by containing toxic and mutagenic effects, parasiticides present higher environmental risk than antibiotics (Rico & Van den Brink, 2014). In study of most important aquaculture region (Asia), disinfectants showed acute toxicity for all three evaluated taxonomic groups (primary producers, invertebrates, and fish). The highest ecological risks were calculated for the par-

asiticides deltamethrin and ivermectin, the disinfectants trichloroisocyanuric acid and dibromodimethylhydantoin, and the antibiotics amoxicillin, levofloxacin, and sulfadiazine (Rico & Van den Brink, 2014).

Antibiotics are another class of products widely used in combating important fish diseases. Florfenicol and oxytetracycline are the best-known molecules in Brazil, and their use in aquaculture has been approved for controlling bacterial diseases in several species of fish (US Food & Drug Administration, 2012). However, several other antimicrobials have been used in aquaculture (Rico *et al.*, 2013), even without registration and approval. Inappropriate use of antibiotics in treatment and prophylaxis for bacterial fish diseases is associated with increasing of problems related to selection of resistant bacteria. Resistance is not unique to the bacteria of fish, but occurs in relation to all bacteria present in the environment or in other animals and even humans, thus constituting a serious public health problem (Sapkota *et al.*, 2008; Burridge *et al.*, 2010).

More than 15 years ago, Burka *et al.* (1997) described 'off-label' use of disinfectants, pesticides, and antibiotics in treating diseases within aquaculture. This has continued to be a widely used practice up to the present day, and it is characterized by use of substances in larger doses than those that have been approved, nonapproved administration routes, and even use in treatments for other diseases in different animal species. Athanassopoulou *et al.* (2004) reported that this practice occurs, for example, through extrapolation of treatments approved for diseases of salmonid species and other different diseases. Given the deleterious effects of certain drugs used in aquaculture, 'off-label' use poses an even greater risk to the environment and to animals and humans. Table 1 highlights the disinfectants, pesticides, and antibiotics used in treating fish diseases in Brazil. Except for the antibiotics florfenicol and oxytetracycline, the other products have not been legalized in this country for this purpose. This means that the dosage, exposure time, withdrawal period, toxicity, and environmental impact have not been fully established for most fish species.

Phytotherapy: alternative treatment. Phytotherapy, by definition, consists of treatment using products obtained from medicinal plants, or derivatives thereof, with prophylactic, curative, or palliative purposes (Brazil, 2011). Medicinal plants have broad antiparasitic, antibacterial, and antifungal activity proven by scientific studies on animals and humans (Silva & Fernandes-Júnior, 2010). Although herbal medicines have been used as therapy for human diseases for centuries, their potential for use in aquaculture has only recently been discussed. This has come about because of the demand for products to replace the current drugs, which pose great risks to the aquatic environment.

Regarding the antimicrobial properties of substances derived from plants, studies on controlling parasitic infections in humans and animals, including fish, have been stimulated (Abd El-Galil & Aboelhadid, 2012). Their use in fish farming can contribute toward reducing the use of chemotherapeutic parasiticides, thereby providing greater sustainability for fish production, with reduced environmental risks, while having the capacity to treat fish diseases and consequently avoid mortality and therefore economic losses. A few years ago, phytotherapeutics were studied in relation to treatments for some fish parasites (Ekanem *et al.*, 2004a,b; Chitmanat *et al.*, 2005; Steverding *et al.*, 2005). They have gained prominence over more recent years through the publication of numerous papers in important aquaculture journals.

An extensive search for studies in which herbal medicines were used to treat fish parasites was conducted, and the data from this search have been presented in Table 2. These data may help future researchers in selecting herbal medicines to deepen the research and development studies on new products. It can be seen that herbal medicines against *I. multifiliis* parasites and various species of monogeneans have been widely studied. This is mainly because of the great importance of these fish parasites in aquaculture worldwide and the lack of safe treatments, and this information is discussed in this review.

The main pathway for treating parasitic infections consists of bathing the fish in water containing the phytotherapeutic agent. However, use of herbal medicines in water for large-scale treatments is difficult (i.e., in commercial fish farming), which may explain the lack of commercial products. In contrast, these substances are already in use in fishkeeping, where the treatment pathway of bathing in water is facilitated. Some products made from medicinal plants are already found in aquarium shops around the world.

In view of the great diversity of types of herbal products, methodologies, and target pathogens, comparisons between studies are complicated. The present review revealed that few studies have been conducted regarding the effects of phytotherapeutics for treating parasitic diseases when these are added to the diet. The effectiveness of these molecules when added to the diet can increase the feasibility of their use in aquaculture. Therefore, research on this administration route should be encouraged, especially in relation to endoparasites or ectoparasites that have part of their cycle in the host tissue, like *I. multifiliis*. This type of treatment is a challenge, as it requires technical knowledge, so that the treatment starts at the first signs of the disease, when the fish are still feeding.

Table 1. Some of the major substances used in the treatment of fish diseases in Brazil

Disinfectants	Formaldehyde
	Potassium permanganate
	Copper sulfate
	Chloramine-T
Pesticides	Diflubenzuron
	Malathion
	Methyl parathion
Antibiotics	Florfenicol
	Oxytetracycline
	Enrofloxacin
	Chloramphenicol
	Erythromycin
	Trimethoprim + Sulfametoxazol

Table 2. Phytotherapics with greatest potential for use in aquaculture to treat parasitic diseases

		Phytotherapic		Most effective treatment				
Parasite	Host	Type/Active compound	Plant	Route	Period	Concentration	Results	Authors
Argulus spp.	*Carassius auratus*	Piperine	*Piper longum*	Water	48 h	9.0 mg/L	100% efficacy compared to control	Kumar *et al.* (2012)
Dactylogyrus intermedius	*Carassius auratus*	Osthol and isopimpinellin	*Fructus cnidii*	Water	48 h	Osthol 1.6 mg/L and isopimpinellin 9.5 mg/L	100% efficacy compared to control	Wang *et al.* (2008)
Dactylogyrus intermedius	*Carassius auratus*	Methanol extract	*Semen aesculi*	Water	48 h	10 mg/L	100% efficacy compared to control	Liu *et al.* (2010)
		Aqueous extract				12 mg/L	100% efficacy compared to control	
Dactylogyrus intermedius	*Carassius auratus*	Dioscin and polyphyllin D	*Paris polyphylla*	Water	48 h	Dioscin EC50* = 0.44 mg/L and polyphyllin D EC50 = 0.70 mg/L	1.5–3.0 times more effective than the positive control, mebendazole	Wang *et al.* (2010a)
Dactylogyrus intermedius	*Carassius auratus*	Gracillin	*Dioscorea zingiberensis*	Water	48 h	0.9 mg/L	High antiparasitic activity; almost 10 times more effective than the positive control, mebendazole	Wang *et al.* (2010b)
Dactylogyrus intermedius	*Carassius auratus*	Sanguinarine	*Macleaya microcarpa*	Water	48 h	0.7 mg/L	100% efficacy compared to control	Wang *et al.* (2010c)
Dactylogyrus intermedius	*Ctenopharyngodon idella*	Chelerythrine	*Chelidonium majus*	Water	48 h	1.60 mg/L	100% efficacy compared to control	Li *et al.* (2011)
Dactylogyrus intermedius	*Carassius auratus*	Osthole	*Radix angelicae pubescentis*	Water	48 h	1.6 mg/L	100% efficacy compared to control	Wang *et al.* (2011a)
Dactylogyrus intermedius	*Carassius auratus*	Bruceine A and bruceine D	*Brucea javanica*	Water	48 h	1 mg/L	Bruceine A = 97% efficacy and bruceine D = 91.2% efficacy; 2–2.5 times more effective than the positive control, mebendazole	Wang *et al.* (2011b)
Dactylogyrus intermedius	*Carassius auratus*	Methanol extract	*Radix Bupleuri chinensis*	Water	48 h	10 mg/L	100% efficacy compared to control	Wu *et al.* (2011)
Dactylogyrus intermedius	*Carassius auratus*	Aqueous extract	*Cinnamomum cassia*	Water	48 h	30 mg/L	100% efficacy compared to control	Ji *et al.* (2012)
		Methanol extract				40 mg/L	100% efficacy compared to control	
Dactylogyrus intermedius	*Carassius auratus*	Methanol extract	*Dryopteris crassirhizoma*	Water	48 h	60 mg/L	100% efficacy compared to control	Lu *et al.* (2012)

Table 2. (continued)

Parasite	Host	Phytotherapic		Most effective treatment			Results	Authors
		Type/Active compound	Plant	Route	Period	Concentration		
Gyrodactylus spp.	*Gasterosteus aculeatus*	Essential oil	*Melaleuca alternifolia*	Water	48 h	30 ppmv	90% efficacy compared to control	Steverding *et al.* (2005)
Gyrodactylus elegans and *Dactylogyrus extensus*	*Carassius auratus*	Methanol extract	*Piper guineense*	Water	96 h	1.5 mg/L	Approx. 45% efficacy against *Dactylogyrus* and 75% against *Gyrodactylus* compared to control	Ekanem *et al.* (2004b)
Gyrodactylus turnbulli	*Poecilia reticulata*	Freeze-dried	*Allium sativum*	Water	One time	0.03 mg/L	95% efficacy compared to control	Schelkle *et al.* (2013)
Ichthyobodo necator	*Oncorhynchus keta* and *Oncorhynchus masou*	Epigallocatechin gallate	*Camellia sinensis*	Water	5 min	0.9%	100% efficacy compared to control	Suzuki *et al.* (2006)
Ichthyophthirius multifiliis	*Carassius auratus*	Methanol extract	*Mucuna pruriens*	Water	72 h	200 mg/L	92% (skin) and 91% (gill) efficacy; Fish mortality decreased 45% compared to control	Ekanem *et al.* (2004a)
		Petroleum-ether extract	*Carica papaya*		96 h	250 mg/L	92% efficacy (skin); Fish mortality decreased 30% compared to control	
Ichthyophthirius multifiliis	*Ctenopharyngodon idella*	Sanguinarine	*Macleaya cordata*	Water	48 h	0.9 mg/L	96.8% efficacy (gill) compared to control	Yao *et al.* (2010)
Ichthyophthirius multifiliis	*Squaliobarbus curriculus*	Dihydrosanguinarine and dihydrochelerythrine	*Macleaya microcarpa*	Water	48 h	dihydrosanguinarine 5.18 mg/L and dihydrochelerythrine 9.43 mg/L	Both showed high antiparasitic activity (EC50*: 5.19 and 9.43 mg/L, respectively)	Yao *et al.* (2011a)
Ichthyophthirius multifiliis	*Carassius auratus*	Aqueous extract	*Capsicum frutescens*	Water	4 h	1:32 and 1:64 (v:v)	Decreases parasite prevalence (13.3 % and 40 %, respectively) compared to 100 % in control	Ling *et al.* (2012)
Ichthyophthirius multifiliis	*Carassius auratus*	Methanol extract	*Magnolia officinalis* and *Sophora alopecuroides*	Water	1 h	*M. officinalis* 40 mg/L and *S. alopecuroides* 320 mg/L	Decreases tomont survival (24.7 % and 44.7 %, respectively) compared to 91.7 % in the control	Yi *et al.* (2012)

Table 2. (continued)

Parasite	Host	Phytotherapic		Most effective treatment			Results	Authors
		Type/Active compound	Plant	Route	Period	Concentration		
Ichthyophthirius multifiliis	*Ictalurus punctatus*	Pentagalloylglucose	*Galla chinensis*	Water	10 days	20 mg/L	93.3 % survival of infected catfish compared to 0 % in the control	Zhang *et al.* (2013b)
Ichthyophthirius multifiliis	*Carassius auratus*	Chelerythrine and chloroxylonine	*Toddalia asiatica*	Water	72 h	1.8 mg/L chelerythrine and 8 mg/L chloroxylonine	Decreases parasite prevalence compared to control (50 % and 60 %, respectively)	Shan *et al.* (2014)
Ichthyophthirius multifiliis	*Piaractus mesopotamicus*	Essential oil	*Melaleuca alternifolia*	Water	2 h / day for 5 days	50 µl/L	99.8 % (skin) and 98.8 % (gill) efficacy; 56.33 % fish survival compared to 0 % in the control	Valladão *et al.* (2015)
Monogenea	*Heterobranchus longifilis*	Ethanol extract	*Artemisia annua*	Water	1 h	200 mg/L	85 % efficacy compared to control	Ekanem and Brisibe (2010)
Myxobolus sp.	*Diplodus puntazzo*	Essential oil	*Origanum minutiflorum*	Oral (Feed)	35 days	8 mL 5/Kg of biomass	Decreases parasite prevalence (37 to 39 % compared to control) and infestation intensity	Karagouni et al. (2005)
Neobenedenia sp.	*Lates calcarifer*	Allicin	*Allium sativum*	Water	1 h	15.2 µl/L	Decreased significantly hatching (approx. 90 % compared to control group) and longevity of oncomiracidium	Militz *et al.* (2014)
Pseudodactylogyrus	*Anguilla anguilla*	Ginkgolic acid C13:0 and C15:1	*Ginkgo biloba*	Water	48 h	C13:0 (2.5 mg/L) and C15:1 (6.0 mg/L)	100 % efficacy compared to control group	Wang *et al.* (2009)
Trichodina sp.	*Oreochromis niloticus*	Crude extract	*Allium sativum* and *Terminalia catappa*	Water	48 h	800 mg/L	Decreases 100% of the parasites on the skin and fins	Chitmanat *et al.* (2005)
Trichodina sp.	*Oreochromis niloticus*	Aqueous extract	*Camellia sinensis*	Water	5 min	0.9%	Decreases 95% of the parasites on the skin and fins	El-Deen (2010)
Trichodina sp.	*Parabramis pekinensis*	Chelidonine, chelerythrine, and sanguinarine	*Chelidonium majus*	Water	48 h	chelidonine 1.0 mg/L, chelerythrine 0.8 mg/L, and sanguinarine 0.7 mg/L	EC50* 20–30 times lower than positive control (formalin)	Yao *et al.* (2011b)

Table 2. (continued)

Parasite	Host	Phytotherapic		Most effective treatment			Results	Authors
		Type/Active compound	Plant	Route	Period	Concentration		
Trichodina sp. and *Gyrodactilus* sp.	*Oreochromis niloticus*	Crushed garlic cloves	*Allium sativum*	Water	indefinite	300 mg/L	Reduced 23% of parasitized fish compared to control group	Abd El-Galil and Aboelhadid (2012)
Uronema sp.	*Pampus argenteus*	Essential oil	*Melaleuca alternifolia*	Water	30 min/day for 5 days	20 ppm	Healing of skin lesions	Al-Yaqout and Azad (2010)

*EC50, Effective concentration to kill 50% of parasites.

A wide range of phytotherapeutics is also known to have antibacterial activity against human pathogens (Ushimaru *et al.*, 2012) and animal pathogens (Dal-Pozzo *et al.*, 2011). Replacement of the current antimicrobials used in aquaculture, with herbal products, is not a utopian concept given that several medicinal plants have shown activity against important pathogenic bacteria of fish, such as *Aeromonas hydrophila* (Muniruzzaman & Chowdhury, 2008; Harikrishnan *et al.*, 2009, 2010c), *Streptococcus iniae* (Abutbul *et al.*, 2004; Zilberg *et al.*, 2010), *Streptococcus agalactiae* (Zilberg *et al.*, 2010), *Flavobacterium columnare* (Rattanachaikunsopon & Phumkhachorn, 2010), *Pseudomonas fluorescens* and *Edwardsiella tarda* (Muniruzzaman & Chowdhury, 2008). There is great concern with the emergence of bacterial strains of the aquatic environment that are resistant to antibiotics, and this tends to increase with the wrong use in aquaculture, mainly with its use as a prophylactic (Cabello, 2006). On the other hand, phytotherapics are less prone to development of bacterial resistance (Kulkarni *et al.*, 2013) because of their mode of action affecting several targets at the same time (Bakkali *et al.*, 2008). The main phytotherapeutics used in treating bacterial fish diseases have been reviewed in Table 3.

The most common administration routes for herbal medicines used in treating bacterial diseases are by immersion or orally. Because of the large number of studies, many types of phytotherapeutics, and different forms of treatment, comparison between the data in the literature is difficult. This review table may foster development of future research in the field of alternative treatments against bacterial fish diseases.

Oral treatment has been showing excellent survival results in fish with bacterial disease. Batches of sick fish need to be treated even if some of them are not eating, because within the same fish cages or ponds, there are many subclinically infected fish and/or newly infected fish, which have a high chance of cure. Therefore, use of herbal medicines in the diet is promising for treating large batches of fish that are suffering from bacterial diseases, so as to be able to avoid large losses.

Studies on the effects of herbal medicines in treating fungal diseases in aquaculture were also reviewed here. Use of eucalyptus extract at 100 mg/L showed significant fungus growth inhibition and a high hatching rate for *Rutilus frisii* eggs (Najafi & Zamini, 2013). An extract of *Radix sanguisorbae* was considered to be a promising phytotherapeutic for treating eggs that were experimentally infected with *Saprolegnia australis* (Cao *et al.*, 2013). Use of herbal medicines in water for treating fungi in eggs is facilitated in incubators or small tanks, compared with their use in ponds or cages. This is because it is possible to have high density and large numbers of eggs in incubators or small tanks, with easy management. In these cases, the efficacy of the substance is not affected by the massive presence of debris and organic matter that is seen in the cultivation system and thus it is certain that the target organism (in this case, the eggs) will be exposed to the product. The major studies involving treatment of fungal diseases within aquaculture are shown in Table 4.

Table 3. Phytotherapics with greatest potential for use in aquaculture to treat bacterial diseases

		Phytotherapic		Most effective treatment				
Bacteria	Host	Type/Active compound	Plant	Route	Period	Concentration	Results	Authors
Aeromonas hydrophila	*Channa punctatus*	Ethyl acetate extract	*Solanum nigrum*	Water	10 min/day for 30 days	1 g/L	Recovery of the injuries; potential treatment of ulcer	Rajendiran *et al.* (2008)
Aeromonas hydrophila	*Carassius auratus*	Ethanol extract	*Azadirachta indica* + *Curcuma longa* + *Ocimum sanctum*	Oral (Feed)	30 days	2.5 g/kg	100% survival compared to 5% of untreated group	Harikrishnan *et al.* (2009)
Aeromonas hydrophila	*Cyprinus carpio*	Aqueous extract	*Azadirachta indica* + *Curcuma longa* + *Ocimum sanctum*	Oral (Feed)	30 days	0.1% of diet	35% lower mortality rate than in control group	Harikrishnan *et al.* (2010a)
Aeromonas hydrophila	*Carassius auratus*	Concoction†	*Azadiracta indica* + *Ocimmun sanctum* + *Curcuma longa*	Water	5 min/day for 45 days	1%	26.6% lower mortality rate than in control group	Harikrishnan *et al.* (2010c)
Aeromonas hydrophila and *Pseudomonas fluorescens*	*Barbodes gonionotus*	Bulb extract	*Allium sativum*	Oral (Feed)	10 days s.i.d.**	3% LW* of feed mix	100% and 90% recovery (*A. hydrophila* and *P. fluorescens*, respectively) compared to 0% in control group	Muniruzzaman and Chowdhury (2008)
Edwardsiella tarda	*Pangasius hypophthalmus*	Decoction‡	*Calotropis gigantea*	Oral (Feed)	10 days s.i.d.**	3% LW* of herbal mix	96.66% recovery compared to 0% in control group	Muniruzzaman and Chowdhury (2008)
Flavobacterium columnare	*Oreochromis niloticus*	Aqueous extract	*Centella asiatica*	Water	Single bath two days postinfection	100 mg/L	100% survival compared to 50% in control group	Rattanachaikunsopon and Phumkhachorn (2010)

*LW, liveweight; **s.i.d., once a day; †Combination of various phytotherapics macerated into a liquid; ‡Phytoterapic extracted by boiling.

Table 4. Phytotherapics with greatest potential for use in aquaculture to treat fungal diseases

Fungus	Host	Phytotherapic		Most effective treatment			Results	Authors
		Type	Plant	Route	Period	Concentration		
Aphanomyces invadans	*Cyprinus carpio*	Aqueous extract	*Azadirachta indica*	Water	5 min/day for 24 days	1%	Complete recovery of the injuries and the hematological values were near normal when compared with the control group	Harikrishnan *et al.* (2005)
Aphanomyces invadans	*Cirrhina mrigala*	Ethanol extract	*Azadirachta indica*	Oral (Feed)	30 days	0.2%	Untreated group showed higher white blood cell count compared to treated group	Harikrishnan *et al.* (2010b)
Saprolegnia australis	*Carassius gibelio* eggs	Aqueous extract	*Radix sanguisorbae*	Water	Single bath	1280 mg/L	88% survival (37% higher than that achieved with a concentration of 0.2 mg/L of malachite green)	Cao *et al.* (2013)
Fungus unspecified	*Rutilus frisii* eggs	Essential oil	*Eucalyptus* spp.	Water	1 h/day for 3 days	100 mg/L	High hatching rate of treated eggs (approx. 30% higher than control group) and significant growth inhibition of the fungus	Najafi and Zamini (2013)

Negative effects of using phytotherapeutics. Deleterious effects and contraindications cannot be ignored when discussing the exposure of living organisms to chemical molecules, whether these are natural or not. The toxic effect of herbal medicines on aquatic organisms has been poorly studied. These effects vary significantly according to the type of phytotherapeutic and the species of the exposed organism. Some authors have described cases of acute and chronic toxicity of phytotherapeutics in fish. For acetone extract of *Morus alba*, the 96-h LC50 for grass carp was approximately eight times higher than the 4-h EC50 of nonencysted tomonts of *I. multifiliis*. For ethyl acetate extract, the 96-h LC50 for grass carp was 29 times higher than the 4-h EC50 of nonencysted tomonts of the same parasite (Fu *et al.*, 2014), which represents a wide safety margin for its use for fish. These studies are essential, because the lack of such information can be an obstacle when developing new products.

Nonetheless, studies on the toxicity of herbal medicines in nontarget organisms are highly encouraged by the authors of the present review. Although these substances are natural and biodegradable, their use in water can expose nontarget organisms that are more sensitive than fish, such as microcrustaceans and molluscs. Among the few studies that have been conducted, Conti *et al.* (2014) reported that the essential oil of *Melaleuca alternifolia* presented acute toxicity against arthropod nontarget *Daphnia magna*, with a 24-h LC50 of 80.64 ppm, while the LC50 for killing the target *Aedes albopictus* was about three times higher. In contrast to that study, Valladão *et al.* (2015) were successful in treating severe cases of ichthyophthiriasis in *Piaractus mesopotamicus* fish using two-hour daily baths of the essential oil of *M. alternifolia* for five days, using a concentration of 50 ppm, which shows that this has great potential for use in aquaculture.

CONCLUSION

Medicinal plants have broad antimicrobial activity against important fish pathogens. Further studies on chronic and acute toxicity and on the deleterious effects of herbal medicines on treated organisms, nontarget organisms, and the environment are encouraged. Most studies on effectiveness have been based on *in vitro* testing or have even been conducted under laboratory conditions. Therefore, further practical and economic studies are needed to enable replacement of the current treatments. Therefore also, joint work between the supply chain, industry, and researchers is paramount.

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A medicinal herb-based natural health product improves the condition of a canine natural osteoarthritis model: A randomized placebo-controlled trial

Maxim Moreau [a,b], Bertrand Lussier [a,b], Jean-Pierre Pelletier [b], Johanne Martel-Pelletier [b], Christian Bédard [c], Dominique Gauvin [a,b], Eric Troncy [a,b,*]

[a] Research Group in Animal Pharmacology of Quebec (GREPAQ), Department of Veterinary Biomedical Sciences – Faculty of Veterinary Medicine, Université de Montréal, P.O. Box 5000, Saint-Hyacinthe, Quebec J2S 7C6, Canada
[b] Osteoarthritis Research Unit, University of Montreal Hospital Research Centre (CRCHUM), Tour Viger, 900 St-Denis Street, Montreal, Quebec H2X 0A9, Canada
[c] Department of Pathology and Microbiology – Faculty of Veterinary Medicine, Université de Montréal, P.O. Box 5000, Saint-Hyacinthe, Quebec J2S 7C6, Canada

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ABSTRACT

An oral herb-based natural health product (NHP) was evaluated in the canine natural osteoarthritis model. At baseline, the peak vertical force (PVF, primary endpoint) and case-specific outcome measure of disability (CSOM) were recorded in privately-owned dogs. Dogs (16/group) were randomized to receive NHP formulations or a negative control. The PVF was measured at week (W) 4 and W8. Daily locomotor activity was recorded using accelerometer. The CSOMs were assessed bi-weekly by the owner. The NHP-treated dogs ($n = 13$) had higher PVF at W4 ($p = 0.020$) and W8 ($p < 0.001$) when compared to baseline. The changes at W8 were higher than control dogs ($n = 14$, $p < 0.027$) and consistent with Cohen's d effect size of 0.7 (95% confidence interval: 0.0–1.5). The NHP-treated dogs had higher locomotor activity at W8 ($p = 0.025$) when compared to baseline. No significant change was observed for the CSOM. The NHP improved the clinical signs of osteoarthritis in this model.

1. Introduction

Osteoarthritis (OA) is by far the most common human musculoskeletal disease, affecting millions worldwide (Lawrence et al., 2008). The prevalence of OA in dogs is also high, particularly in geriatric animals, being estimated to be five times that observed in mature adults (Shearer, 2011). In dogs, OA results mainly from traumatic insults to the cranial cruciate ligament (CCL), and hip or elbow dysplasia (McLaughlin, 2001; Roush, 2001). Cascades of biological and biomechanical events then merge to induce and perpetuate structural changes at the level of the entire joint, which, as in humans, lead to crippling pain, disability and poor quality of life (Cook, 2010; Johnston, 2001; Madsen and Svalastoga, 1994; Martinez, 1997; Martinez and Coronado, 1997).

Naturally-occurring models of OA have been proposed to accelerate the development of human therapeutics (Pelletier et al., 2010), and a recent review of experimental data underlined the high translationability of outcomes obtained from canine OA models, in particular the response to treatment (Moreau et al., 2013). Undertaking a trial in privately-owned dogs afflicted by natural OA would provide preclinical data and additional evidence on the therapeutic potential of new compounds under development. Of note, the potential of several therapeutic approaches has been tested in different randomized controlled trials (RCTs) in the canine natural OA model using force platform gait analysis as an outcome measure of pain-related functional impairment. These tested compounds include non steroidal anti-inflammatory drugs (NSAIDs) (Budsberg et al., 1999; Moreau et al., 2003, 2007), therapeutic diets (Moreau et al., 2012b; Rialland et al., 2013; Roush et al., 2010) as well as natural substances (naturaceuticals) used to restore or maintain good health status (Hielm-Bjorkman et al., 2009; Moreau et al., 2004, 2012a). The latter therapeutic class is considered by the authors as natural health products (NHPs) which originate from plants, fruits and vegetables, animals, microorganisms and marine sources.

Address of the institution where the work was carried out: This study was conducted at the Research Group in Animal Pharmacology of Quebec (GREPAQ), Department of Veterinary Biomedical Sciences – Faculty of Veterinary Medicine, Université de Montréal, P.O. Box 5000, Saint-Hyacinthe, Quebec J2S 7C6, Canada, in collaboration with the Osteoarthritis Research Unit, University of Montreal Hospital Research Centre (CRCHUM), Tour Viger, 900 St-Denis Street, Montreal, Quebec H2X 0A9, Canada.

* Corresponding author. Department of Veterinary Biomedical Sciences – Faculty of Veterinary Medicine, Université de Montréal, P.O. Box 5000, Saint-Hyacinthe, Quebec J2S 7C6, Canada. Tel.: +1 450 773 8521 ext. 8399; fax: +1 450 778 8103.
E-mail address: eric.troncy@umontreal.ca (E. Troncy).

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Currently, no effective therapy seems able to alleviate the clinical signs of OA in humans or dogs. As relief of pain and the preservation of joint structure cannot be claimed with certainty for currently approved treatments, there is a need for effective strategies to improve the condition of afflicted patients.

Medicinal herbs have long been used in traditional medicine and there is considerable evidence that such NHP and their derivatives may play beneficial roles in OA (Mobasheri, 2012). *Harpagophytum procumbens*, also known as devil's claw, is a South African plant which includes harpagoside as one of its major biologically active phytochemical compounds. A large body of evidence supports the efficacy of harpagoside and related extracts in alleviating symptoms of OA in humans (Gagnier et al., 2004). Resin extracts from the *Boswellia serrata* tree have been demonstrated to be effective in alleviating the clinical signs of OA in humans (Kimmatkar et al., 2003) and dogs (Reichling et al., 2004). Active phytochemical compounds isolated from *Ribes nigrum* leaves showed anti-inflammatory properties *in-vivo* in chrondrocyte assays (Garbacki et al., 2002), while its seed oil was an effective treatment for active rheumatoid arthritis (Leventhal et al., 1994). *Salix alba* extracts have recently been reported to have *in-vitro* chondroprotective properties in primary canine articular chondrocyte culture (Shakibaei et al., 2012). These extracts seem also to be potent in counteracting low back pain in humans (Gagnier et al., 2007). In rodent models of inflammation, an extract from *Tanacetum parthenium* demonstrated antinociceptive and anti-inflammatory effects (Jain and Kulkarni, 1999). Classified as a herb, bromelain is a digestive enzyme found in the stem and the fruit of *Ananas comosus.* This herb has been shown to have anti-inflammatory properties mediated through prostaglandin synthesis (Lotz-Winter, 1990). Finally, curcumin, which is the main biologically active phytochemical compound of *Curcuma longa*, showed inhibitory actions against major inflammatory mediators (Aggarwal et al., 2013; Henrotin et al., 2013; Mathy-Hartert et al., 2009; Mobasheri et al., 2012) while being effective in reducing pain in OA knee patients (Kuptniratsaikul et al., 2009; Madhu et al., 2013). In agreement with those findings, a recent Cochrane systematic review concluded to potential benefits of oral herbal medicines, being more effective than placebo (Cameron and Chrubasik, 2014). However, as also highlighted, further high quality, fully powered studies are required to gain insight in the therapeutic potential of medicinal plants as well for other NHPs (Vandeweerd et al., 2012).

These studies suggest that NHP formulations containing the aforementioned medicinal herbs as principal ingredients might be useful in the management of OA. Whether or not such formulations are effective against the functional impairment that prevails in a model of natural OA needs to be scrutinized rigorously. With the scope of providing strong evidence-based findings, the aim of this RCT was to assess NHP formulations in the canine natural OA model when compared with dogs receiving a placebo over an 8-week duration.

2. Materials and methods

2.1. Design and subject selection

This study was a randomized, double-blind, parallel-group, placebo-controlled trial. Dogs were evaluated over either 56 or 61 days depending on the balanced attribution of locomotor activity recording (see Section 2.3). The trial was conducted under the approbation of the Institutional Animal Care and Use Committee (#Rech-1437) in accordance with the guidelines of the Canadian Council on Animal Care. All owners provided written informed consent.

Adult dogs weighed more than 20 kg and had radiographic evidence of OA exclusively at the hip or stifle joints. Radiographs (hips, stifles, and elbows) were obtained under sedation as previously described (Moreau et al., 2010). Hind limb lameness in association with the presence of OA was confirmed by veterinary surgeons. At the time of screening, all dogs were free of any compound purported to relieve the clinical signs of OA according to washout periods ranging between 4 and 12 weeks. Hence, a 4-week washout period was respected for oral NSAIDs and a 6-week period for NHPs including fatty acid supplements, OA therapeutic diets or treats. Dogs having received injectable pentosan polysulfate sodium or corticosteroid 1 year before the screening visit were not eligible. A 12-week washout period was requested for injectable polysulfated glycosaminoglycan and hyaluronan, and for oral or topical corticosteroid. During the study, dogs were exempted from the administration of any type of medication except those prescribed for exo- and endoparasite control. Additional exclusion criteria were as follows: dogs with surgical repair of the cranial cruciate ligament within 1 year prior to study initiation, dogs suffering from neurologic or other musculoskeletal lesions, dogs that underwent orthopedic surgery within the past year and dogs with CCL disease having gross instability (positive drawer motion upon orthopedic examination).

2.2. Complete blood count and biochemistry panel

To ensure that some parameters were within normal limits during the study, each dog underwent routine blood hematological and biochemical analyses in order to evaluate health status at study initiation (baseline, day 0) as well as at week 4 (day 28) and week 8 (day 56). A veterinary clinical pathologist examined all blood counts and biochemistry panels.

Many herbs can increase the risk of bleeding through anti-platelet properties (Samuels, 2005). The buccal mucosal bleeding time is a simple test commonly used in the clinical setting to detect platelet dysfunction in dogs (Callan and Giger, 2001). Each dog underwent a buccal mucosal bleeding time procedure at baseline and at week 8. Mucosal punctures were performed on the upper labial mucosa, using a disposable, fully automated incision device (Surgicutt® Bleeding Time device, International Technidyne Corporation, USA). This device provided a controlled incision of 1.0 mm (depth) per 3.5 mm (length). The time of incision was noted, and circular filter paper (Whatman®, USA) was held 1–2 mm away from the incision to blot the blood, taking care not to disrupt the clot, or to allow blood to drip into the dog's mouth. The end point was when the incision stopped bleeding. Normal buccal mucosal bleeding time is defined to be less than 3 minutes.

2.3. Randomization, blinding and therapy regimen

Thirty-two privately-owned dogs were randomly allocated in two equal groups (placebo or NHP) according to a permuted-block randomization procedure, which included six blocks of four treatment possibilities (A or B) distributed in a 1-to-1 ratio (i.e. AABB, ABAB, ABBA, BBAA, BABA and BAAB). Among those blocks, eight were randomly selected using random integers to define the treatment allocation sequence. Also, seven blocks were randomly selected using random integers to allocate seven motor activity recordings to treatment A and seven others to treatment B. The 32 treatment allocations (with or without locomotor activity recording) were transcribed on individual cards in sequentially numbered, sealed, opaque envelopes to ensure concealment. A third party was responsible for the randomization process and for the treatment preparation. At the trial site, both treatments were labeled exclusively as treatment A or treatment B and were encapsulated identically. The trialists, the animal health technicians and all dog owners were blinded to which treatment (A or B) was given to each dog. The key code revealing what referred to treatments A and B remained confidential with the third party and was revealed only after study completion and preliminary analyses.

Table 1
Ingredients include in each natural health product formulations.

Ingredients (mg/capsule)	Formulations		Minimal contents
	Alpha	*Beta*	
Medicinal herbs			
Harpagophytum procumbens	240.0	60.0	Harpagosides 2.7%
Boswellia serrata	240.0	180.0	Boswellic acid 79.2%
Ribes nigrum	60.0	60.0	Rutines 1%
Salix alba	50.0	–	Salicin 1%
Tanacetum parthenium	50.0	–	Parthenolide 0.2%
Ananas comosus	–	40.0	2000–2500 GDU
Curcuma longa	–	35.0	Curcuminoids 95%
Omega-3 PUFA			
Total	40.0	40.0	
Eicosapentaenoic acid	0.4	0.4	
Docosahexaenoic acid	9.0	9.0	
Others			
Glucosamine sulfate	–	300.0	
Methylsulfonylmethane	–	90.0	
Chondroitin sulfate	–	60.0	
L-glutamine	–	30.0	
Hyaluronic acid	–	15.0	
Excipient	228.0	280.0	
Total weight/capsule	908.0	1190.0	

GDU, Gelatin digesting unit; PUFA, Polyunsaturated fatty acids.

The ingredients of the NHP formulations are described in Table 1. Dogs allocated to the NHP formulations received the *Alpha* formulation from day 1 to day 29, and then received the *Beta* formulation from day 29 to day 56. The dosing regimen was as follows: one capsule for dogs <25.0 kg; two capsules for dogs 25.0–39.9 kg; three capsules for dogs 40.0–49.9 kg; four capsules for dogs 50.0–59.9 kg and five capsules for dogs >59.9 kg. Dogs allocated to the negative (placebo) control received capsules filled of excipient to match the amount of the NHP formulations. The negative control (placebo) was given under the same dosing regimen as for the NHP formulations.

2.4. Force platform measurement

Peak of the vertically-oriented ground reaction force (PVF) was measured at baseline (day 0), week 4 (day 28) and week 8 (day 56) at the trot (1.9–2.2 m/s) using a force platform, as previously described (Moreau et al., 2010). The PVF was defined as the primary endpoint of the study. Normalized PVF values in percentage of body weight (%BW) from the first five valid trials were used for statistical purposes. To be eligible, dogs must have at least one-hind limb with PVF value lower than 66.0 %BW. This value was consistent to –1 standard deviation (SD) of the PVF value measured in normal dogs (Madore et al., 2007). When unilateral or bilateral lameness was observed, the hind limb having the lowest PVF value determined which one was selected for evaluation. This limb was defined as the most affected limb and was used in the subsequent follow-up of the study. The hind limb selected for evaluation must have been in accordance with orthopedic examination findings, otherwise the dog was excluded. The change in PVF was the mean difference between week 8 – baseline values.

2.5. Locomotor activity recording

Accelerometer-based motor activity recording was accomplished using the Actical® system (Bio-Lynx Scientific Equipment Inc., Canada) as described (Rialland et al., 2012). According to the balanced attribution of motor activity recording, collar-mounted accelerometers were worn by 14 dogs for the entire treatment duration (61 days, 24 h/day) which included a baseline period (day –4 to day 0) that preceded the initiation of treatment administration. This period was used to establish baseline level of locomotor activity recording before treatment. Over the 61 days, the motion was continuously recorded every 2 minutes, giving 720 recordings per day. Daily duration of active period (DDAP) referred to the time spent (expressed in hour per day) when the recording exceeded 30 (no unit) in term of intensity. This cut-off value was based on intern data and was used to discern active from inactive period (Moreau et al., 2011; Rialland et al., 2012). Among the 61 days of continuous recording, three periods were predefined: baseline (day –4 to day 0), week 4 (day 26 to day 28) and week 8 (day 54 to day 56). Owners of dogs allocated to the locomotor activity recording were requested to attend an additional fourth appointment.

2.6. Case-specific outcome measure of disability (CSOM)

Assessment of at-home functional disability was accomplished using CSOM as previously described (Moreau et al., 2012a; Rialland et al., 2012, 2013). Owners assessed the ability of their dogs to perform two to five activities, and scored on a five-point Likert-type scale for each activity that ranged from no problem (zero) to full incapacity (four). Each activity was selected by the owner according to his/her own perception of what characterized the disability of the dog. Assessments were conducted twice weekly using a specific form that remained in the possession of the owner. For each dog, median of the activities scores was determined for each assessment, giving a total of 17 median CSOM scores over the study. Among all the assessments, three periods were predefined: baseline (assessment on day 0), week 4 (assessments on days 24, 28 and 31) and week 8 (assessments on days 49, 52 and 56).

2.7. Statistical analysis

All statistical tests were two-tailed with significance determined by reference to a 5% threshold. Normality of the data was tested using Shapiro–Wilk test. Data were log-transformed when requested to assure transformed data Gaussian distribution. Equality of efficacy was the null hypothesis based on the PVF (primary endpoint) as measured for the hind limb having the lowest value. Per trial log-transformed PVF values were analyzed with a repeated-measures general linear mixed model that included two fixed factors (time and group) and their interaction (time × group interaction), with trials and dogs nested in treatment group as random effects. The change in log-transformed PVF values (week 8 – baseline) were analyzed with a repeated-measures general linear mixed model that included group as fixed factor with trials as random effect. Log-transformed DDAP were analyzed similarly to PVF (period and group as fixed factors) and their interaction (period × group interaction) with days and dogs nested in treatment group as random effects. A repeated-measures generalized linear model was used to analyze median CSOM data under Poisson distribution function using independent working matrix. Fixed factors were period and group and their interaction (period × group interaction) with assessments and dogs nested in treatment group as random effects. Scale factor was estimated by Pearson's chi-square. Covariance structures were defined as recommended (Littell et al., 2000). All *post hoc* analyses were conducted with appropriate Bonferroni adjustments. Data are presented as mean (SD).

2.8. Sample size calculation

According to previous works conducted under similar conditions (Moreau et al., 2007), a sample size of 16 dogs/treatment group ensured that a difference of 4.2%BW in the primary endpoint (PVF) between groups could be detected assuming 75% power, a SD of 4.5 and a 5% significance threshold.

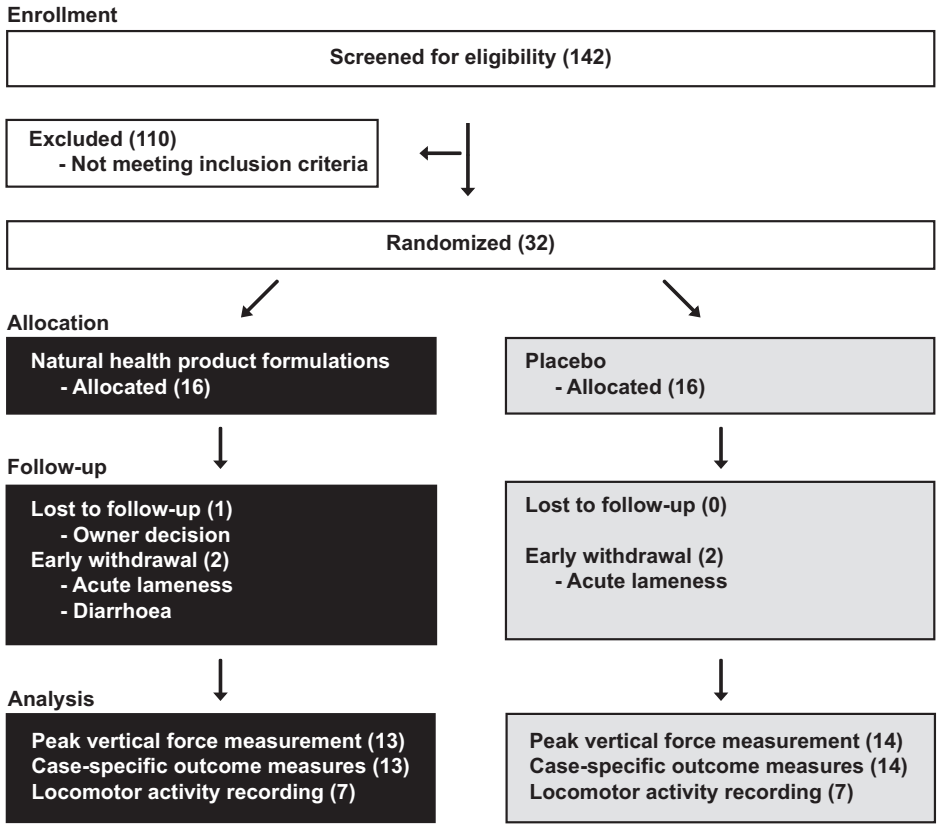

Fig. 1. Flow chart of the study enrolment, allocation, follow-up and analysis.

3. Results

3.1. Animal description

No clinically relevant changes were obtained from hematological and biochemical analyses in the entire study cohort. In addition, abnormal buccal mucosal bleeding times were not observed during the study. The numbers of dogs screened, randomly assigned, and analyzed in each group are detailed in Fig. 1. The NHP dog with persistent diarrhea was diagnosed to have gastrointestinal intolerance. Complete CCL rupture ($n = 2$) and humeral bone inflammation resulted in acute lameness and consequently, to the withdrawal of these dogs.

Baseline characteristics of the dogs stratified per group are presented in Table 2. Groups were well balanced according to the outcomes of interest, as significant difference was not observed for the level of PVF, DDAP and CSOM recorded at baseline. It should be noted that in each group, the dogs did not experience significant change in BW over time.

Table 2
Baseline characteristics of the dogs stratified per group.

Characteristics	Groups ($n = 16$/group)	
	Placebo	Natural health product formulations
Age (months)	71.1 (22.6)	70.8 (33.5)
Sex (male/female)	7/9	10/6
Body weight (kg)	40.7 (8.5)	39.7 (10.8)
Peak vertical force (% body weight)	56.5 (6.2)	56.9 (5.3)
Daily duration of active period (h/day)	6.7 (1.7)	6.9 (2.4)
Case-specific outcome measure of disability	1.6 (0.6)	1.6 (0.6)
Osteoarthritis-afflicted joint (most affected limb)		
Hip (count)	3	4
Stifle (count)	6	8
Hip and stifle (count)	7	4

3.2. Peak vertical force measurement

The PVF generated by the disabled hind limb was increased in the overall study cohort (time effect; $p = 0.016$), without significant group effect ($p = 0.299$) (Fig. 2). Increment in PVF was mostly attributed to the changes observed in the NHP-treated dogs. Hence, a significant time × group interaction ($p < 0.001$) was observed which indicates that groups evolved distinctively from baseline to the end of the study. More specifically, analyses revealed that the PVF of NHP-treated dogs ($n = 13$) was significantly increased at week 4 [58.9 (5.4)%BW, $p = 0.020$] and at week 8 [59.8 (6.3)%BW, $p < 0.001$], when compared to baseline 57.3 (4.9)%BW. Placebo dogs ($n = 14$) did not have significantly different values at week 4 [56.4 (5.8)%BW] or week 8 [56.9 (6.8)%BW] than baseline [57.2 (4.5)%BW]. Both groups did not differ significantly at week 8. Fig. 3 presents the respective individual changes in PVF recorded over the study (*i.e.*, week 8 – baseline) as well as the mean change denoted in each group. The mean changes in PVF values were significantly different between groups ($p = 0.027$).

3.3. Locomotor activity recording

The analysis of DDAP indicated no significant period ($p = 0.862$), or group ($p = 0.414$) effect, but a significant period × group interaction ($p < 0.001$). Analyses revealed that the week 4 period [7.3 (1.9) h/day] in NHP-treated dogs ($n = 7$) was not significantly different

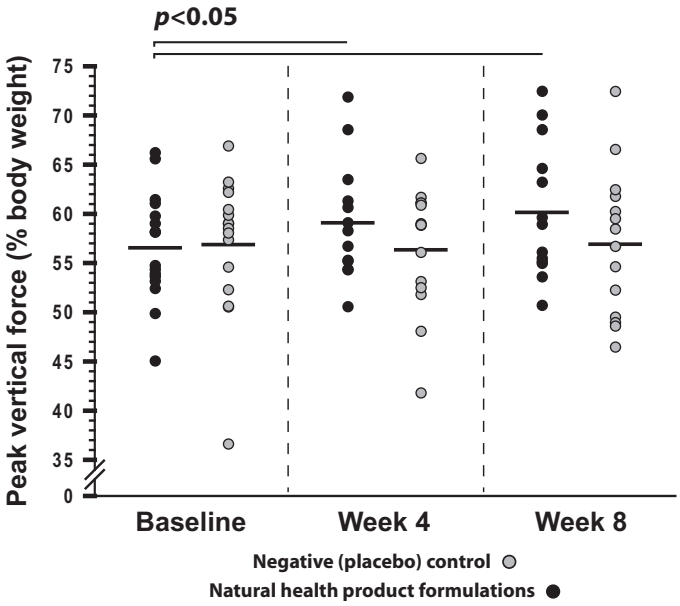

Fig. 2. Peak vertical force. Individual peak vertical force values recorded in dogs having received either natural health product formulations or a negative (placebo) control. Peak vertical force values are expressed as percentage of body weight. The short horizontal lines denote mean group values. For the natural health product formulations group, values at week 4 and week 8 were significantly different ($p < 0.05$) than baseline.

to the baseline, reaching significant increase for the week 8 period [8.2 (3.4) h/day, $p = 0.025$] (Fig. 4). The DDAP values of placebo dogs ($n = 7$) at the week 4 [6.7 (2.1) h/day] and week 8 [6.0 (2.3) h/day] periods were not significantly different than the baseline (Fig. 4). A statistical trend ($p = 0.064$) was observed for a difference in DDAP values between-groups over the study (*i.e.*, week 8 – baseline).

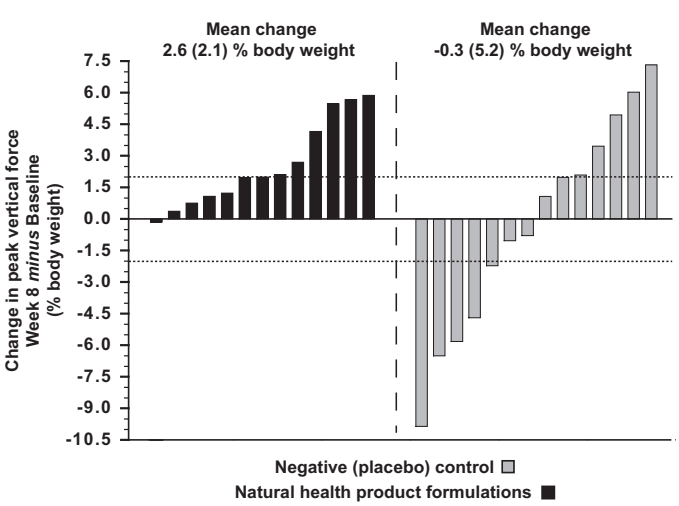

Fig. 3. Changes in peak vertical force. Individual changes in peak vertical force recorded in dogs having received either natural health product formulations or a negative (placebo) control over 8 weeks. Changes are the differences between week 8 *minus* baseline. Negative changes represent a decrease in peak vertical force values at week 8 (*i.e.*, worsening). Dotted lines delineate responders *versus* non-responders according to the minimal detectable change at 95% confidence interval (Moreau et al., 2013).

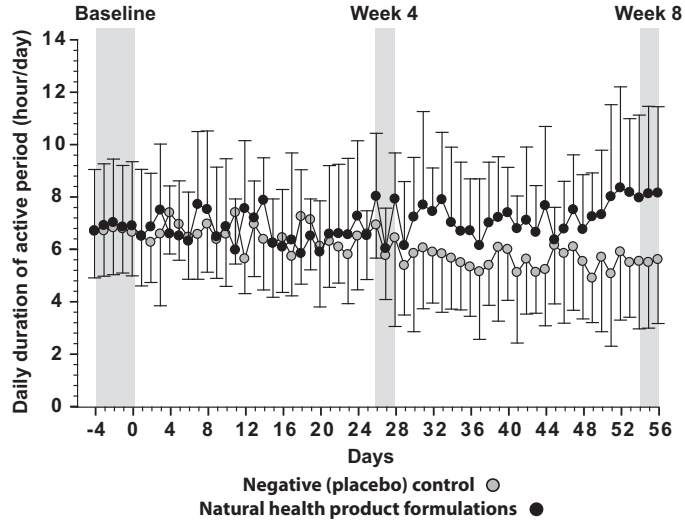

Fig. 4. Locomotor activity recording. Temporal evolution of the locomotor activity recorded in dogs having received either natural health product formulations or a negative (placebo) control over 61-day duration. The daily duration of active period is expressed as mean (h/day) with positive (natural health product formulations) or negative (placebo) standard deviation. Periods are baseline (day4 to day 0), week 4 (day 26 to day 30) and week eight (day 52 to day 56) for the statistical analysis. For the natural health product formulations group, values at week 8 were significantly higher than baseline ($p < 0.05$).

3.4. Case-specific outcome measure

The CSOM analysis revealed no significant period ($p = 0.053$), group ($p = 0.960$) and period × group ($p = 0.524$) effect. Fig. 5 presents the evolution of the CSOM recorded over the entire study duration.

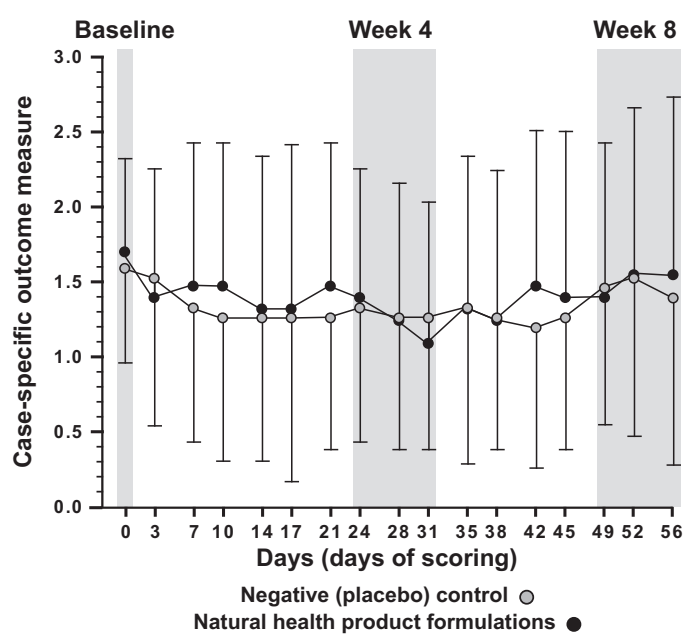

Fig. 5. Case-specific outcome measures of disability. Temporal evolution of the case-specific outcome measures of disability (CSOM) recorded in dogs having received either natural health product formulations or a negative (placebo) control over 8 weeks. Data are expressed as mean with positive (natural health products formulations) or negative (negative control) standard deviation. Periods are baseline (score on day 0), week 4 (scores on days 24, 28 and 31) and week 8 (scores on days 49, 52 and 56) for the statistical analysis.

4. Discussion and conclusions

Current therapeutic approaches used to manage OA-afflicted patients remain largely palliative, NSAIDs being the first line of treatment (Bennell et al., 2012). The effect sizes reported for therapeutic modalities range from small to moderate (Bjordal et al., 2004; Zhang et al., 2007). Therefore, there is an opportunity for novel and effective therapeutics to alleviate pain for the OA-afflicted patient. As naturally-occurring models of OA have recently been proposed to accelerate the development of human therapeutics (Pelletier et al., 2010), and since canine OA models have a high translational value to human OA (Moreau et al., 2013), this randomized, double-blind, placebo-controlled trial was undertaken in the canine natural OA model to assess the efficacy of novel phytotherapeutics for human use. A recent systematic review concluded that NHPs had poor therapeutic potential for the treatment of companion animals affected by OA (Vandeweerd et al., 2012). This disappointing conclusion was largely based on the limited number of rigorous RCTs developed to challenge the proposed therapeutic efficacy of NHP. The quality and quantity of current research studies were also criticized for oral herbal medicines purported to alleviate the clinical signs of human OA (Cameron and Chrubasik, 2014). The present trial was undertaken with the second intention to provide rigorous evidences regarding the therapeutic potential of medicinal herb-based NHP formulations to alleviate the clinical signs of canine OA, and to identify the occurrence of adverse effects with multi-NHP preparations.

According to the present trial, medicinal herb-based NHP formulations improved the functional ability in dogs afflicted by naturally-occurring OA to a higher degree than placebo-control animals. When given once daily, improvements were noted as early as 4 weeks after the initiation of the *alpha* formulation administration, and were even better when the *beta* formulation was given for an additional 4-week duration. It has to be noted that the NHP dosing regimen in this trial was not constant across the entire dog's body mass observed (*i.e. alpha* formulation 58 (10) mg/kg, *beta* formulation 76 (13) mg/kg). The manufacturer's limitations in producing capsules with variable content in multi-NHP preparations support the necessary use of dosing by intervals.

The study primary endpoint was selected as the PVF measured using a force platform. Such an objective evaluation tool was previously used to measure the disability that characterized human OA patients as well as their response to treatment (Detrembleur et al., 2005; Gok et al., 2002; Messier et al., 1992; Schnitzer et al., 1993). Similarly, alterations from normality were detected in OA dogs based on the measurement of the PVF (Madore et al., 2007) while strong improvements in the pain-related limb disuse were reported for several therapeutic approaches including NSAIDs (Budsberg et al., 1999; Moreau et al., 2003), a dual inhibitor of cyclooxygenase and 5-lipoxygenase enzymes (Moreau et al., 2007), therapeutic diets (Moreau et al., 2012b; Rialland et al., 2013; Roush et al., 2010) and NHPs (Hielm-Bjorkman et al., 2009; Moreau et al., 2004, 2012a).

The change over the initial condition [*i.e.*, 2.6 (2.1)%BW] provided by the medicinal herb-based NHP formulations is similar to common therapeutic approaches as recently reviewed (Moreau et al., 2012a). It outweighs the 95% minimal detectable change (MDC_{95}), calculated as 2.0%BW for PVF in canine OA (Moreau et al., 2013). The MDC_{95} can be interpreted as the change magnitude, below which there are more than 95% chances that the change has occurred as a result of measurement error (Kovacs et al., 2008). Outside this cut-off point (*i.e.*, lower than –2.0 or higher than 2.0 %BW), the change does reflect a real difference in the functional impairment toward worsening or improvement, in the canine natural OA model. Establishing such a cut-off point fulfils the requirement to define the magnitude of the measurement that corresponds to a clinically recognizable improvement in the individual animals, as previously criticized in a recent review (Sharkey, 2013).

The MDC_{95} can also serve as a responder criteria, similar to that developed for humans by the OARSI Standing Committee for Clinical Trials Response Criteria Initiative (Pham et al., 2004). According to Fig. 3, 46% (6/13) of the medicinal herb-based NHPs treated dogs were positive responders while negative responders were absent. At the opposite, 36% (5/14) of placebo-control dogs had more severe clinical signs while 36% (5/14) had improved.

In the present study, statistical analyses revealed a significant difference between groups according to the changes in PVF values with a statistical power of 60%. The magnitude of the therapeutic benefits was consistent with a moderate Cohen's *d* effect size of 0.7 (95% confidence interval: 0.0–1.5). The effect size is recognized as a simple and straightforward index to quantify the effects of an intervention relative to a comparator (Coe, 2012). However, effect sizes are not commonly reported in canine models of OA, which compromise comparisons among studies. Nevertheless, the effect size reported herein was similar to other therapeutic approaches including a therapeutic diet rich in omega-3 fatty acids of fish origin (Moreau et al., 2012b) as well as a plant extract from *Brachystemma calycinum* D don (Moreau et al., 2012a).

As previously demonstrated in this model of natural OA (Brown et al., 2010; Moreau et al., 2012a; Rialland et al., 2012, 2013), the usefulness of the continuous monitoring of daily locomotor activity was sustained in the present study. After an 8-week period of treatment with the NHP formulations, the DDAP was increased, reaching more than 1.5 h/day of additional time spent on daily life activities. This finding is consistent with a recent review of experimental data aimed to determine the relationship between the limb function (as reflected by the measurement of the PVF) and the locomotor activity recording (Moreau et al., 2013). Hence, the effect of an additional 54 minutes/day of activity is expected to be mirrored confidently by an increase in PVF measurement exceeding the MDC_{95} (Moreau et al., 2013). As reported herein, the effects of the medicinal herb-based NHP formulations might have been translated into more active dogs, being able to rehabilitate their pain-related limb disuse toward a better muscular strength. This increase in limb use led to dogs more willing to accentuate their limb support by an average of 1.0 kg. These findings sustain the beneficial role of activity in OA dogs. Nevertheless, the level of activity has to be low to moderate to avoid an exacerbation of lameness as reported after intense running (Beraud et al., 2010).

Unlike the objective measures of function, the CSOM did not document an improvement in NHP-treated dogs. The CSOM is a validated proxy method of assessment, which was shown to complement the information provided by the measurement of the PVF (Rialland et al., 2012, 2013). Hence, the CSOM reflects the behavioral aspects of the OA disease affliction as perceived by the owner based on day-to-day environment and situation. The CSOM was used in the present study as an attempt to mirror the dog's quality of life over the 8 weeks. This was done however without knowing the level of functional improvement required to be translated into a better quality of life. The present results suggest at first glance a need for more effective therapy based on owner perception, recognized as less sensitive and more prone to placebo response bias (Conzemius and Evans, 2012; Moreau et al., 2013) or to changes in behavior or perception when being utilized as a proxy assessor. On the other hand, as OA is a lifelong disease, the limb impairment which occurs over several years may have compromised the sensitivity of the owner to detect an improvement in their dog. This is also supported by the relatively low value of CSOM at baseline, compared to other similar population samples (Rialland et al., 2012, 2013), inducing a risk of floor effect for CSOM masking the responsiveness to NHP treatment. Therefore, much time may be required by the owner to appreciate a better quality of life concomitantly to a functional improvement, as previously denoted in OA dogs after a 13-week treatment duration (Moreau et al., 2012b).

Our results indicate that treating with the medicinal herb-based NHPs did not result in a significant buccal mucosal bleeding time prolongation. This indicates that the platelet function was not affected by the treatment. Moreover, the NHP-treated dogs did not demonstrate clinically significant hematological or biochemical alterations when administered for 8 weeks. This result is encouraging for promoting the clinical use of multi-NHP preparations, but would require further confirmation on larger sample size.

Several limitations to this clinical trial study need to be acknowledged. First, the study duration was 8 weeks despite the chronic nature of OA. Second, the content and strength of the NHP capsules were based on empirical evidences (intern data files) suggesting anti-inflammatory and anti-nociceptive potential in rodent models of inflammation and pain. Whether or not the content and strength of the NHP capsule were optimal for dogs afflicted by naturally-occurring OA was unknown. Third, the design of the study did not allow conclusions about the respective potential of each NHP formulation (*i.e.*, *alpha versus beta* formulation). Therefore the efficacy of the medicinal herb-based NHP formulations should be considered as a whole therapeutic regimen involving *alpha* followed by the *beta* formulations. Finally, whether or not the improvement denoted in OA dogs is consistent with disease modifying effects is unknown and should be addressed. Of note licofelone, a dual inhibitor of cyclooxygenase and 5-lipoxygenase enzymes, demonstrated similar functional improvement than the one observed with the NHP formulations in addition to a reduction in the progression of structural changes in experimental dog OA model induced by CCL sectioning (Boileau et al., 2002; Moreau et al., 2006).

This RCT provided evidence of the efficacy of a medicinal herb-based NHP in alleviating the clinical signs of canine OA. The present findings provide relevant and new information about the potential of medicinal phytochemical compounds as a therapeutic modality for human OA. Such NHP appears also interesting for the management of canine OA as not only clear benefits were demonstrated on the function, but also this NHP mixture (with low grade dosage of each component) was not associated with any clinical toxicity.

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An open-field study comparing an essential oil-based shampoo with miconazole/chlorhexidine for hair coat disinfection in cats with spontaneous microsporiasis

Simona Nardoni[1], Angela Grazia Costanzo[1], Linda Mugnaini[1], Francesca Pisseri[2], Guido Rocchigiani[1], Roberto Papini[1] and Francesca Mancianti[1]

Abstract

Objectives The goal of the present study was to compare the antifungal efficacy of an essential oil (EO) shampoo proven to be effective against *Microsporum canis* with miconazole/chlorhexidine for topical hair coat disinfection in cats treated concurrently with oral itraconazole.

Methods Cats received treatment with oral itraconazole (Itrafungol) at a dose of 5 mg/kg/day pulse administration for 1 week, every 2 weeks for at least 6 weeks and were washed twice a week with a neutral shampoo with added EOs of *Thymus serpyllum* (2%), *Origanum vulgare* and *Rosmarinus officinalis* (5% each) for the period of systemic treatment. This protocol was compared with a conventional treatment (oral itraconazole + 2% miconazole/2% chlorhexidine shampoo).

Results The treatment was well tolerated and adverse effects were not recorded. All cats were clinically negative at week 11. With respect to animals with extensive lesions, the speed of resolution was higher in cats with focal lesions. The animals showing diffuse lesions required more than a course of treatment to achieve a mycological cure. There was no significant difference between the number of weeks to obtain mycological cure for cats treated with EOs and animals treated conventionally.

Conclusions and relevance The treatment appeared to be effective and well appreciated by the owners. The use of shampoo with the added EOs of *T serpyllum, O vulgare* and *R officinalis* would seem an interesting, natural alternative to conventional topical treatment.

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Introduction

Microsporum canis, a zoophilic and zoonotic dermatophyte that is highly infectious and has a broad host range, is the main responsible agent for dermatophytosis in cats worldwide. The infection, even if not life threatening, is highly contagious and spontaneous healing can require several months.[1]

Therapeutic measures of feline microsporiasis should include the combination of systemic and topical treatment.[2] The main goal of local drug administration is to minimise the spreading of infective arthrospores, which are the source of both reinfection and new infections. Topical therapy is needed to disinfect the hairs, as systemic therapy combined with the host immune response eradicates the infection from the coat. Repeated disinfection is needed as the hair coat is reseeded with infective arthrospores until this occurs.

The most recent systemic treatment protocol licensed for use in cats in Europe is based on oral itraconazole 5 mg/kg/day pulse administration for 1 week, every 2 weeks,[3] with a total treatment period of 6 weeks, while most commonly recommended topical options include rinses and shampoos. Many commercial rinse formulations containing enilconazole, lime sulfur, accelerated hydrogen peroxide and miconazole/chlorhexidine are available. These products have been tested and reviewed

[1]Department of Veterinary Science, University of Pisa, Pisa, Italy
[2]Cimi Koiné School, Rome, Italy

Corresponding author:
Simona Nardoni DVM, PhD, Department of Veterinary Science, University of Pisa, viale delle Piagge 2, Pisa, 56124, Italy
Email: simona.nardoni@unipi.it

both in vitro and in vivo, with excellent results,[4–10] and as no rinsing is required, their administration is advisable in multiple cat situations. Some (enilconazole, lime sulfur) are indicated as first-choice options.[1] However, shampoo combines the antimycotic effect and the physical act of shampooing,[8] helping to remove fungal propagules, and is recommended in animals kept as pets. A combined 2% miconazole/chlorhexidine shampoo is largely available on the market and has been proven to be effective.[8–10]

In recent years, the interest in selecting sustainable products from landscape plants has increased and some data are available for *M canis*, indicating that a number of chemically defined essential oils (EOs) from several plants can yield antifungal activity both in vitro and in vivo.[11–13] In particular, EOs derived from *Thymus serpyllum, Origanum vulgare* and *Litsea cubeba* have shown strong efficacy against several dermatophyte species.[12,13] Such plant-derived compounds are of interest as they have not yet been manipulated by pharmaceutical industry. Herbal remedia are usually well accepted by pets and owners, and many owners are interested in alternative treatments.

The goal of the present study was to compare the antifungal efficacy of an EO-based shampoo with miconazole/chlorhexidine for topical hair coat disinfection in cats treated with oral itraconazole.

Materials and methods

Cats

Fourteen symptomatic cats affected by spontaneous dermatophytosis caused by *M canis* were included in the study after obtaining informed consent from the owners. The animals were of both sexes, of different breeds (11 domestic shorthairs and three Persians), with ages ranging from 3 months to 8 years.

Fungal infection was confirmed by direct hair examination, Wood lamp examination when possible and culture. Mycotic load was evaluated by counting colony-forming units (CFUs) as previously described, indicating each infection as heavy (⩾50 CFUs/plate), mild (49–5 CFUs) and low (<5 CFUs).[14]

Inclusion/exclusion criteria

Inclusion criteria were the presence of local or generalised lesions due to *M canis*, associated with positive culture (heavy or mild) of hair drawn with the brush technique, achieved on Sabouraud CAF agar + actidione (Liofilchem). Animals treated from less than 6 months before the inclusion day and/or with concomitant dermatoses were not admitted to the study.

Scoring and monitoring

Skin lesions were evaluated at day 0 and scored.[9] In detail were considered ease of epilation (1 = within normal limits; 2 = mild but excessive; 3 = moderate; 4 = severe and extensive), degree of seborrhoea (1 = none; 2 = mild; 3 = moderate; 4 = severe) and extent of the primary lesions (1 = none; 2 = single, small area; 3 = more than one small area; 4 = extensive lesions). The three scores were then added to give a total lesion score. The number of skin lesions ranged from four to 12 for the two groups. The occurrence of human infection (n = 7) was also recorded. The owners of Persian cats were advised to clip their cats. Detailed data are reported in Tables 1 and 2.

Study design

Diagnosis was achieved in different private clinics. Once diagnosed the cats enrolled in the present open study were assigned to two different groups at one time. The treatment took place at home. Cats in group 1 received oral itraconazole (Itrafungol; Eli Lilly Italia) at a dose of 5 mg/kg/day pulse administration for 1 week, every 2 weeks, with a total treatment of at least 6 weeks and they were washed twice a week with about 5 ml of a neutral shampoo with the added EOs of *T serpyllum* (2%), *O vulgare* and *R officinalis* (5% each). The EOs were provided by Flora; their chemical composition and proven antimycotic activities have been reported elsewhere.[11] The shampoo was immediately removed by rinsing after the application.

Cats in group 2 received oral itraconazole at the same dose plus 2% miconazole/2% chlorhexidine shampoo (Malaseb; Eli Lilly Italia) twice a week for the period of systemic treatment. The shampoo was left on the hair coat for 10 mins before rinsing with warm water.[8] All treatments were administered by one of the authors (AGC). Cats were examined weekly to evaluate an improvement of their clinical status and a fungal culture was achieved by brush technique. The animals were treated until they had two negative consecutive weekly cultures,[7] and the protocol treatment was stopped at week 44.

Statistical analysis between the groups was performed by means of Wilcoxon, and Mann–Whitney tests to evaluate significant differences in weeks needed to obtain mycological cure. Statistical significance was defined as $P < 0.01$. When not all study subjects reach mycological cure, the adjusted Kaplan–Meier method was applied to evaluate the probability of healing.

To avoid passive contamination of hair coat, environmental mycotic pollution was monitored by the use of both an air sampler (Sas super-100 Air Sampler; PBI) and contact plates, as previously reported.[15] The owners were advised to clean the environment thoroughly by vacuuming followed by a deep clean with disinfectants available commercially in Italy (accelerated hydrogen peroxide, bleach, ammonium quaternary compounds and enilconazole). Cultural controls were repeated weekly.

Table 1 Anamnestic and clinical data in treated cats, and lasting outcome of therapies

Cat	Group	Age (months)	Breed	Sex	Dermatological lesions	CFU	Human cases	Courses (n)	Clinical outcome (weeks)	Culture outcome (weeks)	Clipping	Hair microscopy	Wood's lamp
1	1	3	DSH	F	Focal periocular	++	No	1	4	7	No	+	+
2	1	5	DSH	F	Multifocal pinnal, alopecia, erythema	++	No	2	5	14	No	+	–
3	1	96	P	F	Diffuse	+++	Yes	6	11	42	Yes	+	NE
4	1	3	DSH	M	Diffuse	+++	Yes	2	5	14	No	+	+
5	1	3	DSH	F	Focal elbow	++	No	1	3	7	No	+	NE
6	1	3	DSH	M	Focal nose	++	No	1	3	7	No	+	–
7	1	13	P	F	Diffuse, furforaceous	+++	Yes	2	11	14	No	+	NE
8	2	4	DSH	M	Diffuse	++	Yes	2	6	14	No	+	+
9	2	3	DSH	M	Diffuse	+++	No	3	6	21	No	+	–
10	2	3	DSH	M	Forelimb multifocal	++	No	2	5	14	No	+	+
11	2	6	P	M	Diffuse	+++	Yes	6	10	>42	No	+	–
12	2	3	DSH	F	Focal pinnal	++	Yes	1	3	7	No	+	+
13	2	6	DSH	M	Multifocal head	++	Yes	2	6	14	No	+	–
14	2	12	DSH	F	Focal pinnal, alopecia, furforaceous	++	no	1	5	7	No	+	–

(++) 5–49 CFUs per plate; (+++) ⩾50 CFUs per plate
CFU = colony-forming unit; DSH = domestic shorthair; F = female; P = Persian; NE = not executed; M = male

Table 2 Clinical score data before and after treatment in cats from groups 1 and 2

Cat	group	Ease of epilation		Degree of seborrhoea		Extent of the primary lesions		Total score	
		Day 0	Week 11	Day 0	Week 11	Day 0	Week 11	Day 0	Week 11
1	1	1	3	1	3	2	3	4	3
2	1	3	3	1	3	3	3	7	3
3	1	4	3	4	3	4	3	12	3
4	1	4	3	4	3	4	3	12	3
5	1	1	3	1	3	2	3	4	3
6	1	2	3	1	3	2	3	5	3
7	1	3	3	1	3	4	3	8	3
8	2	4	3	4	3	4	3	12	3
9	2	4	3	3	3	4	3	11	3
10	2	3	3	1	3	3	3	7	3
11	2	4	3	1	3	4	3	9	3
12	2	1	3	1	3	2	3	4	3
13	2	1	3	1	3	4	3	6	3
14	2	1	3	1	3	2	3	4	3

Results

All enrolled cats had never been treated with antimycotic drugs, except for cat 3 (group 1), who had been treated with griseofulvin and then with ketoconazole, without any improvement of clinical and mycological features. Antimycotic treatments had been stopped about 1 year before the beginning of the present study.

Both treatments were well tolerated and adverse effects were not recorded. In group 1, two animals were clinically healthy at week 3 post-treatment, while all other cats were clinically healthy at week 11. All cats were culturally negative at the end of the trial. In group 1 cats, mean time to clinical and mycological cure was 6 weeks (median 4 weeks, range 3–11 weeks) and 15 weeks (median 14 weeks, range 7–42 weeks), respectively.

One cat in group 2 was dermatologically normal by week 3, while all cats were clinically cured at week 10. By the end of the study 6/7 animals were negative on fungal culture. For cats in group 2, the mean time to clinical and mycological cure was 5.9 weeks (median 6 weeks, range 3–10 weeks) and 12.8 weeks (median 6 weeks, range 7–21 weeks), respectively.

With respect to cats with extensive lesions, as expected, the speed of resolution was higher in cats with focal lesions, ranging from 3 to 4 weeks in group 1 cats and from 3 to 5 weeks in group 2 cats. Cats with diffuse lesions required more than one course of treatment (requiring 2–6 treatments for both groups) to achieve a mycological cure. A reduction in CFUs was observed by week 3 post-treatment in all examined cats. All cats with focal lesions had healed culturally at week 7. After the start of treatment no cases of new human infection or reinfection were reported. Two Persian cats out of three were randomly assigned to group 1; only one was clipped (the other Persian cat from group 2 was clipped as recommended).

With regard to aetiological cure, a significant difference between treatments was not observed (z score –0.1429; U = 19.5), even if the probability of healing at week 42 was 46% more for cats in group 1.

Detailed data on treatment outcome are reported in Tables 1 and 2.

The correct cleaning instructions given to owners led to environmental clearance from fungal propagules from week 1 post-treatment in all cases.

Discussion

Treatment with shampoo with added EOs yielded results comparable with conventional therapy. Owing to the lack of contact time required, it was particularly appreciated by the owners; the miconazole/chlorhexidine shampoo had to be left on the cats' coats for 10 mins, and some cats exhibited nervous behaviour during this time.

Studies on the use of antifungal rinses to control dermatophytosis have been conducted on cats, both naturally and experimentally infected, living in catteries and other communities.[4–10] To the best of our knowledge, our trial is the first study carried out on owned cats. We are aware of our small sample size, but this was due to the difficulty in the simultaneous recruitment of pet cats that met the inclusion criteria. Although the epidemiology and clinical situation in shelters is more controlled and homogeneous, such studies can not be fully applicable to indoor cats, living in very close contact with people, including children, in an environment with furniture, curtains, cushions and other household items. The best treatment protocol is difficult to identify, depending on the number of cats involved, the owner's resources and global health

of cats,[1] so the use of shampooing in such animals is advisable, while in catteries the rinse is more useful.

In the present study the determination of CFUs was applied to evaluate the efficacy of local treatment and the capacity of active compounds to limit the spreading of arthrospores. Both local treatments were able to decrease heavy and mild mycotic loads until elimination of arthrospores on the hair coat. An effective topical treatment together with correct management of environmental disinfection are of primary importance to cure dermatophytoses and to avoid reinfection and/or new infections. Therefore, in our study no relapses or new cases of human infections were recorded during the observation period.

Considering their potential toxicity, EOs should be carefully administered in animals, especially in cats. Oils from *Thymus* species are toxic when administered orally,[16] and carvacrol and thymol, the main components of both *Thymus* and *Origanum* oils are skin sensitisers and antigens,[17] so the use of these oils undiluted should be avoided. Rosemary oil is considered safe for mammals, although chronic exposure to rosemary oil at high concentrations has rarely been reported to cause contact dermatitis; acute toxicity of rosemary oil has not been reported.[18] In general, toxicity testing is concerned with pure single oils rather than mixtures.[19] In the present study, EOs as a mixture were administered to optimise their efficacy and to minimise toxic effects.

Nevertheless, even if EOs, properly diluted, are generally safe, attention must be paid to use chemically defined compounds,[20] under the supervision of a skilled phytotherapist.

Conclusions

On the basis of our observations the use of shampoo with the added EOs of *T serpyllum*, *O vulgare* and *R officinalis* would seem a natural and interesting alternative to conventional topical treatment.

Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Veterinary and Comparative Oncology

Original Article

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In vitro effects of *Yunnan Baiyao* on canine hemangiosarcoma cell lines

K. A. Wirth, K. Kow, M. E. Salute, N. J. Bacon and R. J. Milner

Department of Clinical Sciences, University of Florida, Gainesville, FL, USA

Abstract

Yunnan Baiyao is a Chinese herbal medicine that has been utilized for its anti-inflammatory, haemostatic, wound healing and pain relieving properties in people. It has been utilized in the veterinary profession to control bleeding in dogs with hemangiosarcoma (HSA) and has been anecdotally reported to prolong survival times in dogs with this neoplasm. This study evaluated the *in vitro* activity of *Yunnan Baiyao* against three canine HSA cell lines after treatment with increasing concentrations of *Yunnan Baiyao* (50, 100, 200, 400, 600 and 800 μg mL^{-1}) at 24, 48 and 72 h. Mean half maximum inhibitory concentration (IC_{50}) at 72 h for DEN, Fitz, SB was 369.9, 275.9 and 325.3 μg mL^{-1}, respectively. Caspase-3/7 activity increased in correlation with the IC_{50} in each cell line which was confirmed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, APO-BRDU Kit; BD Biosciences, San Jose, CA, USA) assay. VEGF in cell supernatant was also quantified. Overall, the study found that *Yunnan Baiyao* causes dose and time dependent HSA cell death through initiation of caspase-mediated apoptosis, which supports future studies involving *Yunnan Baiyao*.

Keywords
canine hemangiosarcoma, Chinese herbal medicine, *Yunnan Baiyao*

Correspondence address:
Kelvin Kow, DVM, MS, ACVIM
Department of Small Animal Clinical Sciences
University of Florida
2015 SW 16th Avenue
PO Box 100126, Gainesville
FL 32610-0126, USA
e-mail:
kowk@ufl.edu

Introduction

Hemangiosarcoma (HSA) is a highly malignant neoplasm of vascular endothelial cell origin. HSA is a relatively common neoplasm in the dog, accounting for up to 21% of all soft tissue sarcomas and 0.3–2% of all malignant tumours in this species.[1–4] The incidence of disease is significantly higher in large breed dogs such as German Shepherds, Golden and Labrador Retrievers.[4–7] HSA can affect any tissue in the body; however, the spleen is the most common site of tumour development, accounting for 50–65% of all canine HSAs.[2] HSA is also the most common primary cardiac tumour and tumours of the right atrium account for 3–25% of all HSAs in the dog.[8] Other common sites include the subcutaneous tissues (13–17%) and the liver (5–6%).[9] Canine HSA is an aggressive malignancy, characterized by pathologic angiogenesis and early, aggressive metastasis that is poorly chemo-sensitive.[3,9–19] Previously reported prognostic factors for canine HSA include location (cutaneous versus viscera), histological grading and stage.[5,9–19] Despite available multi-modal therapies to address local and systemic disease, few patients survive beyond 6 months with most succumbing to symptoms associated with metastatic disease.

Malignant tumours of the vascular endothelium are rare in humans; however, this type of cancer is extremely aggressive when it does occur. HSA, also called angiosarcoma, accounts for approximately 2% of soft tissue sarcomas in humans and most commonly occurs in liver, spleen, breast and scalp. As in dogs, this tumour frequently metastasizes and despite multimodal treatment, 5-year survival rates remain between 10 and 35%.[20–24]

The lack of effective adjuvant therapies warrants the investigation of novel treatment options and in recent years, traditional Chinese medicine (TCM) has been receiving increased attention for the treatment of malignant neoplasia. *Yunnan Baiyao* is an herbal TCM that has been used frequently by veterinarians and their clients as an adjunctive treatment

for canine HSA. It has been anecdotally reported to prolong survival times and control bleeding in dogs with this aggressive neoplasm.

Yunnan Baiyao is a well-known Chinese herbal patent formula that has been utilized for its anti-inflammatory, haemostatic, wound healing and pain relieving properties in people for over 100 years. It was developed in the Yunnan Province of China around 1902 and gained popularity among Chinese soldiers during World War II for use as a haemostatic agent on the battlefield.[25,26] *Yunnan Baiyao* has been shown to improve clotting and enhance platelet function.[26–30] This may benefit canine patients with HSA due to the frequency of clotting abnormalities and potential for fatal haemorrhage although this was not evaluated in this study.

Yunnan Baiyao is a class-1 protected TCM and the exact herbal formula is a trade secret. Due to this protected status, component analysis and quality control measures for *Yunnan Baiyao* have been slow to develop; however, due to international demand for quality assurance and the development of Good Manufacturing Practice (GMP), the product is now labelled to identify its major components per 0.5 g serving.[31] The following ingredients are listed based on 2011 manufacturer's label: 200 mg Tienchi ginseng root (*Panax notoginseng*), 85 mg Ajuga forrestii Diels plant, 66.5 mg Chinese yam root, 57.5 mg Dioscoreae nipponica Makino root, 36 mg Erodium stephanianum and Geranium wilfordii plant, 30 mg Dioscoreae parvilora ting root and 25 mg Inula cappa plant (*Yunnan Baiyao*; Yunnan Baiyao Group, Kunming, China).

There is a vast body of scientific literature showing that components of *Yunnan Baiyao* have various anti-cancer properties; however, studies on *Yunnan Baiyao* itself as an anti-cancer therapy have not been previously performed.[32–37]

Panax notoginseng root extract (NGRE), which is a major component of *Yunnan Baiyao*, showed significant growth inhibition and increased apoptosis of SW480 human colorectal cancer cells *in vitro*. NGRE also enhanced cell growth inhibition when combined with either 5-fluorouracil or irinotecan.[32] The saponin ginsenoside Rd, isolated from *P. notoginseng*, was shown to inhibit proliferation of human cervical cancer (HeLa) cells *in vitro* and induce apoptosis by upregulation of Bax, downregulation of Bcl-2 and activation of the caspase-3 pathway.[33] Additionally, *P. notoginseng* has been documented to inhibit DNA synthesis and cell proliferation in human umbilical vein endothelial cells (HUVEC) *in vitro*.[34,35]

Wild yam root (*Diosocoreae spp.*), another major component of *Yunnan Baiyao*, was shown to have the most potent effects on cell viability and induction of apoptosis in a murine malignant neuroblastoma cell line when compared with 373 other naturally derived herb, seed, root, plankton and fungi extracts.[36] Wild yam root has also been shown to induce anti-proliferative and pro-apoptotic effects in a range of tumour cells by G2/M arrest, downregulation of NF-κB, Akt, cyclin D, c-myc and initiating PARP cleavage/DNA fragmentation.[36] *Dioscoreae nipponica* extract exerted dose dependent inhibition on the invasion, motility, secretion of MMPs and u-PA in murine melanoma (B16F10) and human melanoma (A2058) cells *in vitro*.[37] It was also shown to inhibit activation of NF-κB and increase expression of I-κB in the B16F10 cells *in vitro*. Additionally, lung metastasis formation was significantly reduced in mice treated with the extract versus the control group *in vivo* in the same study.[37]

Novel therapeutic options are needed if we hope to improve outcomes associated with canine HSA. Studies on the anti-cancer properties of *Yunnan Baiyao* components combined with anecdotal evidence to its efficacy suggest that it may enhance the traditional medical approach to treatment of canine HSA. This study aims to take the first step in evaluating the biological activity of *Yunnan Baiyao* against canine HSA cells *in vitro*. We studied *Yunnan Baiyao's* ability to inhibit growth of canine HSA cells and to induce apoptosis. Cell survival assays were performed for HSA cell lines exposed to *Yunnan Baiyao*. Apoptosis was investigated by measuring caspase-3/7 activity and the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Changes in cell cycle kinetics were evaluated using flow cytometry. Due to the association of increased VEGF levels in dogs with HSA,[38] we also investigated levels of VEGF found in supernatant from untreated and *Yunnan Baiyao* treated HSA cells. The information gained from this

study will be used to establish a proof-of-concept for clinical use as well as support for further *in vitro* investigation of the use of *Yunnan Baiyao* as a novel anti-cancer agent.

Materials and methods

Cell cultures

Three established canine HSA cell lines were evaluated: DEN-HSA, Fitz-HSA (provided by Dr Ilene Kurzman, University of Wisconsin, Madison, WI, USA) and SB-HSA (provided by Dr Stuart Helfand, Oregon State University, Corvallis, OR, USA). DEN was established from a renal HSA from a Golden Retriever, Fitz was from a splenic HSA of a Golden Retriever and SB was obtained from a subcutaneous HSA of a German Shepherd dog.[39,40] It has recently been shown that DEN and Fitz were derived from the same source.[40] This does not mean that DEN and Fitz might not show differences in drug sensitivity as they have been cultured as separate cell lines for several years now and may have 'drifted' apart. All cell lines were cultured under standard conditions (37 °C, 5% CO_2, humidified air). DEN and Fitz were maintained in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Mediatech, Manassas, VA, USA). SB was maintained in Roswell Park Memorial Institute (RPMI, Buffalo, NY, USA) medium supplemented with 10% heat-inactivated FBS, sodium pyruvate, L-glutamine, HEPES, penicillin AND streptomycin.

Yunnan Baiyao preparation

Yunnan Baiyao (Yunnan Baiyao Group) was generously provided as a stock powder (4 g per vial) by Dr Shen Huisheng Xie (University of Florida, Gainesville, FL, USA). A 200 mg mL^{-1} stock solution was prepared in 0.1% dimethylsulfoxide (DMSO) at room temperature, vortexed for 5 min and filtered with a 0.22 μm filter. Aliquots of the stock solution were stored at −20 °C and protected from light. Dilutions of the stock solution were prepared immediately prior to use in cell culture medium such that the DMSO concentration did not exceed 1%.

Evaluation of cell viability

The DEN and Fitz cells were plated at 5000 per well and SB cells were plated at 10 000 per well in 100 μL media in 96-well flat-bottom plates (Falcon, Becton Dickinson Bedford, MA, USA). The plates were incubated under standard conditions for 24 h. After 24 h, *Yunnan Baiyao* was added to the wells at increasing concentrations (50, 100, 200, 400, 600 and 800 μg mL^{-1}) in 100 μL media solution. Control wells were prepared for each assay containing media with 1% DMSO only or 800 μg mL^{-1} *Yunnan Baiyao* in 100 μL media solution. After incubation times of 24, 48 or 72 h, the relative viable cell number was assessed using a one-step tetrazolium-based (MTS) colorimetric assay (CellTiter-Blue® Cell Viability Assay, Promega, Madison, WI, USA) in accordance with the manufacturer's specifications. Fluorescence was quantified with a fluorescence plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Relative viable cell number was assessed by means of triplicate wells for each drug concentration and triplicate wells for each control, and each experiment was repeated three times.

Effect of *Yunnan Baiyao* on apoptosis

To measure and characterize cell death, the effects on caspase-3/7 activity were assessed as an important signalling and effector step in the apoptotic cascade. The DEN and Fitz cells were plated at 5000 per well and SB cells were plated at 10 000 per well in 100 μL media in 96-well flat-bottom plates (Falcon, Becton Dickinson). The plates were incubated under standard conditions for 24 h. After 24 h, *Yunnan Baiyao* was added to the wells at increasing concentrations (50, 100, 200, 400, 600 and 800 μg mL^{-1}) in 100 μL media solution. Control wells were prepared for each assay containing cells and media with 1% DMSO only or 800 μg mL^{-1} *Yunnan Baiyao* in 100 μL media solution. After incubation for 24, 48 or 72 h, caspase-3/7 activity was measured using a commercial assay (Apo-ONE® Homogeneous Caspase-3/7 Assay; Promega) performed in accordance with the manufacturer's specifications. Fluorescence was quantified with a fluorescence plate reader at an excitation wavelength of 485 nm and emission

wavelength of 528 nm. All samples were analysed in triplicate, and each experiment was repeated three times with each of the cell lines.

TUNEL assay

Detection of fragmented DNA, one of the later steps in apoptosis, was performed using a TUNEL assay (APO-BRDU Kit; BD Biosciences, San Jose, CA, USA). Cells were plated into six-well plates (50 000 per well DEN, 75 000 per well Fitz and 100 000 per well SB) and placed in the incubator under standard conditions for 24 h. After 24 h, *Yunnan Baiyao* was then added to the wells (50, 100, 200, 400, 600 and 800 μg mL^{-1}). Control wells were prepared for each assay containing cells and media with 1% DMSO. After incubation, the cells were fixed with 1% (w/v) paraformaldehyde in phosphate buffered saline (PBS) and kept at −20 °C until assayed. The commercial assay was performed in accordance with the manufacturer's specifications. The APO-BRDU kit is a two-colour staining method for labelling DNA breaks and total cellular DNA in order to detect apoptotic cells by flow cytometry. Apoptotic cells with exposed 3′-hydroxyl DNA ends were labelled with brominated deoxyuridine triphosphate nucleotides (BR-dUTP). FITC labelled anti-BrdU mAb provided by the commercial kit was then used to stain apoptotic cells. Propidium iodide (PI) was used as a counterstain to label total cellular DNA for cell cycle analysis. Flow cytometry was performed using a flow cytometer (FACSort; BD Biosciences) with a green fluorescence (520 nm) and a red fluorescence (623 nm) detection. Data were processed by use of Cell Quest software (Cell Quest software, version 3.3; BD Biosciences). The samples were pooled and this assay was performed as a single run for all three cell lines at 24, 48 and 72 h. The percentage of apoptotic cells and cell cycle kinetics were evaluated.

VEGF enzyme linked-immunosorbent assay

A commercial enzyme linked-immunosorbent assay (ELISA) kit (Quantikine Canine VEGF ELISA Kit; R&D systems, Minneapolis, MN, USA) was used to measure VEGF levels in the cell culture supernatants before and after treatment with *Yunnan Baiyao*. The kit contains Sf21-expressed, recombinant VEGF and antibodies raised against the recombinant protein. Results obtained for naturally occurring canine VEGF show linear curves that are parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural canine VEGF.[39] In brief, HSA cell lines were plated into six-well plates (50 000 per well DEN, 75 000 per well Fitz and 100 000 per well SB) and placed into the incubator under standard conditions for 24 h. Then *Yunnan Baiyao* (50, 100, 200, 400, 600 and 800 μg mL^{-1}) was added to the wells. Untreated (control) wells containing cells only were also plated. After incubation for an additional 24, 48 or 72 h the supernatant was removed, centrifuged and stored at −20 °C until assayed. The samples were added in duplicate to a 96-well plate and the VEGF immunoassay was performed in accordance with the manufacturer's specifications. All samples were run in duplicate and calibration on the microtitre plate included a standard series of dilutions of recombinant human VEGF. The optical density of the standard solutions was plotted against their corresponding concentrations to generate a standard curve and allow determination of all VEGF concentrations. All samples were analysed at the same time. This assay has been previously validated for measurement of canine VEGF.[39]

Statistical analysis

Statistical analyses were performed with Sigma-Plot software (SigmaPlot for Windows, version 12.5; Systat Software, Erkrath, Germany). Cell survival data were fitted to a four-equation regression model to determine the mean half maximum inhibitory concentration (IC_{50}) for each cell line. The IC_{50} was defined as the drug concentration that caused 50% cell death compared with the control. For the cell viability assay and caspase-3/7 assay, a two-way analysis of variance (ANOVA, two-factor repetition) was used to determine whether time and concentration had an effect on cell viability and caspase-3/7 activity, and pair-wise multiple comparisons procedures (Hom-Sidak method) were performed for *post hoc* analysis. To account for changes in cell number which may influence

levels of apoptosis, the reading was normalized to the cell viability of non-untreated cells at the same time-point under investigation. For the VEGF assay, a one-way ANOVA was used to determine if time had an effect on median VEGF concentrations of controls incubated for 24, 48 and 72 h. A two-way ANOVA was then used to analyse if *Yunnan Baiyao* concentration had an effect on mean VEGF levels for all three cell lines treated at 72 h. To account for changes in cell number which may influence VEGF levels, the reading was normalized to the cell viability of non-treated cells at the same time-point under investigation. Overall significance was set at $P = 0.05$.

Results

Effects of *Yunnan Baiyao* on cell viability

For all three canine HSA cell lines, cell viability decreased after incubation with higher concentrations of *Yunnan Baiyao* at 24, 48 and 72 h. (see Fig. 1A–C). For the DEN cell line, a significant decrease in cell viability was found at $\geq 400\ \mu g\ mL^{-1}$ concentrations at 24 h, and at $\geq 200\ \mu g\ mL^{-1}$ concentrations at 48 and 72 h ($P < 0.001$). For the Fitz cell line, a significant decrease in cell viability was found at $\geq 400\ \mu g\ mL^{-1}$ concentrations at 24 and 48 h, and at $\geq 200\ \mu g\ mL^{-1}$ concentrations at 72 h ($P < 0.001$). For the SB cell line, a significant decrease in cell viability was found at $\geq 400\ \mu g\ mL^{-1}$ concentrations at 24 and 48 h, and at $\geq 200\ \mu g\ mL^{-1}$ concentrations at 72 h ($P < 0.001$).

Cell viability data were fitted to a four-equation regression model in order to determine the IC_{50} for each cell line (see Table 1). The IC_{50} values at 72 h were 275.9 and 325.3 $\mu g\ mL^{-1}$ for the Fitz and SB cell lines, respectively. The IC_{50} was slightly higher at 369.3 $\mu g\ mL^{-1}$ for the DEN cell line at 72 h. The correlation coefficient or R^2 value was evaluated to determine the goodness of fit of the derived values for each dose response curve. The mean R^2 value for DEN, Fitz and SB was 0.98 at 72 h where unity is considered a perfect correlation.

The duration of *Yunnan Baiyao* incubation time (24, 48 and 72 h) was found to be a significant factor ($P < 0.001$) in mean cell viability for all three cell lines, with the proportion of cell viability of *Yunnan Baiyao* treated cells to cell viability of the control samples decreasing with time. Time was found to be a significant factor for concentrations $\leq 200\ \mu g\ mL^{-1}$ for the DEN and Fitz cell lines and at $\leq 100\ \mu g\ mL^{-1}$ for the SB cell line. Time was no longer a factor at concentrations $\geq 400\ \mu g\ mL^{-1}$ for all three cell lines.

Effects of *Yunnan Baiyao* on apoptosis

Caspase-3/7

Overall, the duration of *Yunnan Baiyao* incubation time and concentration were significant ($P < 0.001$) factors in the mean caspase-3/7 activity (apoptosis) for all cell lines (see Fig. 2A–C). For the DEN cell line, significant increases in caspase-3/7 were found at $\geq 400\ \mu g\ mL^{-1}$ concentrations ($P < 0.001$) at 24, 48 and 72 h. For the Fitz cell line, significant increases in caspase-3/7 activity were found at $\geq 400\ \mu g\ mL^{-1}$ for 24 and 48 h, and at $\geq 200\ \mu g\ mL^{-1}$ at 72 h ($P < 0.001$). For the SB cell line, significant increases in caspase-3/7 activity were found at $\geq 600\ \mu g\ mL^{-1}$ for 24 h, $\geq 400\ \mu g\ mL^{-1}$ at 48 h, and at $\geq 200\ \mu g\ mL^{-1}$ at 72 h ($P < 0.001$). This suggests that the SB cell line may be more sensitive to the effects of *Yunnan Baiyao* than the other two cells lines.

Of note is that the caspase-3/7 activity relative to the number of viable cells increased significantly compared with the control sample at close approximation with the IC_{50} of each cell line (see Table 1 and Fig. 2A–C). The duration of incubation time with *Yunnan Baiyao* was also a significant factor in caspase-3/7 activity in all three cell lines ($P < 0.001$).

TUNEL assay

Flow cytometry was used to detect the number of TUNEL-positive cells as a measure of percentage of apoptotic cells in the population (see Table 2 and Fig. 3). Samples for all three cell lines at 24, 48 and 72 h were pooled and the TUNEL assay was performed as a single experiment in order to minimize cost and sample processing time. No appreciable levels of apoptosis were noted in the control samples or in cells treated at *Yunnan Baiyao* concentrations $\leq 100\ \mu g\ mL$. However, there was a statistically significant ($P < 0.001$) increase in the percentage of apoptotic cells at concentrations $\geq 200\ \mu g\ mL^{-1}$ for all cell lines incubated for 72 h (see Table 2). Specifically, at the 200 $\mu g\ mL^{-1}$

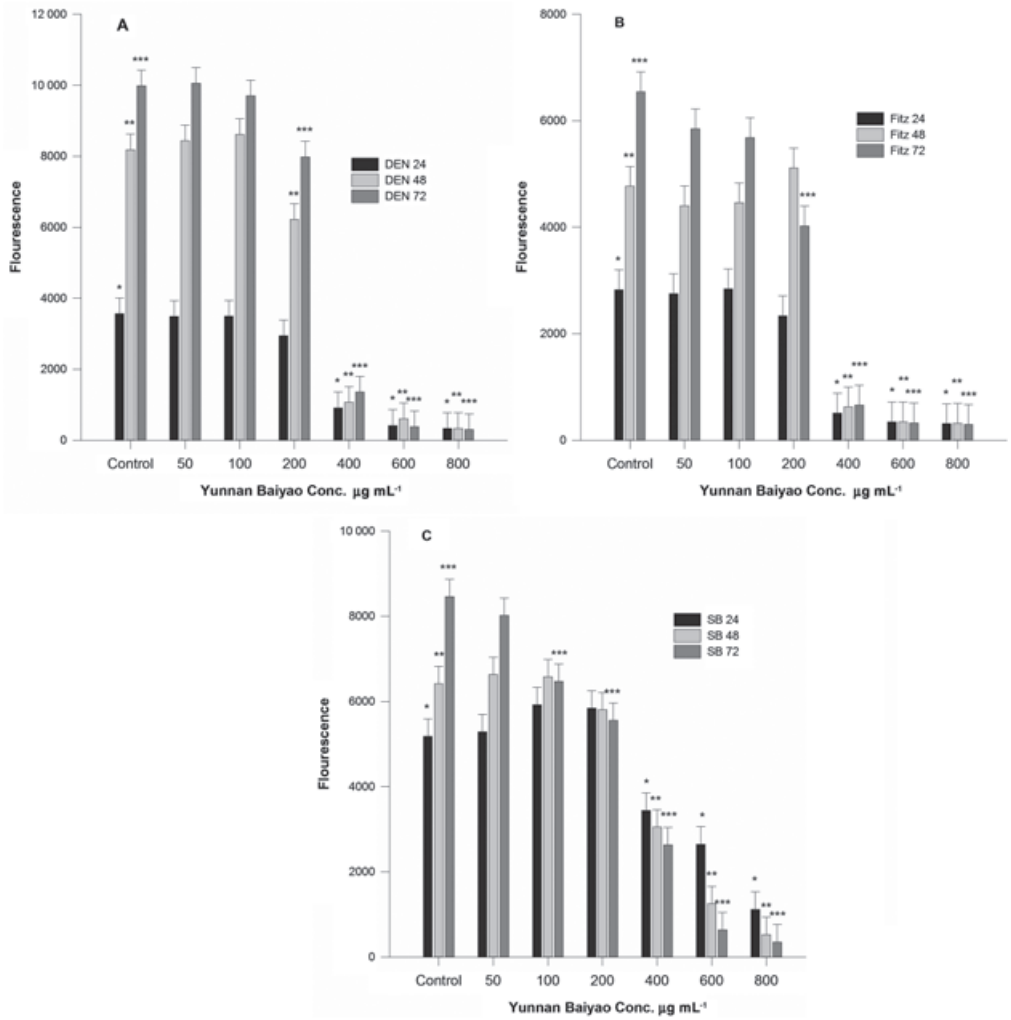

Figure 1. *Yunnan Baiyao* causes a concentration dependant decrease in HSA cell viability over time as measured by the CellTiter-Blue Cell Viability Assay. An increase in fluorescent signal is correlated with an increase in viable cells. Control samples are designated as 24 h (*), 48 h (**) and 72 h (***). Error bars represent standard deviation (SD). A statistically significant decrease in cell viability compared with untreated control sample at the corresponding time point is represented on the graph by *, ** and *** for 24, 48 and 72 h, respectively. (A) DEN cell line treated with increasing concentrations of *Yunnan Baiyao*. (B) Fitz cell line treated with increasing concentrations of *Yunnan Baiyao*. (C) SB cell line treated with increasing concentrations of *Yunnan Baiyao*.

concentration the percentage of apoptotic cells (TUNEL-positive) was 19.63, 56.34 and 86.47% for DEN, Fitz and SB, respectively. The percentage increased to 90.63, 99.69 and 98.98% for DEN, Fitz, and SB (respectively) at the 400 µg mL^{-1} concentration; then remained high at 600 and 800 µg mL^{-1} (see Table 2). Curiously, there was also a statistically significant ($P < 0.001$) increase in percentage of apoptotic cells at the 50 and 100 µg mL^{-1} concentrations at 24 h for the SB cell line which was not noted in the other cell lines (data not shown).

Overall, the greatest percentage change in apoptosis occurred between 200 and 400 µg mL^{-1} for DEN, Fitz and SB which correlates with the calculated IC_{50} for all three cell lines (see Table 1 and Fig. 3).

Cell cycle analysis

Cell cycle analysis was performed on data recorded for all three cell lines at 24, 48 and 72 h (see Fig. 4A–C). The DEN cell line (see Fig. 4A) when

Table 1. Cell viability data were fitted to a four-equation regression model in order to determine the IC_{50} for each cell line

Cell line	24 h	48 h	72 h
DEN (μg mL^{-1})	313.4	313.4	369.3
Fitz (μg mL^{-1})	356.5	285.8	275.9
SB (μg mL^{-1})	497.6	414.4	325.3

The IC_{50} for the Fitz and SB cell lines decreased with increasing exposure time to *Yunnan Baiyao*. The IC_{50} for the DEN cell line slightly increased at 72 h when compared with the 24 and 48 h time points.

compared with control cells at 24 and 48 h, showed a moderate increase in G1- and G2-phases and a corresponding decrease in S-phase at concentrations ranging from 50 to 400 μg mL^{-1}. In contrast, at 72 h the DEN cell line showed moderate decreases in G1- and G2-phases with a corresponding increase in S-phase at concentrations ranging from 50 to 400 μg mL^{-1}. Both Fitz and SB cell lines showed moderate increases in G2-phase compared with control cells at all time points at concentrations ranging from 50 to 400 μg mL^{-1}, with G1-phase being the predominant cell phase.

Remarkably, all cell lines at concentrations >200–400 μg mL^{-1} and at all time points the cell phases disappeared and were replaced by DNA debris (see Fig. 4A–C). This DNA debris, found to the left of the G1 peak in the cell cycle histogram, is considered a sign of late apoptosis due to endonuclease cleaving of DNA. This is an unusual finding, but consistent with the TUNEL assay's recording of virtually 100% (see Fig. 3) apoptotic cells at concentrations >200–400 μg mL^{-1}.

Effects of *Yunnan Baiyao* on VEGF concentrations

In the DEN, Fitz and SB cell lines, the untreated (control) cells expressed increasing levels of VEGF over time (see Table 3). The DEN cell line expressed the highest levels of VEGF followed by Fitz and then SB at all time points. Of note is that the untreated SB cells expressed a VEGF level that remained below the detectable threshold of the standard curve (<19.5 pg mL^{-1}) at 24 h.

VEGF levels were only measured in cells that were treated with increasing concentrations of *Yunnan Baiyao* at 72 h because of the cost of test kits. To account for changes in cell number that may influence VEGF levels, the reading was normalized to the cell viability of non-treated cells at the same time-point under investigation (see Fig. 5). No significant VEGF changes from baseline were found for the DEN cell line at 72 h for any concentration of *Yunnan Baiyao*. The SB cell line showed a statistically ($P < 0.001$) significant fold (×) increase from baseline in VEGF levels at 50 (×2.9 ± 1.1), 100 (×64.5 ± 3.7), 200 (×19.1 ± 1.9) and 600 μg mL^{-1} (×2.9 ± 0.4) of *Yunnan Baiyao*. The maximum increase in VEGF concentration by the SB cell line at 100 μg mL^{-1}, was 3170 ± 0 pg mL^{-1}, which was significant considering the control cell concentration was only 58.1 ± 2.2 pg mL^{-1} (see Table 3). The Fitz cell line also showed a statistically ($P < 0.001$) significant fold increase from baseline in VEGF levels when compared with control cells at 100 (×3.7 ± 0.1), 200 (×4.5 ± 0.1), 600 (×3.8 ± 0.8) and 800 μg mL^{-1} (×4.0 ± 0.3) of *Yunnan Baiyao*. Nevertheless, the fold increase for Fitz was lower when compared with the SB cell line. Both SB and the Fitz cell line lacked statistical significance at 400 μg mL^{-1} of *Yunnan Baiya*. The SB cell line also lacked significance at 800 μg mL^{-1} which was due to the wide standard deviation from the mean (see Fig. 5).

Discussion

This study showed that *Yunnan Baiyao* causes time and concentration dependant death of canine HSA cells. The Cell Titer Blue results showed a decrease in cell viability with increasing concentrations of *Yunnan Baiyao*. Results from the APO-ONE caspase-3/7 and TUNEL assays suggested that this decrease in cell viability occurred due to apoptosis. Caspase-3 activation occurs downstream of both the extrinsic and intrinsic apoptotic pathways; thus, should reflect the amount of apoptosis occurring regardless of the pathway. In this study, caspase-3/7 activity was shown to increase in correlation with the IC_{50} consistently in each cell line which was confirmed by the TUNEL assay. These results suggest that caspase-mediated apoptosis is a mechanism of cell death in all three cell lines. The TUNEL assay showed an increase in the percentage of cells undergoing apoptosis as

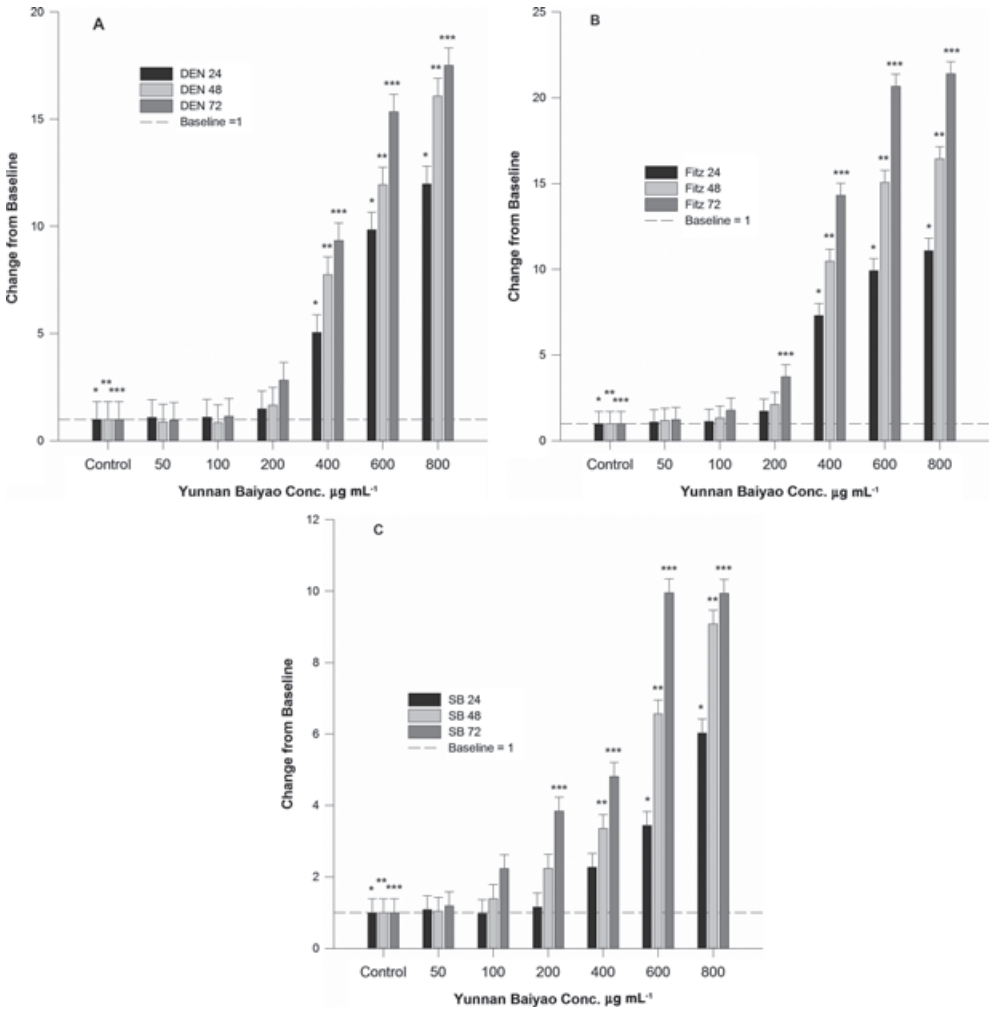

Figure 2. *Yunnan Baiyao* causes a concentration dependant increase in caspase-3/7 activity in HSA cells over time as measured by the Apo-ONE Homogenous Caspase-3/7 Assay. An increase in fluorescent signal is correlated with an increase in caspase-3/7 activity which is an important signalling and effector step in the apoptotic cascade. The results are expressed as a ratio of change compared with the baseline apoptosis measured in the control at 24, 48 and 72 h (dotted line represents baseline of 1). Control samples are designated as 24 h (*), 48 h (**) and 72 h (***). Error bars represent standard deviation (SD). A statistically significant increase in caspase-3/7 activity compared with the untreated control sample at the corresponding time point is represented on the graph by *, ** and *** for 24, 48 and 72 h, respectively. (A) Level of apoptosis measured in the DEN cells treated with increasing concentrations of *Yunnan Baiyao*. (B) Level of apoptosis measured in the Fitz cells treated with increasing concentrations of *Yunnan Baiyao*. (C) Level of apoptosis measured in the SB cells treated with increasing concentrations of *Yunnan Baiyao*.

the concentration increases in correlation with the APO-ONE caspase-3/7 results. This suggests that later mechanisms in the apoptotic cascade, such as DNA fragmentation are also involved in inhibition of HSA cell growth by *Yunnan Baiyao*. The mechanism by which *Yunnan Baiyao* causes apoptosis has not been elucidated. It is possible that *Yunnan Baiyao* could cause blockage of a receptor that triggers initiation of apoptotic pathways through downregulation of anti-apoptotic factors or upregulation of apoptotic factors which has been previously shown with *P. notoginseng*.[33] Another possibility is that it may directly alter downstream signalling proteins in the apoptotic pathway.

Cell cycle analysis did show some minor change in cell cycle kinetics. The changes were not inconsistent with normal cell cycling. No evidence was found to indicate cell cycle arrest was present in

Table 2. This table demonstrates the percentage of apoptotic cells as detected by the APO-BRDU Kit for cells incubated for 72 h at increasing *Yunnan Baiyao* concentrations

***Yunnan Baiyao* concentration (mg mL^{-1})**	**Den (%)**	**Fitz (%)**	**SB (%)**
0	0.29	0.20	0.08
50	1.91	2.76	0.41
100	4.68	7.01	6.47
200	19.63[a]	56.34[a]	86.47[a]
400	90.63[a]	99.69[a]	98.98[a]
600	100.00[a]	99.81[a]	98.78[a]
800	100.00[a]	99.78[a]	99.84[a]

A significant increase in apoptosis occurred at *Yunnan Baiyao* concentrations ≥200 μg mL^{-1} in each cell line (DEN, Fitz and SB).
[a]Represents a statistically significant increase in apoptotic cells compared with the untreated control sample.

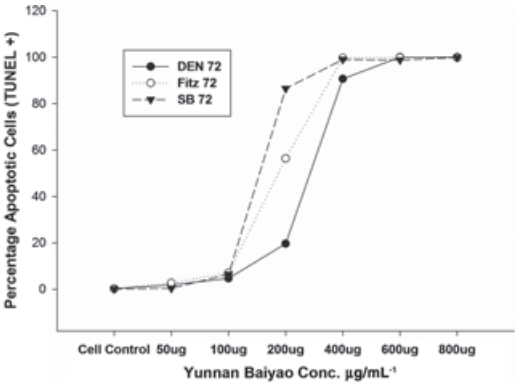

Figure 3. This graph demonstrates the percentage of apoptotic cells as detected by the APO-BRDU Kit for cells incubated for 72 h at increasing *Yunnan Baiyao* concentrations. A significant increase in apoptosis occurred at *Yunnan Baiyao* concentrations ≥200 μg mL^{-1} in each cell line (DEN, Fitz and SB).

either G1- or G2-phases of the cell cycle. This differs from previous data which showed that wild yam root arrested cells in the G2/M phase.[36] This may be due to the fact that *Yunnan Baiyao* consists of a combination of herbs which has different bioactivity than the individual components. Nonetheless all three cell lines at concentrations >200–400 μg mL^{-1} and at all time points showed induction of apoptosis (DNA debris) to the exclusions of all cell phases (see Fig. 4A–C). This DNA debris, found to the left of the G1 peak in the cell cycle histogram, considered a sign of late apoptosis is due to endonuclease cleaving of DNA. This unusual finding, was supported by the TUNEL assay's recording of virtually 100% (see Fig. 3) apoptotic cells at concentrations >200–400 μg mL^{-1}. These cells were still metabolically active since Cell Titer Blue activity was recorded for all cell lines at concentrations >200–400 μg mL^{-1}, although at significantly reduced levels (see Fig. 1A–C). Further investigation is required to explain the rapid induction of nuclear endonuclease activity by *Yunnan Baiyao*.

VEGF levels in cell supernatant were measured in untreated (control) cells and found to increase over time for all three cell lines. Although the VEGF levels for SB were negligible at 24 and 48 h, it was significant at 72 h (see Table 3). We have previously reported *in vitro* VEGF concentrations for these cell lines at 24, 48 and 72 h and the findings from this study are consistent with the variation found in our previous report.[41] We then evaluated the ability of *Yunnan Baiyao* to modulate VEGF levels in the cell supernatant for all three cell lines at 72 h. *Yunnan Baiyao* did cause some significant increases in VEGF levels in Fitz and SB cell lines, but not in the DEN cell line (see Fig. 5). The increases in VEGF occurred at concentrations of *Yunnan Baiyao* that were approaching the IC_{50} for Fitz and SB, namely 275.9 and 325.3 μg mL^{-1}, respectively. Moreover, these concentrations of *Yunnan Baiyao* were also consistent with the induction of apoptosis in the cell lines. These findings are not unlike our previous findings from a report that showed the induction of VEGF when mastinib concentrations approached the IC_{50} for Fitz and SB.[41] However, in the report by Lyles *et al.* the DEN cell line was marginally affected, but similar to this study the SB cell showed the greatest fold increase of VEGF. This needs to be investigated further by examining the effects of these drugs on cellular pathways involved in VEGF signalling and production, e.g. hypoxia inducible factor 1α (HIF1α). Interestingly, human cancer patients treated with anti-angiogenic tyrosine kinase inhibitors show increased plasma levels of VEGF and placental growth factor in the face of clinical efficacy.[42] The relationship between cell supernatant concentration and *in vivo* plasma concentration of VEGF is not clear. In the study by Clifford C *et al.*, median VEGF concentrations actually

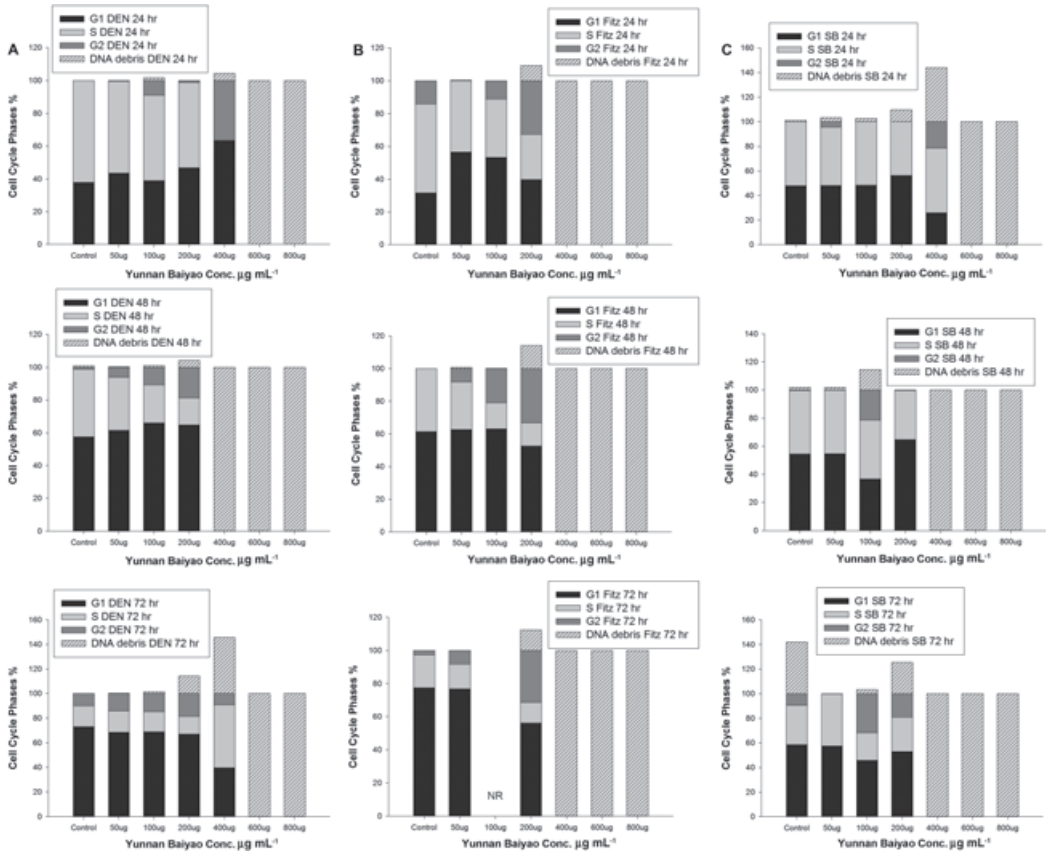

Figure 4. Cell cycle analysis was performed using flow cytometry and propidium iodide counter staining and data were recorded for all three cell lines at 24, 48 and 72 h. Remarkably, all cell lines at concentrations >200–400 µg mL^{-1} and at all time points the cell phases disappeared and were replaced by DNA debris. This DNA debris is considered a sign of late apoptosis due to endonuclease cleaving of DNA which correlates with the noted increase in caspase-3/7 activity. (A) Cell cycle kinetics measured in the DEN cells treated with increasing concentrations of *Yunnan Baiyao*. (B) Cell cycle kinetics measured in the Fitz cells treated with increasing concentrations of *Yunnan Baiyao*. (C) Cell cycle kinetics measured in the SB cells treated with increasing concentrations of *Yunnan Baiyao*.

decreased with increasing stage of disease and 4 of 17 dogs with HSA did not have detectable VEGF levels in the plasma.[38] This may be due to the fact that VEGF can differ within the tumour versus in circulation or this may not be the primary factor involved with progression of HSA in dogs.

This is the first documentation of *Yunnan Baiyao's* ability to cause a decrease in cell viability via apoptosis in canine HSA cells. It lends evidence to the anecdotally reported improvement in survival times in canine patients with HSA receiving this medication.

Pharmacokinetic studies on *Yunnan Baiyao* itself have not been performed; however, studies of the major component, *P. notoginseng*, have been performed. A pharmacokinetic study of intravenous panaxatrol disuccinate sodium, a ginsenoside derivative, was performed in healthy human volunteers and human patients with advanced solid tumours. The steady-state peak concentration,

Table 3. The untreated (control) cells expressed increasing levels of VEGF over time

Mean VEGF (pg mL^{-1})	DEN	Fitz	SB
24 h	437.0 ± 24.3	107.7 ± 2.0	0 ± 0
48 h	1192.6 ± 34.9	241.7 ± 3.1	25.7 ± 16.8
72 h	2532.7 ± 86.9	392.0 ± 5.0	58.1 ± 2.2

All samples were run in duplicate and the mean VEGF values ± standard deviation are reported here. VEGF levels were only statistically increased for the 72 h time point compared with the 24 h baseline ($^{*}P < 0.001$).

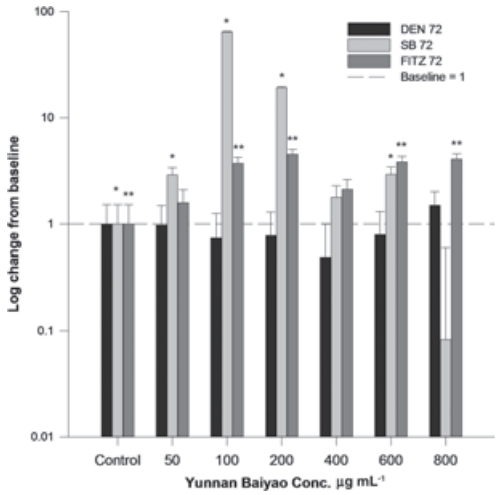

Figure 5. VEGF levels were measured in canine HSA cell supernatant after treatment with increasing concentrations of *Yunnan Baiyao* at 72 h. A logarithmic scale has been used due to the wide variation in VEGF levels among cell lines (dotted line represents baseline of one). The DEN cell line had no significant increases or decreases ($P < 0.001$) in VEGF levels. Significant increases ($P < 0.001$) in VEGF levels were found in the Fitz (**) cells treated with 100, 200, 600 and 800 µg mL^{-1} *Yunnan Baiyao*, and for the SB (*) cells with 50, 100, 200 and 600 µg mL^{-1} *Yunnan Baiyao*.

average concentration and mean steady state AUC in plasma were 13.96±15.48, 0.15±0.29 and 148.00±117.18 mg L^{-1}, respectively. An intravenous injection at a dose of 100 mg m^{-2} has been suggested for further phase II clinical trials.[43] We can not necessarily correlate what is achievable *in vitro* to *in vivo* availability based upon our study as we are not examining an individual component of *Yunnan Baiyao*. However; the average IC_{50} for the three cell lines across the three time points (24, 48 and 72 h) was 350.17 µg mL^{-1} (equal to 350.17 mg L^{-1}). This value does exceed the above noted steady state peak concentration and average concentration in plasma but is in a similar range with the mean steady state concentration achieved in plasma.[43] On the basis of dosing of other chemotherapeutic medications in veterinary medicine and the results of this *in vitro* study, this would appear to be a clinically attainable dose in the canine patient. It should also be noted that the IC_{50} data presented here is based on the entire *Yunnan Baiyao* compound and separation of the individual ingredients is more likely to result in even more comparable data. Pharmacokinetic studies in canine patients on the individual components as well as whole compound *Yunnan Baiyao* are needed to have a better understanding of clinically achievable levels. Ginsenosides have also been identified as pharmacokinetic markers in the serum of rats after oral administration of *P. notoginseng*.[44] *Panax notoginseng* may serve as a marker of *Yunnan Baiyao* plasma concentration in the future.

Novel medications for the treatment of canine HSA are needed and Chinese herbal medications are being studied at an increasing rate for the purposes of cancer treatment in people. Increased demand for herbal medications worldwide as well as voluntary use of Good Agricultural Practice (GAP) has advanced knowledge as well as safety of these medications.[31] A nutrient and metal analysis on various marketed herbal products showed that contaminants such as Ni, Pb and Cd were equal to or lower than previously reported. Concentrations of these minerals were also below National Research Council proposed tolerances at recommended dosing.[45] Another study performed HPLC specifically on different *Yunnan Baiyao* batch preparations and showed that the total content of 13 saponins varied insignificantly (<4.78%) for different batches of powder and capsule forms when purchased from the *Yunnan Baiyao* Group.[46] On the basis of these studies, *Yunnan Baiyao* also appears to be a safe medication for further study in the canine and human patient.

In conclusion, *Yunnan Baiyao* induces both time-dependant and concentration-dependant cell death through apoptosis in canine HSA cells *in vitro*. This is the first study to document *Yunnan Baiyao's* ability to induce apoptosis in canine HSA cells and the associated IC_{50} values. VEGF expression was also documented in untreated (control) and treated HSA cells. The information gained from this study supports the further investigation of *Yunnan Baiyao* in treatment of canine HSA in the laboratory and clinical settings.

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Conflicts of interest

The authors have declared no conflicting interests.

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The effect of a spot-on formulation containing polyunsaturated fatty acids and essential oils on dogs with atopic dermatitis

M. Blaskovic [a], W. Rosenkrantz [b], A. Neuber [c], C. Sauter-Louis [d], R.S. Mueller [a,*]

[a] Clinic of Small Animal Medicine at the Centre for Clinical Veterinary Medicine, Ludwig-Maximilian University, Veterinärstrasse 13, 80539 Munich, Germany
[b] Animal Dermatology Clinic, Tustin, CA 92780, USA
[c] Derm4Pets Clinic, Chiltern Referral Services, Chalfont St. Giles, Buckinghamshire HP8 4AB, UK
[d] Clinics for Ruminants at the Centre for Clinical Veterinary Medicine, Ludwig-Maximilians University, Sonnenstrasse 16, 85764 Oberschleissheim, Germany

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ABSTRACT

Recent studies have shown that immunological aberrations and epidermal barrier defects could be important in the pathogenesis of canine atopic dermatitis (CAD) and that oral polyunsaturated fatty acids (PUFAs) might influence the epidermal barrier. The aim of this study was to evaluate the effects of a spot-on formulation containing PUFAs and essential oils on pruritus and lesions caused by CAD. Forty-eight privately owned dogs of different breeds, ages and genders diagnosed with atopic dermatitis were included in a randomized, double-blinded, placebo-controlled, multicentre clinical trial. Dogs were treated with a spot-on formulation containing PUFAs and essential oils or placebo on the dorsal neck once weekly for 8 weeks. Before and after the study, CAD extent and severity index-03 (CADESI-03) and pruritus scores were determined by veterinarians and owners, respectively.

There was significantly more improvement in CADESI-03 and pruritus scores in the treatment group than in the placebo group ($P = 0.011$ and $P = 0.036$, respectively). Additionally, more dogs improved by at least 50% in CADESI-03 and pruritus scores in the treatment group than in the placebo group ($P = 0.008$ and $P = 0.070$, respectively). No adverse reactions were observed. The topical preparation containing PUFAs and essential oils was a safe treatment and beneficial in ameliorating the clinical signs of CAD.

Introduction

Canine atopic dermatitis (CAD) is a commonly presented disease in veterinary practice (Scott and Paradis, 1990) and is associated with pruritus (Saridomichelakis et al., 1999; Griffin and DeBoer, 2001) and skin lesions (Griffin and DeBoer, 2001; Favrot et al., 2010). It is diagnosed by history, clinical signs and the exclusion of differential diagnoses, and clinical diagnostic criteria have been recently introduced (Favrot et al., 2010). In CAD, a hypersensitivity response against environmental or food allergens develops due to a genetic predisposition and could be associated with disturbances in the skin barrier function (Merryman-Simpson et al., 2008; Sandilands et al., 2009; Wood et al., 2009). Allergens involved in the pathogenesis of non-food-induced CAD include house dust mites, pollens, moulds and insect antigens (Hill and DeBoer, 2001). Allergens can be inhaled or percutaneously absorbed (Olivry and Hill, 2001; Marsella et al., 2006).

Symptomatic treatment for CAD includes antihistamines, glucocorticoids, cyclosporin, topical therapy, and polyunsaturated fatty acids (PUFAs), while specific treatment employs allergen-specific immunotherapy (Olivry et al., 2010). PUFAs cannot be synthesized de novo and need to be ingested pre-formed in the diet. They contain one or more double bonds, and are classified as omega-3 and omega-6 fatty acids, depending on the position of the first double bond relative to the carboxy end of the chain. Important omega-3 fatty acids are α-linolenic acid (in linseed oil), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA; in fish oils). Omega-6 fatty acids are linoleic acid (in sunflower or safflower oil), γ-linoleic acid (in evening primrose oil) and dihomo-γ-linoleic acid.

In vitro, PUFAs are reported to have anti-inflammatory (Ziboh and Chapkin, 1988; Ziboh et al., 2000) and immunomodulating (Stehle et al., 2010) effects. A further possible mechanism of action is improvement of the epidermal barrier function, presumably by changing the composition of epidermal lipids. Oral fatty acid supplementation has been reported to change cutaneous lipids in Beagle dogs (Campbell and Dorn, 1992).

In contrast to many other symptomatic therapies for CAD, oral supplementation with PUFAs rarely causes adverse effects (Olivry

* Corresponding author. Tel.: +49 8921 802654.
E-mail address: R.Mueller@lmu.de (R.S. Mueller).

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et al., 2001; Mueller et al., 2004), although diarrhoea might occur with oral supplementation (Scott et al., 1992). Adverse effects of topically administered PUFA therapy have not been reported (Tretter and Mueller, 2011). Concurrent treatment with PUFAs might permit reduction of the dosage of other anti-inflammatory medications, such as glucocorticoids, and further improvement in clinical signs (Scott and Miller, 1993; Bond and Lloyd, 1994; Saevik et al., 2004).

Studies on the use of oral fatty acid supplementation have been published (Mueller et al., 2004; Saevik et al., 2004), but reports about the efficacy of topically applied PUFAs or ceramides are rare and describe non-blinded and open trials (Piekutowska et al., 2008; Tretter and Mueller, 2011). The aim of this study was to evaluate the efficacy of a commercial spot-on containing PUFAs and essential oils on the clinical signs of CAD in a prospective, placebo-controlled, randomised trial.

Materials and methods

The study was approved by the Ethics Committee of the Centre for Clinical Veterinary Medicine/Ludwig Maximilian University Munich (Approval number 03-051012). Prior to enrolment, dog owners gave their written consent (Appendix A: Supplementary material).

Study design and study objects

This was a randomized, double-blinded, placebo-controlled multicentre study. Three dermatology referral practices in Germany (Centre for Clinical Veterinary Medicine, Ludwig Maximilian University Munich), the UK (Derm4Pets Clinic, Buckinghamshire/Berkshire) and the USA (Animal Dermatology Clinic, Tustin, California) participated.

Forty-eight privately owned dogs with atopic dermatitis were included, of different genders, ages and breeds. The treatment group consisted of 23 dogs classified with either moderate to severe CAD ($n = 12$) or mild CAD ($n = 11$). There were 25 dogs in the placebo group (16 classified with moderate to severe CAD and nine with mild CAD).

Randomization

The dogs were stratified into two subgroups with mild disease characterized prior to treatment by either low lesion scores i.e. a CAD extent and severity index-03 (CADESI-03) < 60 ($n = 20$), or moderate to severe disease (CADESI-03 > 60; $n = 28$; Olivry et al., 2008). Separate randomization schedules for both groups and each study centre were created by the study monitor prior to the study according to a computer-generated randomization list.[1] Medication and identically packaged placebos were sent to each study centre and each package was specifically marked and dispensed according to the randomization list.

Inclusion criteria

All dogs had been diagnosed with environmentally-induced atopic dermatitis based on history, clinical signs and rule-out differential diagnoses by appropriate means, such as skin cytology, skin scrapings, elimination diets and/or ectoparasite control measures. Dogs with mild disease were treated exclusively with topical therapy, either product or placebo. Antihistamines and other topical therapies were discontinued at least 2 weeks prior to starting the study and glucocorticoids and cyclosporin were discontinued at least 6 weeks prior to enrolment.

In the group with moderate to severe CAD, exclusive treatment with placebo or topical fatty acids/essential oils was considered unethical due to the reported limited improvement seen with oral fatty acid supplementation (Olivry et al., 2001; Mueller et al., 2004). Concurrent low dose glucocorticoids, antihistamines and topical therapy were permitted if they had been administered at an unchanged dose for more than 12 weeks prior to inclusion and during the trial. Diet changes were not permitted within 3 months prior to or during the study. Allergen-specific immunotherapy was permitted in dogs that had been receiving it for at least 12 months prior to inclusion. Dogs with a history or clinical signs of flea bite hypersensitivity received fipronil spot on (Frontline, Merial) or selamectin spot on (Stronghold, Zoetis) once monthly.

[1] See: http://graphpad.com/quickcalcs/randomN1.cfm (last accessed 15 October 2013).

Study protocol

All dogs were treated with a spot-on preparation once weekly for 8 weeks. The owners applied the product on the dorsal cervical area after being given detailed instructions on how to spread the hair coat and apply the product directly onto the skin. Dogs received either a product containing PUFAs (6 mg/mL of α-linolenic and 30 mg/mL of linoleic acid), essential oils (neem oil, rosemary extract, lavender oil, clove oil, tea tree oil, oregano extract, peppermint extract and cedar bark extract) and vitamin E (Dermoscent Essential 6 spot-on, LDCA) or a placebo (bio diffusing agents, Dermoscent, LDCA).

Dogs <10 kg received 0.6 mL weekly; dogs weighing 10–20 kg received 1.2 mL weekly, and dogs of 20–40 kg received 2.4 mL weekly. This protocol was according to the manufacturer's recommendations and the same as the protocol used in a previously published pilot study (Tretter and Mueller, 2011). The commercial product has a distinct odour that was absent from the placebo. However, the owners of placebo treated dogs were not aware of this difference. It was previously established that the odour dissipated within 1 week of application and investigators were unable to detect the odour at the time of scoring, thus keeping the integrity of the blinding intact.

Clinical evaluation

A validated lesion score (CADESI-03; Olivry et al., 2007, 2008) was used to determine the severity of skin lesions. If the initial CADESI-03 was ⩽60, dogs were considered to have mild CAD ($n = 20$). If the CADESI-03 was >60, the disease was categorized as moderate to severe ($n = 28$), as previously reported (Olivry et al., 2008). Dogs with moderate to severe disease commenced the study after their clinical signs had improved with other therapies (see above) and they were considered stable. Dogs were evaluated at enrolment and after 8 weeks of treatment. The CADESI-03 score was determined by the clinician at each visit. Similarly, owners completed a validated pruritus score at each visit, scoring pruritus from 0 to 10 using a visual analogue scale combined with features of the behaviour and severity-based scales (Hill et al., 2007; Appendix B).

Statistical analyses

Based on data gathered in a recent pilot study (Tretter and Mueller, 2011), it was calculated that with at least 20 dogs in each group (treatment and placebo), a difference of 6 points in CADESI-03 scores and 2 points in pruritus scores could be determined with a power of 90% and a significance level of $P < 0.05$. To ensure similar groups, initial CADESI-03 scores and pruritus scores were compared using Mann Whitney tests. For the same reason, the age and weight of dogs in both groups were compared with an unpaired t test or (if data were normally distributed) or Mann–Whitney U tests (if data were not normally distributed). Gender distribution was analyzed using Fisher's exact tests. Improvements in pruritus and CADESI-03 scores, respectively, were calculated by subtracting the score at enrolment from the score at the end of the study. This was compared between groups using an unpaired t test with Welsh correction (if data were normally distributed), or a Mann–Whitney U test (if data were not normally distributed). The number of dogs improving by at least 50% and the number of dogs deteriorating in the treatment group compared to the placebo group were compared using Fisher's exact tests.

A one-sided P value was chosen, as a previously published pilot study had shown improvement in both pruritus and CADESI-03 scores with this therapy (Tretter and Mueller, 2011) and thus deterioration was not expected in the treatment group compared to placebo. Significance for all tests was set at $P < 0.05$. The statistical program used was GraphPad Prism 5.0 (GraphPad). Dogs were excluded from the per protocol analysis if they exhibited clinical signs of an adverse reaction to the product, when owner compliance was not satisfactory, or when the clinical signs of atopic dermatitis deteriorated to the point that additional antipruritic therapy was needed. An intention to treat analysis, with the last value carried forward, using all dogs included in the study was performed, as well as a per protocol analysis.

Results

CADESI-03 and pruritus scores

There was no significant difference between treatment and placebo groups with respect to CADESI-03 scores ($P = 0.278$) or pruritus ($P = 0.909$) at enrolment. There was also no difference between groups in age ($P = 0.735$), bodyweight ($P = 0.782$) or gender distribution ($P = 0.785$). Because two dogs did not complete the study, per protocol analysis was performed on 46 dogs. As the results of the intention to treat analysis and that of the per protocol analysis were similar, only the results of the intention to treat analysis are reported here.

The mean and the confidence intervals of CADESI-03 and pruritus scores pre- and post-therapy are shown in Table 1. Individual improvements in CADESI-03 scores and pruritus scores in each dog were significantly higher in the treatment group than in the placebo group (Mann–Whitney U test, $P = 0.011$ and $P = 0.036$, respectively). The numbers of dogs improving by at least 50% or 90% are listed in Table 2. More dogs showed an improvement of ⩾50% in CADESI-03 and pruritus scores in the treatment group than in the placebo group (Fisher's exact test, $P = 0.008$ and $P = 0.07$, respectively). Significantly more dogs deteriorated in the placebo group (15/25) compared to the treatment group (5/23; Fisher exact test, $P = 0.01$). Raw data are shown in Appendix B: Supplementary materials.

Adverse effects and exclusions

All except two dogs completed the study. These had moderate to severe clinical signs and both deteriorated during the first 4 weeks of the study, requiring their concurrent therapy to be changed. One of those dogs was in the treatment group and one was in the placebo group. Adverse effects were not observed in any of the treated dogs.

Discussion

This study demonstrated that the clinical signs of atopic dermatitis in dogs with stable CAD that met the study entry criteria significantly improved after eight weekly topical treatments of a commercially available compound containing PUFAs and essential oils. The degree of improvement was similar to another randomized, placebo-controlled study where 29 dogs received oral fatty acid supplementation for 10 weeks and showed significant improvement (Mueller et al., 2004). That study used a different scale to measure outcomes, as it preceded the use of the CADESI-03 and the visual analogue pruritus scale for use by dog owners (Mueller et al., 2004).

The results of the present investigation support the findings of a recent pilot study using the same product (Tretter and Mueller, 2011) and also studies evaluating oral fatty acid supplementation (Olivry et al., 2010). PUFAs are thought to have lower efficacy than glucocorticoids and cyclosporin (Olivry et al., 2010), but they are a safe alternative to other anti-inflammatory therapies. Adverse effects associated with their use are rare and usually mild (Mueller et al., 2004; Olivry et al., 2010), which is particularly important in the long-term treatment of chronic diseases such as CAD. In this work, no adverse effects were noted with short-term use (8 weeks). Widespread use of the product over longer periods of time is needed to make more definitive statements regarding safety.

As this is the first double-blinded, placebo-controlled study evaluating topical therapy with a commercial product containing essential oils and PUFAs, the only comparison possible is with oral PUFA supplementation. There are many studies using oral PUFA supplementation, but only a few that are placebo-controlled and double-blinded. In one such study, there was a significant improvement of clinical signs with commercially available EPA and DHA preparations (Mueller et al., 2004). In another controlled study based on the measurement of serum arachidonic acid before and after the trial, dogs with early CAD showed more improvement than dogs with a longer history of CAD (Abba et al., 2005).

Direct comparison of an oral and topical product is not possible, but our study provides evidence that the efficacy of topical therapy with essential oils and PUFAs appears to be comparable to that reported for studies evaluating oral PUFAs (Mueller et al., 2004; Saevik et al., 2004). With oral fatty acid supplementation, approximately half of the dogs treated with daily fatty acids improved by 50% or more compared to only 10% in the placebo group in an earlier study (Mueller et al., 2004). In the current work, the corresponding results were in the same range for CADESI-03 and pruritus scores, suggesting both types of treatment are suitable for the treatment of CAD, but the success rate for either one is not as high as that for glucocorticoids or cyclosporin (Steffan et al., 2006; Olivry et al., 2010).

It was recommended a decade ago that PUFA supplementation should be administered for at least 12 weeks before assessing the success of treatment (Olivry et al., 2001). This was based on the pharmacokinetics of oral PUFAs (Campbell and Dorn, 1992; Campbell et al., 1995). However, other authors observed effects with daily PUFA supplementation as early as 2 weeks after the initiation of therapy (Scott et al., 1992, 1997; Olivry et al., 2010). In our study, an 8-week supplementation period was chosen because the clinical effects in a pilot study were noted after 8 weeks of administration (Tretter and Mueller, 2011).

In the present study, a validated pruritus score and a CADESI-03 were used. Pruritus and skin lesions are typically considered the most relevant parameters in studies evaluating CAD (Olivry et al., 2010). The scores for lesions and pruritus have been previously validated (Hill et al., 2007; Olivry et al., 2007, 2008). The number of dogs improving by more than 50% and 90%, respectively, was always higher in the treatment group than in the placebo group for both pruritus and CADESI-03 scores (Table 2). However, in the placebo group more dogs improved in pruritus scores than in CADESI-03. The improvement in pruritus scores could be perceived rather than real and might provide evidence for the more subjective nature of the assessment of pruritus.

PUFAs are considered less efficacious than, for example, glucocorticoids (Olivry et al., 2010) or cyclosporin (Steffan et al., 2006) in the treatment of CAD, but have been shown to be successful

Table 1
Data for CADESI-03 and pruritus scores of dogs with atopic dermatitis treated with either a spot-on formulation containing essential fatty acids/essential oils or placebo.

	Treatment Day 0	Treatment Day 56	Improvement[a] with treatment	Placebo Day 0	Placebo Day 56	Improvement[a] with placebo
Total number of dogs	23			25		
Mean CADESI-03 (95% CI)	46 (29–63)	28 (18–39)	18 (4–32)	78 (41–116)	80 (44–115)	−1 (−19 to 13)
Mean pruritus (95% CI)	5.2 (4.2–6.2)	3.9 (2.7–5.2)	1.3 (0.2–2.4)	5.3 (4.3–6.3)	5.0 (4.1–6.0)	0.2 (−0.7 to 1.2)
Number of dogs with mild AD	11			9		
Mean CADESI-03 (95% CI)	25 (13–36)	15 (9–22)	9 (−1 to 20)	22 (11–32)	34 (21–48)	−13 (−21 to −5)
Mean pruritus (95% CI)	4.8 (2.9–6.6)	4.0 (1.5–6.0)	1 (−0.9 to 2.9)	3.8 (2.1–5.5)	4.0 (2.1–5.9)	−0.2 (−2.6 to 2.2)
Number of dogs with moderate–severe AD	12			16		
Mean CADESI-03 (95% CI)	66 (38–94)	40 (22–58)	24 (−1 to 50)	110 (58–163)	105 (53–157)	6 (−19 to 30)
Mean pruritus (95% CI)	5.6 (4.4–6.8)	4.1 (2.5–5.7)	1.5 (0–3.0)	6.1 (5.1–7.2)	5.6 (4.5–6.8)	0.5 (−0.5 to 1.5)

CADESI-03, canine atopic dermatitis extent and severity index-03; CI, confidence interval.
[a] Improvement = Score at the beginning of the trial − Score at the end of the trial.

Table 2
Numbers of dogs improving by more than 50% and 90% in CADESI-03 and pruritus scores when treated with a spot-on formulation containing essential fatty acids/essential oils or placebo.

	Treatment improvement ⩾50%	Placebo improvement ⩾50%	Treatment improvement ⩾90%	Placebo improvement ⩾90%
CADESI-03 (*n*)	8/23	1/25	1/23	0/25
Pruritus score (*n*)	9/23	4/25	2/23	1/25

CADESI-03, canine atopic dermatitis extent and severity index-03.

as adjunctive therapy (Saevik et al., 2004). For this reason, additional medications were permitted in dogs with moderate–severe atopic dermatitis, as outlined above. In addition, it was considered unethical to treat dogs with more severe disease exclusively with topical fatty acids/essential oils. Dogs with a CADESI-03 of >60 have been classified as having moderate to severe atopic dermatitis (Olivry et al., 2008). Since the clinical signs in those dogs were unlikely to be controlled by sole therapy with PUFAs/essential oils, the use of concurrent medication was considered ethical and justified. As the dose of concurrent medications was not changed for the 12 weeks preceding the study or during the study, those drugs are unlikely to have influenced the study outcome.

It is not clear how well spot-on preparations containing fatty acids distribute in the epidermis and how long possible changes in epidermal ceramide composition last. One study reported a significant increase in free ceramides at the application site 3 days after the last treatment after twice weekly application of a topical spot-on preparation containing ceramides and free fatty acids for 3 weeks (Popa et al., 2012). The clinical improvement seen in dogs with multifocal to generalized skin disease treated with such products further supports some effect on the epidermis, but more studies are needed to provide details regarding the distribution and mechanism of action of topical essential oils and PUFAs in dogs.

Conclusions

Based on the findings in this study, the application of a spot-on containing PUFAs and essential oils was beneficial in alleviating the clinical signs of CAD. As complete remission was not achieved in the vast majority of dogs, it seems most useful as an adjunctive therapy in this disease.

Conflict of interest statement

The study was financed by Laboratoire de Dermo-Cosmetique France, which had no influence on study design, data evaluation or manuscript preparation. Dr. Blaskovic was financially supported by LCDA France. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tvjl.2013.10.024.

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Evaluating the effect of oral administration of *Echinacea* hydroethanolic extract on the immune system in dog

S. Torkan[1], F. Khamesipour[2] & S. Katsande[3]

[1]Department of Small Animal Internal Medicine, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran, P.O. Box: 166, [2]Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran, P.O. Box: 166, [3]Paraclinical veterinary studies, University of Zimbabwe, P. O. Box MP 167 Mt Pleasant, Harare, Zimbabwe

Correspondence:
F. Khamesipour

Summary

1. This study was designed to evaluate the effects of oral administration of *Echinacea* hydroethanolic extract on the dog's immune system.
2. The study was performed on 14 dogs that were referred to the veterinary clinic. These dogs were randomly allocated to two equal treatment groups. The first group received 1 ml of 5% *Echinacea* hydroethanolic extract two times a day for 2 months, and the second group received a placebo (water). To do haematology and immunology tests, the dogs were bled on days 0, 30 and 60. Blood tests, including packed cell volume (PCV), haemoglobin (Hb), red blood cell count (RBC), white blood cell count (WBC), counting neutrophils (Nut), lymphocytes (Lym), monocytes (Mon), eosinophils (Eos), basophils (Baso) and B cell, were performed. Furthermore, safety factor IgM and per cent of phagocytosis and phagocyte were measured from the blood sample.
3. The results showed that in the group which received *Echinacea* PCV, Hb, RBC count, WBC count, Lym, Nut, the per cent of phagocytosis and IgM significantly increased ($P < 0.05$). Moreover, positive effects of *Echinacea* plant on the immune system were observed. There was a significant change in HTC, RBC, Hb over time in the group that received *Echinacea* and the per cent of phagocytosis and IgM ($P < 0.05$).
4. The study establishes that these extracts might have appreciable immunostimulatory activity. However, further studies are required to confirm these findings.

Keywords: *Echinacea*, oral administration, dog, immune system

Introduction

The use of liquid extracts of *Echinacea* has increased recently and most often used as immunostimulating agents for the treatment (Hermann *et al.*, 2003) and prevention of various infectious disorders in human medicine (Wolfram & Hans-Helge, 1999). This is due to an increase in the clinical importance of herbal drugs in modern medicine with considerable attention being paid to the use of plants as a source of immunomodulators being at the centre stage. Several medicinal herbs have shown to promote immunity in different ways; they have shown to augment specific cellular and humoral immune response (Duke, 1985).

Immunomodulators are agents that can modulate the immune response, and their effect may be stimulatory or suppressive (Ghonime *et al.*, 2011). *Echinacea* extracts exhibit that potential. Rehman *et al.* (1999) (Rehman *et al.*, 1999) showed an increase in primary and secondary IgG response in rats treated with *Echinacea*. A few scientific studies have assessed the efficacy of Echinacea *in vivo* with varying results (Grimm & Mu¨ ller, 1999; Turner *et al.*, 2000). Therefore, there is need to assess the effects of *Echinacea* hydroethanolic extract on dogs as an animal model in assessing its efficacy as an immunostimulant. This study was designed to evaluate the effects of oral administration of *Echinacea* hydroethanolic extract on the dog's immune system.

Materials and methods

Animals

All experiments were carried out under the ethical guidelines of the Islamic Azad University of Shahrekord Branch, for the care and use of animals (Ernest *et al.*, 1993).

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The study was performed on 14 male dogs of mixed breeds that were randomly allocated to two equal treatment groups. All dogs were subjected to clinical examination and housed under uniform environment after being treated for internal parasites. The first group received 1 ml of 5% *Echinacea* hydroethanolic extract two times a day for 2 months, and the second group received a placebo (water) instead of *Echinacea* extract.

Preparation method for Echinacea extract

Fifty grams of powdered plant was added to 700 ml of 50% ethanol (350 ml distilled water and 350 ml ethanol), and Soxhlet apparatus was used to prepare hydroethanol extract. The solvent was filtered under reduced pressure. The plant ingredient concentration in the final extract was adjusted to the required concentration by adding distilled water to the dried extract. The extract was prepared each week and stored in a refrigerator.

Sample collection and analyses

The dogs were bled on days 0, 30 and 60 for haematology and immunology tests, and every blood sample was divided into two equal volumes for further analyses. The blood tests that were carried out include packed cell volume (PCV), haemoglobin (Hb), red blood cell count (RBC), white blood cell count (WBC), counting neutrophils (Nut), lymphocytes (Lym), monocytes (Mon), eosinophils (Eos) and basophils (Baso).

Statistical analysis

Statistical analysis focused on mean analysis for repeated measures that is before intervention, 30 days and 60 days. The following model was used:

$$Y_{\mathrm{ijkl}} = \mu + \tau_i + B_j + T_k + (\tau\mathrm{BT})_{\mathrm{ijkl}} + \varepsilon_{\mathrm{ijkl}}$$

where Y_{ij} is the response measured on the ith treatment, i = [PCV, Hb, RBC count, WBC count, counting Nut, Lym, Mon, Eos, Baso, B and Cell], on the jth dog, at the kth time, μ is the overall mean, τ is the mean effect of treatment, B is the random subject effect, T_k is the time effect, τBT is the interaction effect and ε is the error.

Results

The total sample size was 14, and each treatment group comprised of seven dogs. Body temperature and respiratory rate of the two groups were significantly different before the intervention Table 1 ($P < 0.05$). Heart rate was higher in the group that was selected to receive *Echinacea* but not significantly different from the group that received the placebo ($P > 0.05$). The results showed that in the group which received *Echinacea* PCV, Hb, RBC count, WBC count, Lym, Nut, the per cent of phagocytosis and IgM significantly increased (Table 2 and 3) ($P < 0.05$). Also, the results indicated effects of *Echinacea* plant on the immune system. There was a significant change over time in the group that received *Echinacea* on HTC, RBC, Hb and the per cent of phagocytosis and IgM ($P < 0.05$).

Table 1 The mean ± SD of vital signs of two groups before intervention

Variable	Group	Number of dogs	Mean
Heart rate min^{-1}	*Echinacea*	7	87.9 ± 12
	Placebo	7	84.7 ± 8.7
Body temperature °C	*Echinacea*	7	37.76^{b} ± 0.6
	Placebo	7	38.47^{a} ± 0.4
Respiratory rate min^{-1}	*Echinacea*	7	20.1^{b} ± 2.5
	Placebo	7	25.3^{a} ± 2.6

Different superscript letters within the same column indicate significant difference ($P < 0.05$).

Discussion

Burger *et al.* (1997) (Burger *et al.*, 1997) and See *et al.* (1997) (See *et al.*, 1997) observed that extracts from *Echinacea* have non-specific immunostimulatory properties *in vitro* including increased phagocytosis, cytokine production and natural killer cell activity. The plant and its extracts have been shown to stimulate phagocytosis *in vitro* and *in vivo* in murine (Melchart *et al.*, 1995). Roesler *et al.* (1991) (Roesler *et al.*, 1991) confirmed activation of human phagocytic function both *in vitro* and *in vivo*.

Information generated in past research suggests that the immunostimulatory activity of *Echinacea* depends on the combined action of caffeic acid derivatives and alkylamides (Bauer 1998; Hermann *et al.*, 2003). Moreover, many pharmacological compounds have been isolated from *Echinacea* (San Feliciano *et al.*, 1993), and several constituents are alleged to be immunologically active, including polysaccharides and glycoproteins (Bauer *et al.*, 1988). Sloley *et al.* (2001) (Sloley *et al.*, 2001) showed that phenylpropanoid glycosides, which are constituents of certain *Echinacea* species, possess antiviral properties and are antioxidants and free radical scavengers and inhibit Fe^{2+}-induced lipid peroxidation.

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Table 2 Comparison of mean ± SD of blood parameters before and after intervention

Variable	Treatment	Before	1 month	2 month
HTC	*Echinacea*	$43.43^{b} \pm 1.11$*	$47.71^{a} \pm 0.606$**	48.14 ± 0.857**
	Placebo	$47^{a} \pm 3.97$	$46.29^{b} \pm 4.539$	50 ± 1.397
Red blood cell count (RBC)	*Echinacea*	$7.36^{b} \pm 0.143$*	$7.86^{a} \pm 0.053$**	$7.94^{a} \pm 0.084$**
	Placebo	$7.47^{a} \pm 0.465$	$7.50^{b} \pm 0.493$	$7.46^{a} \pm 0.178$
Haemoglobin (Hb)	*Echinacea*	$14.70^{a} \pm 0.235$	$15.34^{a} \pm 0.043$	$15.10^{a} \pm 0.136$
	Placebo	$14.26^{b} \pm 0.612$	$14.56^{b} \pm 0.581$	$14.42^{b} \pm 0.274$
White blood cell count (WBC)	*Echinacea*	$12990^{a} \pm 445.67$*	13290 ± 441.65**	13550 ± 408.54**
	Placebo	$11580^{b} \pm 233.467$	11890 ± 535.079	11950 ± 328.494
Neutrophils (Nut)	*Echinacea*	58.143 ± 3.181*	62.286 ± 1.96**	56.714 ± 2.579*
	Placebo	63.714 ± 3.746*	61.143 ± 3.508*	57.429 ± 3.872**
Band	*Echinacea*	2.286 ± 0.747	1.571 ± 0.297	1.571 ± 0.429
	Placebo	1.714 ± 0.36	1.714 ± 0.184	1.143 ± 0.508
Lymphocytes (Lym)	*Echinacea*	27.143 ± 2.539*	31.714 ± 2.942**	34.286 ± 4.144**
	Placebo	32.571 ± 2.626*	31.714 ± 2.109*	27.143 ± 3.068**
Monocytes (Mon)	*Echinacea*	2.714 ± 0.565	2.857 ± 0.34	3.857 ± 0.34
	Placebo	3.286 ± 0.522	3.429 ± 0.369	2.857 ± 0.261
Eosinophils (Eos)	*Echinacea*	4.143 ± 0.738	3.857 ± 1.122	4.371 ± 0.528
	Placebo	3.714 ± 1.04	3 ± 0.65	4 ± 0.535
Basophils (Baso)	*Echinacea*	$0.313^{b} \pm 0.143$	0.557 ± 0.143	0.571 ± 0.297
	Placebo	$0.429^{a} \pm 0.202$	0.286 ± 0.184	0.571 ± 0.297

Different superscript letters within the same column indicate significant difference ($P < 0.05$).
*; **; *** = significantly different means in the same row.

Table 3 Comparison of phagocytes, phagocytosis and IgM before and after intervention

Variable	Treatment	Before	30 days	60 days
Phagocytes	*Echinacea*	25.857 ± 2.604*	27.286 ± 2.884**	28.462 ± 2.07**
	Placebo	34.286 ± 2.427*	32.571 ± 2.776*	27.286 ± 1.886**
Phagocytosis	*Echinacea*	25.429 ± 3.747	25.143 ± 2.385	23.857 ± 2.064
	Placebo	30.714 ± 4.714	23.143 ± 2.721	26.429 ± 1.478
IgM	*Echinacea*	$138.714^{b} \pm 3.242$*	159.857 ± 13.674**	182.857 ± 13.956***
	Placebo	$150.286^{a} \pm 18.774$	181.714 ± 7.63	186.429 ± 6.679

Different superscript letters within the same column indicate significant difference ($P < 0.05$).
*; **; *** = significantly different means in the same row.

The haemoglobin levels were significantly increased in the trial by *Echinacea* extract. This is supported by Anon (1989) (Anon, 1989) who concluded that *Echinacea* extract behave as an agent that improves the quality of blood by increasing haemoglobin levels and the number of erythrocytes therefore, considered to improve parameters of exercise physiology and performance.

Increase in the number of lymphocytes during the treatment phase of the study support the information generated in other investigations (See *et al.*, 1997; Steinmuller *et al.*, 1993) which suggests that *Echinacea* behaves as an immune system stimulant. In addition, previous studies have demonstrated that *Echinacea* has an enhancing effect on lymphocyte function and proliferation (See *et al.*, 1997). Furthermore, Echinacea extracts showed protection of immunosuppressed mice against systemic infections with stimulation of macrophage and neutrophil function (Steinmuller *et al.*, 1993).

Increase of neutrophil counts was achieved only after the first month and then decreased after the second month on *Echinacea* treatment group, thereby raising the question as to what other external factors may have contributed to the change. However, Melchart *et al.* (1995) (Melchart *et al.*, 1994, 1995) and O'neill *et al.* (2002) demonstrated the effect of *Echinacea* on the capacity of neutrophils to ingest more foreign particles and stimulatory effect on these cells by improving phagocytic function.

The changes that occurred to the blood parameters measured in the current study are strongly due to the effects of *Echinacea* which acted as an immunomodulator by activating cytotoxic effecter cells such as cytotoxic T lymphocytes, natural killer (NK) cells, lymphocytes, macrophages and

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activated neutrophils as observed by Ghonime *et al.* (2011) (Ghonime *et al.*, 2011).

Administration of *Echinacea* showed increased number of the total WBC count. Similar increase in WBC count was obtained by plant extracts of *Silene nocturna, Nigella sativa* and *Matricaria chamomilla* (Ghonime *et al.*, 2011) *and Withania somnifera* (Davis & Kuttan, 2000). This indicates they can stimulate the hemopoietic system. Although significant patterns were observed, nevertheless, one limitation of the study was the insufficient sample to detect small to moderate differences in the parameters measured between the *Echinacea* and the placebo groups. However, the sample size was small and that might have resulted in large discrepancies observed between the two treatments.

Conclusion

The study establishes that these extracts have appreciable immunostimulatory activity. However, further studies are required to confirm these conclusions.

Conflict of interest

The authors declared no conflict of interests with respect to the research, authorship and/or publication of this article.

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Effect of *Bidens pilosa* on infection and drug resistance of *Eimeria* in chickens

W.C. Yang [a,b], Y.J. Tien [c], C.Y. Chung [d], Y.C. Chen [a], W.H. Chiou [d], S.Y. Hsu [d], H.Y. Liu [d], C.L. Liang [e], C.L.T. Chang [d,*]

[a] Agricultural Biotechnology Research Center, Academia Sinica, Taiwan
[b] Department of Life Sciences, National Chung Hsing University, Taiwan
[c] Institute of Statistical Science, Academia Sinica, Taiwan
[d] Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taiwan
[e] Department of Microbiology and Immunology, Institute of Microbiology and Immunology, Chung Shan Medical University, Taiwan

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ABSTRACT

Extensive use of current anti-coccidial drugs together with drug resistance and residue has raised concerns about public health and poultry development. Here, we studied the anti-coccidial properties of *Bidens pilosa*. A phytochemical approach was developed for analysis of *B. pilosa* utilized as a feed additive. The protective effects of *B. pilosa* supplemented chicken diet were evaluated chickens infected with *Eimeria tenella*. *B. pilosa*, at doses of 0.5%, 1% and 5% of the chicken diet, significantly protected against *E. tenella* as measured by reduction in mortality, weight loss, fecal oocyst excretion and gut pathology in chickens. Finally, drug resistance of *E. tenella* to *B. pilosa* was assessed in chickens using the anti-coccidial index. This index showed that *B. pilosa* induced little, if any, drug resistance to *Eimeria* in chickens. Collectively, this work suggests that *B. pilosa* may serve as a novel, natural remedy for coccidiosis with low drug resistance in chickens.

1. Introduction

Coccidiosis is a disease that has a large economic impact on the poultry industry causing high mortality, poor growth and high medical costs (Williams, 1998). In chickens, coccidiosis is caused by parasites of the genus *Eimeria* (Coccidia subclass). Currently, the use of anti-coccidial drugs is one common means to prevent and treat coccidiosis. However, massive and long-time use of anti-coccidial drugs has led to the presence of drug-resistant parasites and residual drugs in chicken products, raising concerns about public health and food safety (Chapman, 1997; McDonald and Shirley, 2009; Orengo et al., 2012). In European countries, the use of anti-coccidial and anti-histomonas drugs as feed additives has been strictly limited since 2006 (Regulation 1831/2003 of the European Parliament) and a full ban has been proposed to be effective in 2021 by the Council Directive of 2011/50/EU published in the Official Journal of the European Union, L 104 of 19 April 2011. The utilization of anti-coccidial vaccines is an alternative means to prevent coccidiosis. Despite the significant progress made over recent years, efficacy, safety and cost effectiveness are still challenges for anti-coccidial vaccines in poultry (Sharman et al., 2010).

Given the concern voiced by consumers and poultry farmers about the use of the present anti-coccidial agents, there is an urgent need for novel and alternative approaches to prevent and treat coccidiosis in fowl. Reports have indicated that the use of effective, edible herbs and natural products as coccidicides in poultry production can be easily appreciated and accepted by consumers (Hassan et al., 2008; Orengo et al., 2012). Plants have been an extraordinary source of food and medicines for humans and animals since antiquity. Over the past decade, over 20 herbs have been tested for anti-coccidial activities (Akhtar et al., 2012; Allen, 2003; Allen et al., 1997; del Cacho et al., 2010; Lee et al., 2011; Naidoo et al., 2008; Orengo et al., 2012; Remmal et al., 2011; Youn and Noh, 2001). Although some plants showed high toxicity or little or no anti-coccidial activity (Nwosu et al., 2011), others were found to exert anti-coccidial function via immune action (Akhtar et al., 2012; Allen, 2003; Lee et al., 2011), suppression of oocyst wall formation (del Cacho et al., 2010), oocyst destruction (Remmal et al., 2011), anti-oxidant action (Allen et al., 1997, 1998; Naidoo et al., 2008; Orengo et al., 2012) and other mechanisms (Youn and Noh, 2001). Phytochemials, saponins and artemisinin have been proposed to be the active compounds against Coccidia (Allen et al., 1997; del Cacho et al., 2010; Mshvildadze et al.,

* Corresponding author. Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University; 250, Kuo Kuang Rd., Taichung 402, Taiwan, ROC. Tel.: +886 4 22859886; fax: + 886 4 22862073.
E-mail address: ltchang@nchu.edu.tw (C.L.T. Chang).

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2000). Despite these initial findings in early studies on anti-coccidial herbs, new anti-coccidial plants are still needed.

B. pilosa (Asteraceae) is an edible plant, commonly utilized as an ingredient in foods and medicines worldwide (Bartolome et al., 2013). The Food and Agriculture Organization of the United Nations advocated the cultivation of *B. pilosa* in Africa because of its high biosafety and easy growth (Young et al., 2010). Around 200 compounds have been identified from this plant including aliphatics, flavonoids, terpenoids, phenylpropanoids, aromatics, porphyrin and many others (Bartolome et al., 2013). The richness and complexity of the phytochemicals in *B. pilosa* may reflect the wide variety of bioactivities that have been reported for this herb, such as antimicrobial, anti-protozoal and many other actions (Bartolome et al., 2013). Nevertheless, the anti-coccidial properties of *B. pilosa* have not been evaluated.

In this study, batch consistency and quality control of a preparation of *B. pilosa* were assessed using phytochemical approaches, and the anti-coccidial activities of *B. pilosa* in chickens, as evidenced by survival rate, body weight loss, oocyst shedding and intestine pathology, were examined. Finally, the drug resistance of *B. pilosa* was evaluated.

2. Materials and methods

2.1. Plant preparation and analysis

The plant processing and analysis were performed similar to a previous publication (Chien et al., 2009). Three batches of the whole plant of *B. pilosa* were collected from Changhua County, Taiwan, and authenticated. After air drying at room temperature, the plant material was ground into a powder and the particles whose size ranges from 0.149 to 0.177 mm were collected for further use. For chemical fingerprint analysis, each batch of the pulverized *B. pilosa* material was extracted in 10-fold volumes of methanol at room temperature for 2 days. The crude extracts were evaporated by a rotary evaporator (Heidolph, Schwabach, Germany). After evaporation, the extracts were dissolved in water and subjected to high pressure liquid chromatography (HPLC) analysis using an RP-18 column (Phenomenex C18), hyphenated with a ultraviolet (UV) photodiode detector at 254 nm or a mass spectroscope (MS). The solvent gradient for HPLC was 0.1% TFA/acetonitrile (B) in 0.1% TFA/H_2O: 10–11% of B for 0–10 min, 11–19% of B for 10–15 min, 19–21% of B for 15 35 min, 21–28% of B for 35–47 min, and 28–100% of B for 47–55 min. Commercial standards, chlorogenic acid and isochlorogenic acid C were purchased from Sigma (St. Louis, MO, USA). The pulverized *B. pilosa* material from batch 1 was selected for the chicken diet formulation as described below.

2.2. Isolation, characterization and sporulation of E. tenella oocysts

Two isolates of *E. tenella* were collected from ceca of infected chickens after sacrifice at local poultry farms. Briefly, to obtain pure lines of *E. tenella*, different individual oocysts were sporulated with potassium dichromate and propagated throughout 2-week old chickens, one sporulated oocyst per chicken. Two isolates (Et C1, and Et C2) of *E. tenella* with ~20 μm in diameter were obtained and identified by microscopic method (Joyner and Long, 1974) and interspecies molecular characterization (Blake et al., 2008). All sporulated oocysts were maintained in the laboratory of the Department of Veterinary Medicine, National Chung-Hsing University for 3 years without exposure to any anti-coccidial drugs. The survival rate of the Lohmann chickens 7 days after challenge with Et C1 or Et C2 strain (1×10^4 sporulated oocysts) was ~60%. The Et C1 strain was used in this study unless indicated otherwise.

2.3. Animal husbandry, feed formulation and oral infection of E. tenella

In Experiment 1, 74 1-day-old disease-free Lohmann chicks from a local hatchery in Taichung, Taiwan, were obtained from a coccidian-free laboratory. To analyze the anti-coccidial action of *B. pilosa*, the chicks were randomly divided into six groups. There were four cages (4, 3, 3 and 3 chicks) in Group **1**, four cages (4, 4, 4 and 3 chicks) in Group **2**, four cages (4, 4, 4 and 3 chicks) in Group **3**, four cages (4, 3, 3 and 3 chicks) in Group **4**, three cages (3, 3 and 3 chicks) in Group **5,** and three cages (3, 3 and 3 chicks) in Group **6**. Chicks in all cages had *ad libitum* access to feeds and water throughout the experiment. Group **1** (UI control) and Group **2** (I control) had daily access to standard chicken diet (63.5% yellow corn, 16% soybean meal, 10% full fat soybean, 3.5% fish meal, 3% bran, 1.2% soybean oil, 1% calcium carbonate, 1.1% dicalcium phosphate, 0.4% salt, 0.2% lysine, 0.02% vitamin premix, 0.08% mineral premix) from day 1 to day 21. Group **3** (I Mad control) had daily access to the same diet supplemented with maduramicin (6 mg/kg diet). Group **4** (Bp5), Group **5** (Bp1), and Group **6** (Bp0.5) had daily access to the diet supplemented with *B. pilosa* powder at a dose of 5% (50 g/kg diet), 1% (10 g/kg diet) or 0.5% (5 g/kg diet), respectively. Chickens were inoculated on day 14. The chickens in Group **1** (UI control) were administered with 2 ml of phosphate buffered saline (PBS) and those in Groups **2** (I control), **3** (I Mad control), **4** (Bp5), **5** (Bp1) and **6** (Bp0.5) were infected with *E. tenella* sporulated oocysts (1×10^4). All animals were handled according to the guidelines of the National Chung Hsing University Institutional Animal Care and Use Committee (IACUC).

2.4. Measurement of survival rate, body weight, oocyst numbers, and gross and microscopic lesion scores in animals

Survival rate and bird appearance were checked daily. All birds in each cage in Experiment 1 were weighed on days 1, 7, 14 and 21 after hatching. Following published protocols in the literature (Conway et al., 1999; Haug et al., 2006), fecal samples were collected daily, from days 3 to 7 post infection, and weighed. Diluted oocyst suspension was prepared by adding water to 1 g of each fecal sample, followed by a serial filtration with W.S. Tyler sieves (1 mm, 250 μm and 45 μm). After centrifugation, the oocysts were suspended in saturated salt solution and mixed thoroughly. The homogenous suspension was transferred into two McMaster chambers for oocyst counts, with three repeats for each sample. Fecal oocyst number was calculated from the average of three counts of each sample. All the chickens in each group were sacrificed on day 21 and their ceca were removed. Gross lesion scores are obtained as described previously (Johnson and Reid, 1970). Briefly, gross lesions in the ceca caused by *E. tenella* were scored based on 5 grades: 0, normal tissue with no gross lesions; 1, very few scattered petechiae on cecal wall with normal cecal contents; 2, more numerous petechiae on thickened cecal wall with normal cecal contents; 3, noticeable cecal cores on greatly thickened cecal wall, large amounts of bloody cecal contents, and 4, greatly distended cecal wall with bloody or large caseous cores or dead birds. Microscopic lesion scores were obtained from the summation of lesion distribution and mucosal severity as published (Goodwin et al., 1998). Briefly, the entire ceca from the birds were fixed with 10% formalin and embedded in paraffin, followed by hematoxylin and eosin staining. The location of cecal lesions and mucosal histology were examined. The distribution of *E. tenella* infection along the observed cecal segment was graded as follows: 0, no *Eimeria* in any microscopic field at 10-fold magnification; 1, *Eimeria* in one field; 2, *Eimeria* in two fields; 3, *Eimeria* in three fields and 4, *Eimeria* in all four fields. The severity score in mucosae was graded as follows: 0, *Eimeria* in 0% of villi; 1, *Eimeria* in < 25% of villi; 2, *Eimeria* in 25 to 50% of villi; 3, *Eimeria*

in 51 to 75% of villi; 4, *Eimeria* in > 75% of villi. The microscopic lesion score is the sum of grades (0–4) found in five section slides per cecum.

2.5. Development and evaluation of drug resistance in E. tenella

In Experiment 2, 169 newly hatched chickens were purchased for drug resistance testing. Drug resistance of *E. tenella* in chickens was induced according to a previously described protocol with slight modification (Bafundo and Jeffers, 1990; Chapman, 1984). Briefly, *E. tenella* was passaged in chickens fed standard diet alone or supplemented with *B. pilosa* (0.5%) from day 0 to day 21 to obtain the first-generation oocysts. Such passage continued until the fifth-generation *Eiemria* oocysts were produced. Similarly, *E. tenella* was passaged in chickens fed a standard diet supplemented with salinomycin (70 ppm) from day 12 to day 21 until the fifth-generation oocysts were obtained. The above three lines were passaged in chickens fed a standard diet alone or supplemented with *B. pilosa* (1%) and salinomycin (140 ppm) for another three rounds, respectively. To assess drug resistance of the lines after eight serial passages, the three *Eimeria* lines were used to infect chickens in Groups **8**, **9** and **10** on day 14. Groups **7** (10 chickens, UI control) and **8** (10 chickens, I control) had daily access to standard chicken diet from day 1 to day 21. Groups **9** (15 chickens, Bp1) was fed daily with the diet supplemented with *B. pilosa* at the dose of 1% (10 g/kg diet) from day 1 to day 21. Group **10** (10 chickens, I Salino control) was given the diet supplemented with salinomycin (140 ppm) from day 12 to day 21. Drug resistance of *E. tenella* in the above experiments were assessed by the anti-coccidial index (ACI) based on the following formula (Li et al., 2004; Wang et al., 2006): ACI = [relative body weight gain (RBWG, %) + survival rate (SR, %)] − [lesion score index (LSI) + oocyst count index (OI)], where ACI ≥160 is defined as sensitive to the anti-coccidial drug, ACI between 120 and 160 is partially resistant to the anti-coccidial drug, and ACI < 120 is resistant to the anti-coccidial drug. RBWG = (100 × BWG per group)/[BWG of the uninfected unmedicated group (Group **7**, UI control)]; SR = (100 × the number of living chickens)/(total number of chickens per group); LSI = 10 × (lesion score per group); and OI = 100 × 0.4 × (oocyst counts per group)/[oocyst counts for unmedicated-infected group (Group **8**, I control)].

2.6. Statistical analysis

Data from nine chickens or more in each group of chickens in Experiments 1 and 2 are presented as mean ± standard error (SE). The cage in each group was used as the experimental unit. Pearson's chi-square test was used to determine whether there was a significant difference in the survival rate between treatment groups and control groups. Data on weight gain were subjected to two way ANOVA with factors group and cage (group) using the GLM procedure of SAS system. The excreted oocyst values were transformed into $\ln(x + 1)$ and, in turn, analyzed by ANOVA using the GLM procedure of SAS system under a normal distribution. Lesion scores were analyzed using a chi-square test after multinomial transformation. Actual *P* values are presented in all experiments.

3. Results

3.1. Chemical fingerprinting techniques for assessment of batch consistency and quality control of B. pilosa added to chicken diets

Batch consistency and quality control of *B. pilosa* in different preparations is important for the success of applications of *B. pilosa* products in chicken diseases. Therefore, we first employed high-performance liquid chromatography (HPLC), ultraviolet (UV) spectroscopy and mass spectroscopy to analyze the chemical fingerprints of three batches of *B. pilosa* preparations. Each preparation was made from a different plant sample. The HPLC profiles of the three batches of *B. pilosa* extracts were highly similar, suggesting good batch consistency among the *B. pilosa* preparations (Fig. 1A). The identity of two peaks, chlorogenic acid (**1**) and isochlorogenic acid C (**2**), in the extracts were confirmed using UV and mass spectroscopy compared with commercial standards (Fig. 1B and Supplementary Figs. S1 and S2). Both compounds can serve as index compounds for quality control of *B. pilosa* preparations. Overall, chemical fingerprint analyses confirmed batch consistency and quality control of *B. pilosa* preparations.

3.2. Effect of B. pilosa on survival rate of chickens following E. tenella challenge

To examine the anti-coccidial effect of *B. pilosa* as a feed additive on chickens, chickens were given daily access (day 1–21) to standard chicken feed or feed containing maduramicin or *B. pilosa* powder (at doses of 0.5%, 1% and 5% of chick feed) (Fig. 2A). The survival rate of chickens with access to standard feed dropped from 100% (Group **1**; UI control) to 60% (Group **2;** I control) after *E. tenella* infection (Fig. 2B). As expected, the survival rate of infected chickens with access to feed containing maduramicin was 93% in Group **3** (I Mad control, Fig. 2B). In contrast, the survival rate was 100% for the infected chickens with access to feed containing 0.5% or more *B. pilosa* (Groups **4** (Bp5), **5** (Bp1) and **6** (Bp0.5), Fig. 2B). Furthermore, we examined the anti-coccidial effect of *B. pilosa* on challenge with a mixture of *E. tenella*, *E. maxima* and *E. acervulina*. We found that *B. pilosa* significantly increased the survival rate of infected chickens (Supplementary Fig. S4).

3.3. Effect of B. pilosa on reduced weight gain of chickens following E. tenella challenge

Next, we monitored body weight of chickens with access to different diets before and after *Eimeria* infection. Instead of using repeated measurement, we used the change in body weight of the chickens as a single measurement variable in the test in which individual chickens with similar initial weights were chosen. This method of measurement avoided time dependent confounding. The body weight gain in chickens of each group from day 21 and day 14 to day 1 is presented in Table 1. Two-way nested ANOVA with factors group and cage (group) was used to compare the body weight gain data. The actual *P* values are indicated in Table 1. There was no significant difference between each cage in each group.

On day 14, there was no significant difference in the body weight gain of the chickens in Group **3** (I Mad control), Group **4** (Bp5) and Group **5** (Bp1) in comparison with Group **1** (UI control) and Group **2** (I control). In contrast, the body weight gain in chickens of Group **6** (Bp0.5) was significantly different from those of the chickens in Group **1** (UI control) and Group **2** (I control). On day 21, there was a significant difference in the body weight gain of the chickens in Groups **3** (I Mad control), Group **4** (Bp5), Group **5** (Bp1) and Group **6** (Bp0.5) in comparison with Group **1** (UI control) and Group **2** (I control). Overall, *B. pilosa* significantly ameliorated reduced weight gain caused by *E. tenella* to a greater degree than maduramicin or control feed alone. Part of this amelioration may be attributed to the weight-gaining effect of *B. pilosa*.

3.4. Effect of B. pilosa on fecal oocyst excretion of chickens following E. tenella challenge

To further determine the anti-coccidial effect of *B. pilosa* in chickens, *Eimeria* oocysts in chicken feces, an indicator of *Eimeria* multiplication, was evaluated. No fecal oocysts were detected in the

Table 1
Body weight gain (BWG) of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 7 days post *E. tenella* infection.

Group[a] cage no. (chickens)	BWG (g)[b]	*P* value[c]	*P* value[d]	BWG (g)[b]	*P* value[c]	*P* value[d]
	Day 14 – 1	Day 14 – 1	Day 14 – 1	Day 21 – 1	Day 21 – 1	Day 21 – 1
1 (*n* = 13) 4(4, 3, 3, 3)	75.0 ± 5.1			145.4 ± 5.5		
2 (*n* = 15) 4(4, 4, 4, 3)	76.1 ± 3.9	>0.05		111.7 ± 5.5	<0.0001	
3 (*n* = 15) 4(4, 4, 4, 3)	77.9 ± 4.0	>0.05	>0.05	120.1 ± 7.9	<0.0001	0.008
4 (*n* = 13) 4(4, 3, 3, 3)	79.0 ± 4.2	>0.05	>0.05	121.1 ± 2.9	<0.0001	<0.0001
5 (*n* = 9) 3(3, 3, 3)	85.9 ± 12.0	>0.05	>0.05	121.8 ± 5.7	<0.0001	0.0023
6 (*n* = 9) 3(3, 3, 3)	86.1 ± 3.9	0.00095	<0.0001	134.5 ± 5.9	<0.0001	<0.0001

[a] The chickens were classified into six groups. Group 1 (UI control) and Group 2 (I control) had daily access to standard chicken diet from day 1 to day 21. Group 3 (I Mad control) were daily given the diet supplemented with maduramicin (6 mg/kg diet). Group 4 (Bp5), Group 5 (Bp1), and Group 6 (Bp0.5) were daily fed with the diet supplemented with *B. pilosa* powder at the dose of 5% (50 g/kg diet), 1% (10 g/kg diet) or 0.5% (5 g/kg diet), respectively. The number (*n*) of chickens in each group, cage number in each group and chicken number in each cage are indicated.
[b] Body weight gain (BWG) was obtained by the formula: body weight on day T (14 or 21) – body weight on day 1.
[c] The difference in body weight gain (g) of the chickens between infected groups (Groups **2**–**6**) and uninfected unmedicated group (Group **1**) is analyzed by nested ANOVA and shown by *P* value.
[d] The difference in body weight of the chickens between the infected medicated groups (Groups **3**–**6**) and infected unmedicated group (Group **2**) is analyzed by nested ANOVA and shown by *P* value.

uninfected unmedicated controls (Group **1** (UI control), Table 2). Fecal oocyst excretion was first detected on day 4 post-infection in all *E. tenella*-infected groups and peaked on day 7 post-infection (Group **2** (I control), Table 2). As expected, the infected maduramicin-fed birds in Group 3 (I Mad control, Table 2) had significantly fewer oocysts per gram of feces than the infected controls in Group 2 (I control, Table 2). Similarly, the infected *B. pilosa* diet-fed birds in Group 4 (Bp5) and Group 5 (Bp1) and Group **6** (Bp0.5) had significantly fewer oocysts per gram of feces than those in Group 2 (I control) as shown in Table 2.

3.5. *Effect of* B. pilosa *on intestinal lesions of chickens following* E. tenella *challenge*

Next, gross examination of the cecum in the animals that had access to different diets was performed 7 days after *Eimeria* infection. The gross cecal lesion score is shown in Table 3. The uninfected unmedicated control chickens (Group **1** (UI control), Table 3) had no lesions in the ceca (score = 0). In contrast, *E. tenella* caused more gross cecal lesions in the gut of unmedicated chickens 7 days post-infection, as evidenced by a lesion score close to 4 (Group **2** (I control), Table 3). Like maduramicin (Group **3** (I Mad control)), *B. pilosa* at different doses (0.5%, 1% and 5%) significantly diminished cecal damage in infected chickens (Groups **4** (Bp5), **5** (Bp1) and **6** (Bp0.5), Table 3) as shown by the gross lesion scores of 2–3. Mucosal damage caused by coccidia was examined by microscope and scored as microscopic cecal lesions based on the distribution and severity of mucosal destruction in chicken cecum (Table 4). No microscopic cecal lesions (score = 0) were observed in the uninfected unmedicated control group (Group **1** (UI control), Table 4), akin to the observation for gross cecal lesions in the same animals (Group **1** (UI control), Table 3). In sharp contrast, the infected unmedicated animals showed serious microscopic lesions (score = 7.8) in ceca 7 days after *Eimeria* infection (Group **2** (I control), Table 4). Severe ulceration, hemorrhage and decreased villi were also observed in ceca (data not shown). Oocysts, gametocytes and schizonts appeared inside the cecal epithelia (data not shown). The infected maduramicin-fed animals (Group **3** (I Mad control), Table 4) showed mild improvement in microscopic lesions (score = 7.3) in the cecum from the infected unmedicated animals (Group **2** (I control), Table 4) post infection. However, the infected *B. pilosa* diet-fed animals (Groups **4** (Bp5), **5** (Bp1) and **6** (Bp0.5), Table 4) showed significantly reduced microscopic lesions (scores of 1.0–1.7) in the cecum. Consistently, *B. pilosa* decreased ulceration and hemorrhage and preserved more mucosae and villi in chicken ceca than control diets (data not shown). *B. pilosa* also decreased the number of oocysts, gametocytes and schizonts inside the cecal epithelia to a greater extent than maduramicin and control diets (data not shown). Overall, *B. pilosa* significantly reduced gut pathology in chickens following *E. tenella* infection.

Table 2
Fecal oocyst excretion of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 3–7 days post *E. tenella* infection.

Group	Days post-infection				
	3	4	5	6	7
	Ln(OPG + 1)	Ln(OPG + 1)	Ln(OPG + 1)	Ln(OPG + 1)	Ln(OPG + 1)
1 (*n* = 13)	0	0	0	0	0
2 (*n* = 15)	0	11.44 ± 0.11[a]	12.18 ± 0.07[a]	13.39 ± 0.11[a]	13.96 ± 0.05[a]
3 (*n* = 15)	0	11.09 ± 0.05[a,b]	11.93 ± 0.09[a,b]	13.04 ± 0.06[a,b]	13.66 ± 0.04[a,b]
4 (*n* = 13)	0	10.53 ± 0.12[a,b]	11.83 ± 0.09[a,b]	12.87 ± 0.06[a,b]	13.55 ± 0.11[a,b]
5 (*n* = 9)	0	10.80 ± 0.07[a,b]	11.72 ± 0.09[a,b]	13.01 ± 0.03[a,b]	13.58 ± 0.02[a,b]
6 (*n* = 9)	0	9.55 ± 0.40[a,b]	11.54 ± 0.16[a,b]	12.87 ± 0.10[a,b]	13.21 ± 0.09[a,b]

The oocysts per gram feces (OPG) of the same chickens from Table 1 in Experiment 1 were counted from day 3 to day 7 post infection. The OPG values ($\times 10^4$) of the chickens in each group were transformed into Ln(OPG + 1) and analyzed with ANOVA using the GLM procedure of SAS system under a normal distribution. The number (*n*) of chickens in each group is indicated.
[a] The difference in OPG in the chickens between the infected groups (Groups **2**–**6**) and uninfected unmedicated group (Group **1**) on the indicated days is statistically significant with a *P* value < 0.05.
[b] The difference in OPG in the chickens between the infected medicated groups (Groups **3**–**6**) and infected unmedicated group (Group **2**) on the indicated days is statistically significant with a *P* value < 0.05.

Table 3
Gross lesion scores in the ceca of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 7 days post *E. tenella* infection.

Group[b]	Gross lesion score[a]					Average[b]	*P* value[c]
	0	1	2	3	4		
1 (*n* = 13)	13	0	0	0	0	0.0 ± 0.0	
2 (*n* = 15)	0	0	0	4	11	3.7 ± 0.4	
3 (*n* = 15)	0	0	3	6	6	3.2 ± 0.8	0.0876
4 (*n* = 13)	0	0	5	5	3	2.8 ± 0.8	0.0003
5 (*n* = 9)	0	1	3	4	1	2.6 ± 0.9	0.0091
6 (*n* = 9)	0	1	5	3	0	2.2 ± 0.7	0.0008

[a] Gross lesion of the ceca of the same chickens from Table 1 in Experiment 1 was examined and scored as described in the Materials and methods section. The numbers represent the number of chickens with cecal gross lesions in five grading categories (0–4) and the number (*n*) of total chickens in each group, respectively.
[b] Average is expressed as the least square mean ± SE.
[c] The difference in the gross lesions of the ceca of chickens between the infected medicated groups (Groups **3**–**6**) and infected unmedicated group (Group **2**) is analyzed by chi-square test after multinomial transformation and shown by *P* value.

3.6. *Drug resistance of* E. tenella *to* B. pilosa *in chickens*

In parallel, we tested drug resistance of *E. tenella* to *B. pilosa* using the ACI, which is a commonly-used index for the assessment of drug resistance (Li et al., 2004; Wang et al., 2006). Weight gain, survival rate, fecal oocyst excretion and lesion scores of the four groups of experimental chickens (Table 5) were used to calculate this index. After eight passages in infected chickens given standard diet for 168 days, the ACI value was 200 and 47, respectively, for the uninfected unmedicated chickens (Group **7** (UI control), Table 5) and infected unmedicated chickens (Group **8** (I control), Table 5). In contrast, the ACI value was 146 and 40, respectively, for chickens given 1% *B. pilosa* (Group **9** (Bp1), Table 5) and 140 ppm salinomycin (Group **10** (I Salino control), Table 5), indicating the high drug resistance to *E. tenella* induced by salinomycin but not *B. pilosa*. The overall data demonstrated that long-term use of 1% *B. pilosa* showed low drug resistance to *E. tenella*, a superior result to that for salinomycin.

4. Discussion

Avian coccidiosis poses a continuous challenge to the poultry industry. Due to unmet efficacy and side effects of anti-coccidial drugs and vaccines, edible plants are considered possible viable alternative substituents to replace current anti-coccidial approaches (Orengo et al., 2012; Remmal et al., 2011). Here, we established spectroscopic methods for chemistry, manufacturing and control of *B. pilosa* (Fig. 1 and Supplementary Figs. S1 and S2). We also demonstrated that *B. pilosa* protects chickens against *Eimeria* infection (Fig. 2 and Tables 1–4) and resistance of *E. tenella* is poorly developed after long-term treatment with *B. pilosa* (Table 5). This study, for the first time, proved the feasibility of the use of *B. pilosa* as an anti-coccidial agent in chickens.

Table 5
Evaluation of drug resistance of *E. tenella* after eight serial passages in chickens given salinomycin and *B. pilosa*.

Group	RBWG (%)	SR (%)	LSI	OI	ACI
7 (*n* = 10)	100	100	0	0	200
8 (*n* = 10)	60.3	60	33.8	40	46.6
9 (*n* = 15)	79.5	100	23.3	10	146.2
10 (*n* = 10)	32.4	80	32.5	40	39.9

In Experiment 2, experimental induction and assessment of drug resistance of *E. tenella* in Groups **7** (uninfected unmedicated chickens, UI control), **8** (infected unmedicated chickens, I control), **9** (infected *B. pilosa*-fed chickens, Bp1) and **10** (infected salinomycin-fed chickens, I Salino control) are described in the Materials and methods section. The formulae for the RBWG, SR, LSI, OI and ACI values are also indicated in the Materials and methods section. The number (n) of chickens in each group is indicated.

Chicken *E. tenella* infection rate and mortality are 20–100% and 20–60%, respectively. The severity of both indices is dependent on chicken genetics and *Eimeria* species (Abu-Akkada and Awad, 2012). For example, the mortality of chickens caused by different isolates of *E. tenella* can reach up to 40% (Dakpogan et al., 2012). In our study, *E. tenella* isolate, Et C1, was isolated and amplified from a single *E. tenella* oocyst in chickens. This *E. tenella* isolate was considered to be a pure strain based on its morphological traits and molecular markers (Supplementary Fig. S3). We found that *E. tenella* used in this study caused ~40% of chicken death (Fig. 2B). Clearly, this high mortality is attributable to the virulence, but not the impurity, of the *E. tenella* isolate.

So far, 20 or so plants have been shown to possess anti-coccidial activities. Nevertheless, some of them showed discrepancies in *in vitro* and *in vivo* anti-protozoal bioactivities (van der Heijden and Landman, 2008a, 2008b). One explanation could be lack or insufficiency of batch consistency and/or quality control in the preparation of the plant products. In this work, we established a protocol by which to prepare and analyze *B. pilosa* extracts using phytochemical techniques (Fig. 1 and Supplementary Figs. S1 and S2). These efforts can ensure the quality of *B. pilosa* as an anti-coccidial formulation.

More importantly, our data showed that *B. pilosa* is prophylactically effective against *E. tenella* in young chickens aged 14 days (Fig. 2 and Tables 1–4). Survival rate, body weight, oocyst shedding, and cecal lesions were used as indicators by which to evaluate the anti-coccidial potential and drug resistance to *E. tenella* of *B. pilosa* using the same protocols as published elsewhere (Awais et al., 2011;

Table 4
Microscopic lesion scores in the ceca of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 7 days post *E. tenella* infection.

Group	Microscopic lesion score[a]									Average[b]	*P* value[c]	
	0	1	2	3	4	5	6	7	8		*Pa*	*Pb*
1 (*n* = 13)	65	0	0	0	0	0	0	0	0	0.0 ± 0.0		
2 (*n* = 15)	0	0	0	0	0	0	1	6	68	7.8 ± 0.4	<0.0001	
3 (*n* = 15)	0	0	0	2	0	4	10	7	52	7.3 ± 1.2	<0.0001	0.0036
4 (*n* = 13)	23	0	24	7	11	0	0	0	0	1.7 ± 1.5	<0.0001	<0.0001
5 (*n* = 9)	16	0	19	2	8	0	0	0	0	1.7 ± 1.5	<0.0001	<0.0001
6 (*n* = 9)	22	0	23	0	0	0	0	0	0	1.0 ± 1.0	<0.0001	<0.0001

[a] Microscopic lesion of the ceca of the same chickens from Table 1 in Experiment 1 was examined and scored as described in the Materials and methods section. The number represent the sum of microscopic lesions (0–4) in the gut samples, five section slides per gut, and the number of the examined gut samples multiplied by 5 per group.
[b] Average = Least square means ± SE.
[c] The difference in microscopic lesion scores of the chickens between infected medicated groups and uninfected unmedicated group (Group **1**) is analyzed with a chi-square test after multinomial transformation and shown by *Pa* value. Similarly, the difference in microscopic lesion scores of the chickens between infected medicated groups (Group **3–6**) and infected unmedicated group (Group **2**) is shown by *Pb* value.

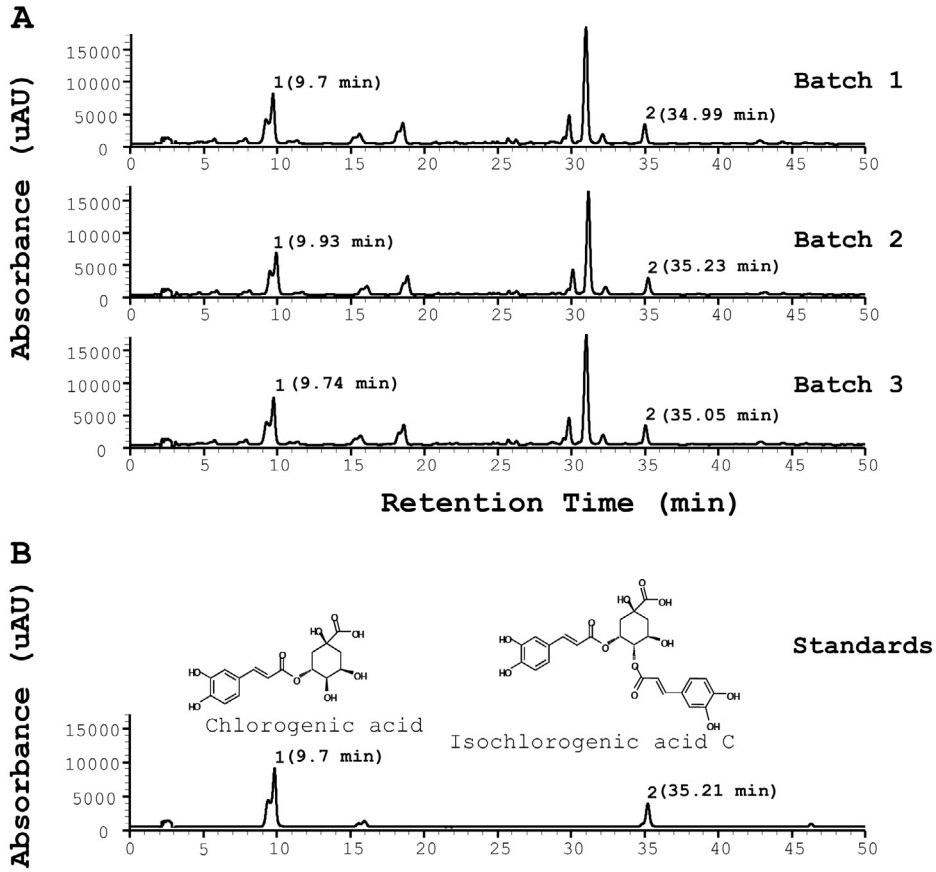

Fig. 1. HPLC chromatograms of *B. pilosa* extracts and chlorogenic acid standards. (A) Three batches of *B. pilosa* extracts were prepared. The extracts underwent HPLC chromatography with UV photodiode detection at 330 nm. Chlorogenic acid (**1**) and isochlorogenic acid (**2**) were identified as index compounds in these extracts. (B) HPLC profile of the mixture of commercial standards, chlorogenic acid (**1**) and isochlorogenic acid C (**2**). Retention time of peaks **1** and **2** in different batches of *B. pilosa* extracts and standards is indicated in the parentheses.

Hassan et al., 2008). Our study demonstrated that *B. pilosa* at the dose of 0.5% of chicken feed or more, conferred 100% protection against *Eimeria* challenge in chickens (Fig. 2B). *B. pilosa* consistently reduced fecal oocyst excretion (Table 2) and degree of intestine destruction (Table 3 and 4). Accordingly, *B. pilosa* treatment improved the reduced weight gain in chickens infected with *Eimeria* (Table 1). This improvement can be attributed to the ability of *B. pilosa* to control *Eimeria* infection and, to some degree, to induce gain weight in chickens (Table 1). The modest weight-gaining effect of *B. pilosa* powder reflects the fact that this plant is used as a food and has nutritional value as described elsewhere (Bartolome et al., 2013). *B. pilosa* at 0.5% of chicken diet is effective against chicken coccidiosis in our experimental system. However, higher doses of *B. pilosa* seems bad for coccidiosis control as evidenced by weight gain, gut pathology and oocyst excretion. These detrimental effects may be associated with the higher viscosity of the gut content when more *B. pilosa* is added to the chicken diet.

Apart from anti-coccidial action, *B. pilosa* has several other advantages over the anticoccidial drugs, maduramicin and salinomycin. First, *B. pilosa* is an edible plant and, therefore, there is little concern about biosafety and drug residence in chicken meat. Second, *B. pilosa* has a novel anti-coccidial action, which is different from that of commercial drugs. Third, long-term use of *B. pilosa* shows much lower drug resistance than that of salinomycin. This application of *B. pilosa* may reduce massive use of anti-coccidial drugs, anti-coccidial drug residue in chicken products, generation of drug-resistant mutants and concerns about public health. This work also expands the medicinal utility of *B. pilosa* in veterinary medicine.

Drug resistance has been reported against almost all anticoccidial drugs and is a major issue for coccidiosis control (Li et al., 2005). The ACI values revealed that drug resistance of *E. tenella* to salinomycin significantly increased in 168-day induction experiments (Table 5). In sharp contrast, its drug resistance to *B. pilosa* was poorly developed (Table 5). Of note, the degree of the drug resistance to *B. pilosa* may be underestimated because the weight-gaining effect of this plant. Nevertheless, the ACI data suggest that *B. pilosa* induced little, if any, drug resistance. The reason for this may be that this plant possesses multiple bioactive compounds, which may simultaneously inhibit different pathways of *E. tenella*. It is not hard to image that *E. tenella* can develop drug resistance to one agent more easily than multiple agents with different chemical structures.

The anti-coccidial mechanism of action of *B. pilosa* is currently unclear and needs to be ascertained in further studies. Direct chemical destruction and attenuation of invasive sporozoites are the primary reasons for decreases in oocyst excretion, induction of precocious lines and control drug resistance (Li et al., 2004; McDonald and Shirley, 2009). Since *B. pilosa* significantly reduced the shedding of fecal oocysts (Table 2) and drug resistance (Table 5), it is plausible that the compounds in *B. pilosa* act to destroy and attenuate Coccidia. Our earlier publications showed that *B. pilosa* can increase Th2 immunity (Chang et al., 2004, 2005), related to eradication of intestinal helminth, and inhibit the propagation of enteric bacteria (Chang et al., 2007a, 2007b). These findings suggest that *B. pilosa* may promote the clearance of Coccidia via immune regulation. In the future, identification of the active compounds in *B. pilosa*

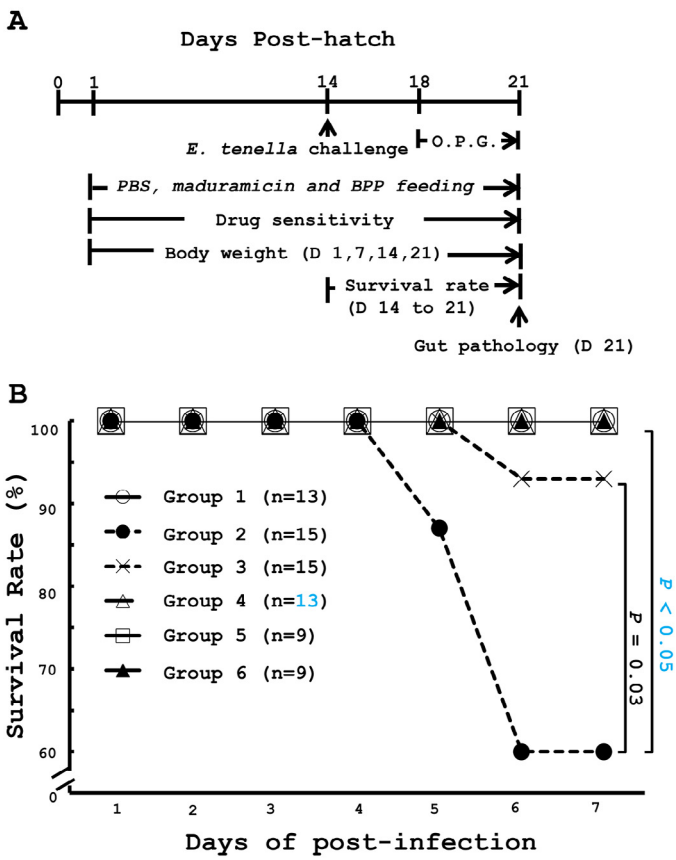

Fig. 2. *B. pilosa* increases survival rate of chickens given *E. tenella* challenge. (A) Summary of the experimental protocol of Experiment 1 in this study. (B) The same chickens as in Table 1 were divided into six groups. They had daily access to standard diet (Group **1** (UI control) and Group **2** (I control)) and the diet containing maduramicin (Group **3** (I Mad control)) or different doses of *B. pilosa* (Group **4** (Bp5), Group **5** (Bp1), and Group **6** (Bp0.5)). On day 14, chickens were infected with PBS or *E. tenella* sporulated oocysts (1×10^4) by gavage. Survival rate was monitored from day 1 to 7 post infection. The number (n) of chicks per group and P values are indicated.

that are active against coccidiosis will be pivotal for unveiling its mode of action.

5. Conclusions

Here, we performed chemical fingerprint analyses to determine batch consistency and quality control of *B. pilosa* preparations, and demonstrated that *B. pilosa*, used as a feed additive can protect against *E. tenella* in chickens by reducing mortality, oocyst excretion and intestinal lesions. In addition, *B. pilosa* decreased the induction of drug-resistant *E. tenella*. In summary, this study illustrates the anti-coccidial potential of *B. pilosa* in chickens.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rvsc.2014.11.002.

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The effects of baicalein on canine osteosarcoma cell proliferation and death

E. C. Helmerick, J. P. Loftus and J. J. Wakshlag

Department of Clinical Sciences, Cornell University College of Veterinary Medicine, Ithaca, NY, USA

Abstract

Flavonoids are a group of modified triphenolic compounds from plants with medicinal properties. Baicalein, a specific flavone primarily isolated from plant roots (*Scutellaria baicalensis*), is commonly used in Eastern medicine for its anti-inflammatory and antineoplastic properties. Previous research shows greater efficacy for baicalein than most flavonoids; however, there has been little work examining their effects on sarcoma cells, let alone canine cells. Three canine osteosarcoma cell lines (HMPOS, D17 and OS 2.4) were treated with baicalein to examine cell viability, cell cycle kinetics, anchorage-independent growth and apoptosis. Results showed that osteosarcoma cells were sensitive to baicalein at concentrations from approximately 1 to 25 μM. Modest cell cycle changes were observed in one cell line. Baicalein was effective in inducing apoptosis and did not prevent doxorubicin cell proliferation inhibition in all the cell lines. The mechanism for induction of apoptosis has not been fully elucidated; however, changes in mitochondrial permeability supersede the apoptotic response.

Keywords
apoptosis, baicalein, chemotherapy, doxorubicin, flavones, osteosarcoma

Correspondence address:
J. J. Wakshlag
Cornell University College of Veterinary Medicine
Veterinary Medical Center
C1-120, Box 34
Ithaca, NY 14853, USA
e-mail: jw37@cornell.edu

Introduction

Osteosarcoma is the most prevalent canine primary bone neoplasia, accounting for 5% of all canine tumours, with a higher incidence in large breed dogs. It carries a poor long-term prognosis because of metastasis. In the majority of cases at the time of diagnosis, clinically non-detectable micrometastasis is likely, thereby reducing survival times. The median survival times range from 165 to 470 days, depending on treatment modalities utilized.[1] Treatment typically involves surgery (amputation of the affected limb or limb-sparing procedures) and follow-up chemotherapy, and a majority of dogs diagnosed with osteosarcoma do not live more than 2 years past initial diagnosis.[2]

In the past 20 years, there has been increased use of nutraceuticals or herbal remedies as a palliative treatment by owners of dogs with cancer.[3] While many of these nutraceuticals have potential antioxidant properties, data regarding the effects on canine cancer are limited. More importantly, it is unknown whether these alternative treatments enhance or diminish current traditional chemotherapies when used in conjunction. Recent *in vitro* data show cytotoxic effects when using carotenoids or isoflavones with *in vitro* canine osteosarcoma and lymphoma cell lines, respectively[4–6]; yet *in vivo* data are lacking. Many of the bioactive components in various nutraceutics and herbal compounds are classified as flavonoids. Flavonoids are polyphenolic compounds found as secondary metabolites of plants, and are ubiquitous in fruits, vegetables and nuts.[7] Specifically, *Scutellaria baicalensis* root, also known as Ma-Huang, has been used for centuries in Eastern medicine for multiple ailments.[8] The bioactive flavones found in this root are the phytochemicals wogonin, baicalin and baicalein.[8]

The flavone baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) in particular has been reported to have a broad spectrum of physiological effects, which include reducing hypertension, decreasing inflammation and inhibiting neoplastic proliferation.[8,9] In many instances, baicalein, as a purified compound, has been shown to be

cytostatic in a number of *in vitro* and xenograft models, and may also induce apoptosis in neoplastic prostate and urinary bladder cell lines.[8–11] The mechanism of baicalein's cytostatic and apoptotic effects appears to be multifaceted. Alterations in mitochondrial-induced apoptosis, p53-induced cell cycle dynamics and MAP kinase signalling cascades are considered targets in baicalein-induced cell senescence and death.[12–15] Although considerable work has been performed on human and mouse cancer epithelial cell lines and xenograft models, the lack of empirical data in sarcoma xenografts or cell lines warrants further investigation. Considering the need for advancements in the treatment in canine osteosarcoma and its prevalence in canine oncology, we sought to assess the effect of baicalein on three canine osteosarcoma cell lines. Cell proliferation assays, soft agar growth, cell cycle analysis and time-course Western blotting for proteins associated with cell cycle alterations, mitochondrial-induced apoptosis and MAP kinase signalling were performed. Additionally, cell proliferation assays using baicalein in conjunction with the chemotherapeutic doxorubicin were performed to observe whether dual treatment would enhance or diminish cell survival.

Materials and methods

Cell culture

Three canine osteosarcoma cell lines were obtained from appendicular osteosarcomas; OS 2.4 cells from Dr Katrina Mealy – Washington State University, HMPOS cells from the Cornell University Comparative Oncology Program and D17 cells from the American Type Culture Collection. A-72 fibroblasts derived from a canine thymoma were also obtained from the American Type Culture Collection. Cells were maintained in complete medium that comprised RPMI 1640 (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Invitrogen) at 37 °C and 5% CO_2 for all experiments and for passage of cells, unless indicated otherwise. Baicalein (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and stored under nitrogen gas at −80 °C for up to 1 month.

Forty-eight-hour MTT proliferation assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assays were performed using D17, OS 2.4, HMPOS and A-72 fibroblasts plated at a density of 2500 cells per well in 96-well tissue culture-treated plates (Costar; Fisher Scientific, Waltham, MA, USA) and incubated overnight. The following day cells were treated with vehicle (DMSO) or various concentrations of baicalein ranging from 0.4 to 25 μM in a serial dilution for 48 h. Briefly, MTT assays were performed after 48 h of treatment by adding 20 μL of MTT dye [5 mg mL^{-1} in phosphate-buffered saline (PBS)] to the incubating cells for an hour at 37 °C in 5% CO_2. Media were then decanted, and the cells were washed with sterile PBS solution, immediately solubilized in 200 μL of isopropanol (Sigma) and evaluated by use of a spectrophotometric plate reader (Epoch; Biotek, Winooski, VT, USA) at a wavelength of 540 nm as previously described.[16] Each concentration was assayed in triplicate during three separate experiments, and a mean ± standard deviation (SD) was calculated for each time point.

Growth curves

Cells were plated at a density of 1000 cells per well in 96-well tissue culture-treated plates in RPMI with 2% FBS and 1% antibiotic/antimycotic and incubated overnight. Cells were then treated with vehicle control (VC; DMSO) or baicalein at 12.5, 6.25, 3.13, 1.57, 0.79 and 0.40 μM. MTT assays were performed every 2 days for a total of 6 days. MTT assays were performed as described above. Each concentration was run in triplicate during three separate experiments, and a mean ± SD was calculated for each time point.

Soft agar analysis

Soft agar analysis was performed with all three cell lines in accordance with the procedure described previously.[17] Briefly, complete medium with 0.3% melted soft agar (Agar VII; Sigma) was plated to create a layer of agar. Once solidified, more soft agar matrix was dissolved in the RPMI medium base, and 5000 cells per well were added with either 1, 5 or

10 μM of baicalein, VC (DMSO) or medium alone. Each treatment was plated in triplicate. Agar with and without baicalein at appropriate concentrations was refreshed every 3 days for 15 days. On day 15, all colonies were counted using the 40× objective of an inverted microscope for each of the 40-mm wells, and a mean ± SD was calculated for each treatment.

Flow cytometry

All three osteosarcoma cell lines were plated in six-well tissue culture dishes in complete medium. Cells were treated with DMSO or 10 μM baicalein for 24 and 48 h. Cells were then trypsinized, washed twice with PBS and fixed with cold ethanol overnight at 4 °C. Fixed cells were stained with 10 μL of a 1 mg mL^{-1} propidium iodide solution (Sigma), treated with bovine pancreatic RNAse (Sigma) and subjected to flow cytometry as previously described using a FacsCalibur with CellQuestPro Software (BD Biosciences, San Jose, CA, USA).[18] Each treatment was performed in triplicate for each cell line. Cell populations were identified by their distinctive position on forward- and side-scatter plots and gated. For each sample, 10 000 gated events were acquired. Total event counts within the sub-G1, G1, S and G_2M phases were used to calculate the percentage of cells within each phase of the cell cycle. Data are presented as the mean ± 1 SD. The exception was sub-G1, which was assessed in comparison with all cells in the cell cycle as a representation of apoptotic debris.

Mitochondrial permeability assays

A MitoPT kit (Immunogenics, St. Paul, MN, USA) was used to evaluate intact non-permeable versus permeable mitochondria. The kit was used in accordance with the manufacturer's recommendations. The HMPOS and D17 cell lines were chosen because of their potential future use in xenograft models. Briefly, 10 000 cells were plated on chamber slides and allowed to incubate in growth media overnight. The next morning, cells were treated with DMSO or 10 μM baicalein. After incubation for 24, 36 and 48 h, 5 μL of MitoPT solution was added to the cell culture media, and cultures were incubated at 37 °C for 15 min. The chamber slides were then removed from the incubator and washed two times with wash buffer. A cover slip was immediately applied to each chamber slide, and slides were examined via a fluorescent microscope using both 595 and 345 wavelength filters. A total of 400 cells were counted for each chamber; cells were quantified on the basis of staining characteristics (i.e. red or green), and the percentage of green cells was determined at each time point for vehicle-treated control cells and baicalein-treated cells. Each experiment was repeated three times, and a data are represented as mean ± 1 SD.

Cell lysis and Western blot analysis

D17 and HMPOS cells were grown in RPMI with 2% FBS and 1% antibiotic/antimycotic and treated for variable amounts of time with vehicle (DMSO) and 10 μM baicalein. DMSO-treated cells at 24 and 48 h were lysed to serve as control time points. Baicalein-treated cells were lysed at 12, 24, 36 and 48 h of incubation. Cells were lysed in accordance with a protocol described previously.[19] Cell lysates were collected, and protein concentration was determined for each sample by use of the Bradford technique. Lysates were equilibrated to a common volume (μg μL^{-1} basis) in lysis and loading buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 8, 12 or 15% SDS-PAGE depending on the kilodalton of the protein of interest by loading 30 μg of total protein per well. The gels were then transferred to polyvinylidene fluoride membranes for 1 h at 300 mA and then placed into 5% milk in Tris-buffered saline/0.5% Tween solution (TBST). Membranes were then transferred to primary antibody solutions including extracellular regulated kinase (ERK), p53 (R&D Biosciences, Boston, MA, USA), Bax, Bcl-2, caspase-3, phospho-ERK, protein kinase B (AKT), ser473 phospho-AKT, jun-n-terminal kinase (JNK) and phospho-JNK (Cell Signaling, Danvers, MA, USA) and β-actin (Sigma) overnight on a rocking platform at 4 °C. All primary antibodies were diluted 1:1000 in TBST, except for anti-β-actin (1:10 000) and anti-ERK (1:5000). Membranes were washed twice with TBST and then incubated at 37 °C for 1 h with 1:5000 dilutions of corresponding anti-rabbit

IgG or anti-mouse IgG horseradish peroxidase-labelled secondary antibodies (Cell Signaling). Blots were again washed three times with TBST and exposed to chemiluminescent reagent (Western lighting reagent; Millipore, Billerica, MA, USA). Digital images were captured using an imaging system (Biospectrum 410; UVP, Upland, CA, USA).

Doxorubicin proliferation assays

Cells were treated with vehicle (DMSO) or various concentrations of baicalein on the basis of previous growth curve evaluations for cell cultures in complete media. All three cell lines were treated with similar concentrations of baicalein as in the aforementioned 48-h MTT cytotoxicity assay. Cells were then incubated with various concentrations of doxorubicin (0.125 μM for OS 2.4, 0.0125 μM for HMPOS and 0.33 μM for D17) to achieve between 40 and 60% proliferation inhibition or DMSO VC for 4 h. Media were then changed to the initial concentrations of baicalein for the remainder of the 48-h incubation. At the end of the 48-h incubation, MTT assays were performed. Optical density for DMSO treatment versus baicalein treatment was compared to doxorubicin with DMSO versus doxorubicin and various concentrations of baicalein to determine if baicalein increased or diminished the proliferative response beyond that of doxorubicin alone. Three replicates of the experiment were repeated in triplicate, and a mean $\pm$ SD was calculated.

Statistical analysis

Data analysis for normality was performed utilizing Shapiro–Wilk testing and revealed normality for a majority of the data; therefore, parametric statistics were used. All soft agar results, 48-h proliferation, growth curves and doxorubicin proliferation were evaluated using one-way analysis of variance compared to VC or control cells with Tukey's *post hoc* comparisons, with an α set at $P \leq 0.05$. All flow cytometry and mitochondrial permeability apoptosis data were compared with VC (DMSO) groups by use of a non-paired Student T-test to determine significant differences at each time point with an α set at $P \leq 0.05$. Additionally, probit analysis was performed for each cell line to determine the 50% inhibitory concentration (IC_{50}) for baicalien alone and in conjunction with doxorubicin.

Results

MTT proliferation assay and growth curve

All three canine osteosarcoma cell lines began to show significant decreases in proliferation when treated with between 0.8 and 1.57 μM baicalein when compared with DMSO VC-treated cells in the 48-h proliferation assay. A-72 fibroblast cells showed significantly diminished cell proliferation at 25 μM. All three osteosarcoma cells showed increased sensitivity with IC_{50}s between 3 and 10 μM (Fig. 1), and A-72 fibroblasts did not reach an IC_{50} using this concentration range. The 6-day growth curve data show a similar range of approximately 2–6 μM IC_{50} for proliferation. The OS 2.4 cell line was most sensitive, with significant decreases in proliferation at 0.8 μM. Higher concentrations were needed to produce a significant decrease in proliferation in HMPOS and D17 cells at approximately 3.13 μM (Fig. 2).

Soft agar analysis

All three cell lines grew successfully in soft agar and had a significant decrease in colony formation when treated with 5 and 10 μM baicalein compared with the VC group. OS 2.4 cells displayed a complete loss of colony formation with 10 μM, indicating that these cells are more sensitive than the other two cell lines (Fig. 3). D17 cells showed no inhibition of colony formation at 1 μM, whereas both OS 2.4 and HMPOS showed significantly diminished colony formation at this concentration ($P < 0.05$).

Cell cycle analysis

Analysis of all three cell lines revealed no pronounced shifts in cell cycle dynamics during $G_{1/0}$, S or G_2M attributable to treatment with the vehicle (DMSO) or 10 μM baicalein, except for the OS 2.4 cells, which showed a mild, yet significant increase in $G_{0/1}$ and a decrease in G_2M (Table 1). All cell lines ranged between 65 and

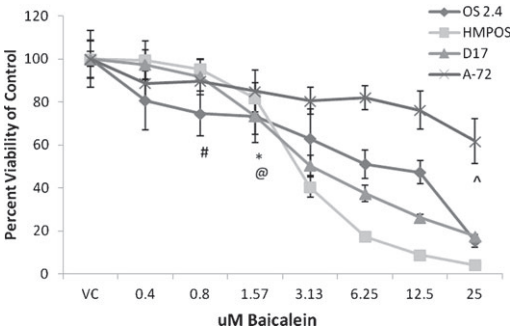

Figure 1. Canine osteosarcoma cells 48-h proliferation MTT assay: significant antiproliferative activity starts at concentration of 0.8–1.57 μM for all osteosarcoma cell lines, with A-72 fibroblasts requiring a higher concentration (25 μM). Lowest concentrations to show significant ($P \leq 0.05$) antiproliferative activity are indicated by # for OS 2.4, * for HMPOS, @ for D17 and ˆ for A-72.

85% for $G_{1/0}$, 6 to 20% for S to 9 and 15% for G_2M. Treatment of OS 2.4 and HMPOS cells with baicalein induced a significant increase in sub-G1 nuclear debris (apoptosis) compared with VC-treated cells (Table 1).

Western blot analysis

The lack of prominent cell cycle changes and only mild increases in $G_{0/1}$ in OS 2.4 cells led to immunoblotting for p53 expression and revealed no expression of p53 with or without baicalein treatment in all osteosarcoma cells. The A-72 cells showed a basal expression of p53; however, there was no observed increase in p53 expression after baicalein treatment (Fig. 4). Western blot analysis for markers of apoptosis in HMPOS and D17 cell lines revealed activation of caspase-3 with evidence of the cleaved caspase-3 fragment at 17 kDa by 36–48 h after treatment with baicalein. The increase in caspase-3 was preceded by a decrease in Bcl-2 expression, which inhibits Bax-induced mitochondrial permeability changes. In contrast, the expression of Bax, the major promitochondrial membrane permeability protein, was not significantly altered throughout treatment (Fig. 5). Western blotting for the MAP kinase activation (ERK and JNK) as well as AKT activation showed no appreciable changes because of baicalein treatment, suggesting that baicalein does not positively or negatively affect these cell signalling events (data not shown).

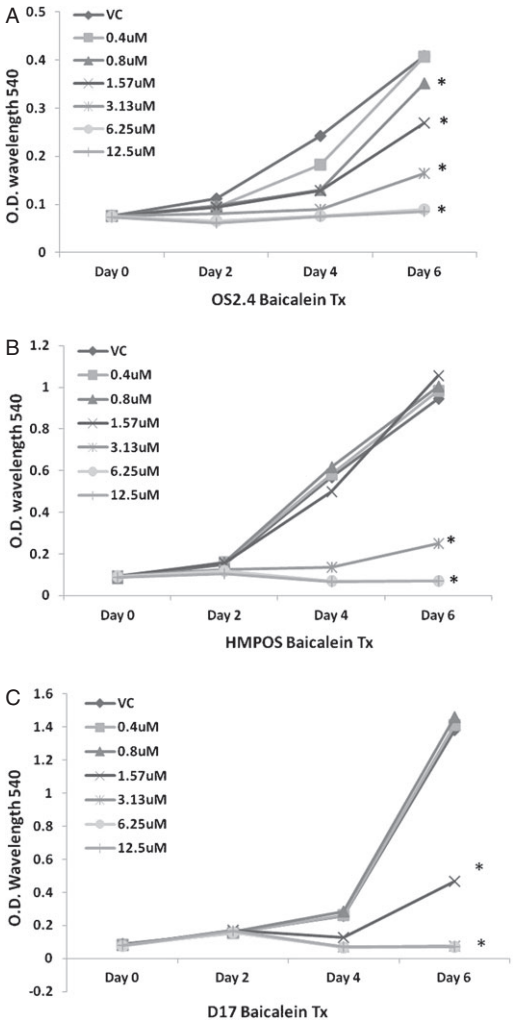

Figure 2. Osteosarcoma cell line 6-day growth curve. Cells were treated every other day with indicated concentrations of baicalein or DMSO as vehicle control (VC) (A) OS 2.4 growth curve showed significant differences between VC and all treatment groups $\geq$0.08 μM ($P < 0.05$). (B) HMPOS growth curve showed significant differences between VC and all baicalein treatment groups $\geq$3.13 μM ($P < 0.05$). (C) D17 growth curve showed significant differences between VC and all concentrations $\geq$1.57 μM baicalein ($P < 0.05$).

Mitochondrial permeability assays

Mitochondrial permeability was assessed using the MitoPT assay and dual immunofluorescence. Under normal cellular conditions, the electrochemical gradient of intact mitochondria results in aggregation of the dye, which fluoresces red. In contrast, fluorescent dye escapes the depolarized mitochondria of apoptotic cells, and the non-aggregated dye fluoresces green (Fig. 6A). VC-treated cells at 24

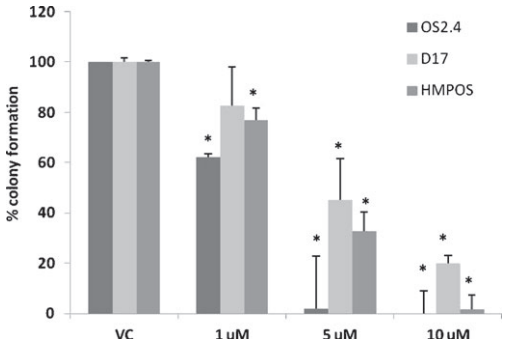

Figure 3. Osteosarcoma soft agar colony formation. Colonies were treated with vehicle control (VC), 1, 5 and 10 μM baicalein. Only HMPOS and OS 2.4 cells showed significant reduction in colony numbers at 1 μM. *indicated $P < 0.05$, with all cell lines showing significant colony reduction at 5 and 10 μM.

Table 1. Cell cycle analysis after 24 h of 10 μM baicalein or vehicle control treatment

Cell cycle analysis	$G_{1/O}$	S	G_2M	Sub-G_1
D17 control	65 ± 4	20 ± 4	15 ± 5	18 ± 5
D17 baicalein 24 h	70 ± 4	18 + 1	12 + 1	22 + 4
OS 2.4 control	72 ± 1	10 ± 2	18 ± 2	15 ± 3
OS 2.4 baicalein 24 h	85 ± 2*	6 ± 2	9 ± 2*	33 ± 5*
HMPOS control	78 ± 3	13 ± 1	9 ± 2	4 ± 1
HMPOS baicalein 24 h	73 ± 1	16 ± 2	11 ± 1	19 ± 1*

Only OS 2.4 cells show a modest significant increase in $G_{1/0}$ and a decrease in G_2M. Both OS 2.4 and HMPOS cells had increased sub-G_1 populations, which likely reflect apoptotic or necrotic nuclear debris. D17 cells show no alterations in the cell cycle. *$P \leq 0.05$ from control cells.

and 36 h showed less than 10% green fluorescing cells when compared with baicalein at 24 or 36 h of treatment which approached 50% by 36 h in both cell lines, which was significantly greater than VC cells at both time points (Fig. 6B).

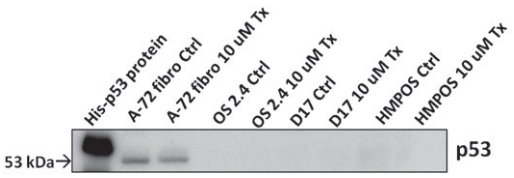

Figure 4. p53 Immunoblot: A-72 fibroblasts and three canine osteosarcoma cells were treated with vehicle control or 10 μM baicalein for 24 h to examine p53 when compared with control protein. The molecular weight of the native protein is approximately 53 kDa, whereas the control protein with the histidine tag is slightly larger at about 56 kDa. Only the A-72 fibroblasts show appreciable p53 protein expression that does not increase with baicalein treatment.

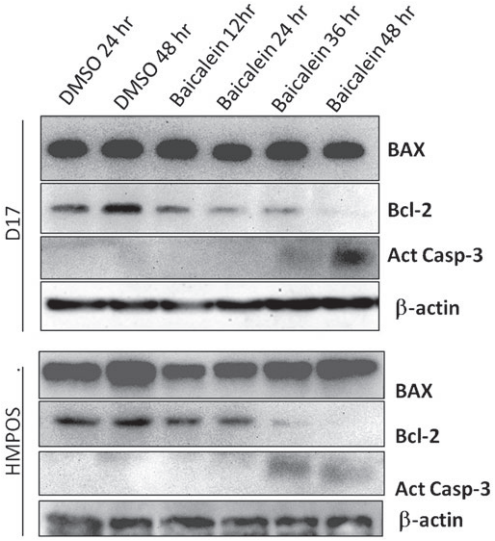

Figure 5. Time-course immunoblotting for markers of apoptosis and mitochondrial permeability in VC and 10 μM baicalein-treated D17 and HMPOS cells. Notice activation of caspase-3 as indication of end-stage apoptosis, while the antiapoptotic mitochondrial protein Bcl-2 decreases within 24 h, and Bax, the proapoptotic mitochondrial protein, appears unchanged compared to vehicle control-treated cells.

Doxorubicin cell proliferation inhibition

Doxorubicin inhibited cell proliferation in all three cell lines within 48 h after the onset of incubation with selected concentrations of doxorubicin to induce approximately 40–60% inhibition of cell proliferation. Concomitant serial dilutions of baicalein surrounding the IC_{50} of all three cell lines showed no protective effects of baicalein treatment because there was no increase in proliferation when treated with both doxorubicin and baicalein (Fig. 7A–C). D17 probit analysis for baicalein alone showed an IC_{50} of approximately 10.0 μM, whereas when treated in conjunction with a sublethal dose of doxorubicin it was 8.9 μM. OS 2.4 cell probit analysis for baicalein alone showed an IC_{50} of 1.9 μM, whereas in conjunction with doxorubicin it was 5.0 μM. Lastly, HMPOS cells when treated with baicalein alone showed an IC_{50} of 3.2 μM, whereas when treated in conjunction with a sublethal dose of doxorubin it was 3.6 μM. Overall, when the concentrations of baicalein reached 3–6 μM in all osteosarcoma cell lines, there was a significant decrease ($P < 0.05$) in cell viability/proliferation regardless of doxorubicin treatment.

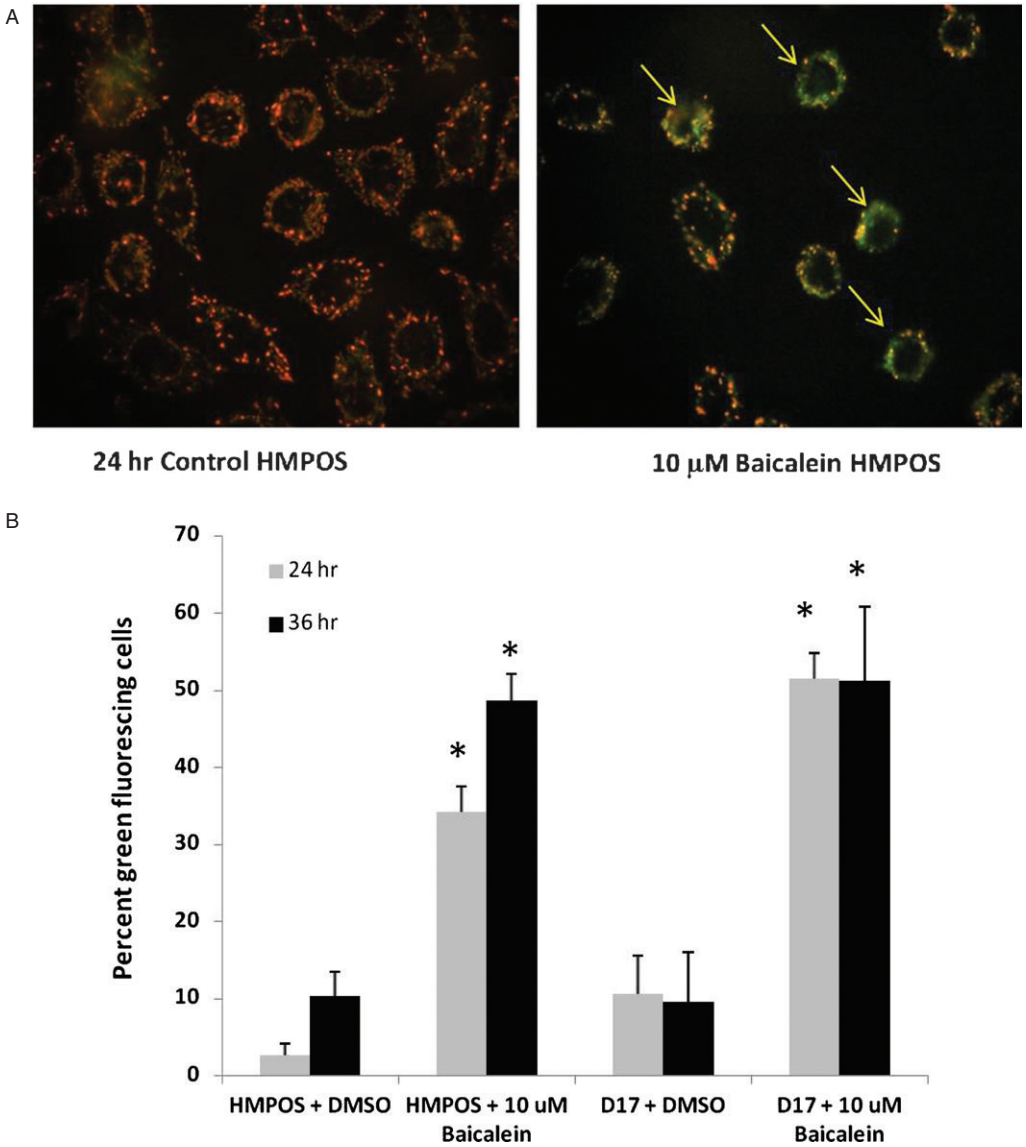

Figure 6. Mito PT staining of HMPOS and D17 cells. (A) Depicting 24-h control and 24 h of 10 μM baicalein treatment. Note the punctate red fluorescence showing intact mitochondria (left panel), while arrows show green fluorescence (right panel) suggesting fluorophore leakage from damaged mitochondrial membranes. (B) Percent green fluorescent cells counted in triplicate (400 cells counted per slide) showing both HMPOS and D17 cells had more green fluorescing cells at time points 24 and 36 h than the vehicle control (VC; DMSO)-treated cells. * indicates $P < 0.05$ when compared with VC cells.

Discussion

The use of flavones *in vitro* has provided ample evidence of antiproliferative effects in many instances; however, the concentrations needed to initiate cytostatic or apoptotic effects tend to be in the mid-to-high micromolar concentrations.[8–12,20–22] Baicalein as a single flavone has been shown to inhibit cell proliferation and chemically induced carcinogenesis in rodent models.[8] Additionally, there have been reports of baicalein being effective in slowing tumour or cellular growth at low micromolar concentrations.[22,23] Our data suggest that canine osteosarcoma cells are sensitive to the cytostatic effects in the 0.8–2 μM range for all three cell lines examined in a 48-h proliferative assay, which is the lowest concentration reported

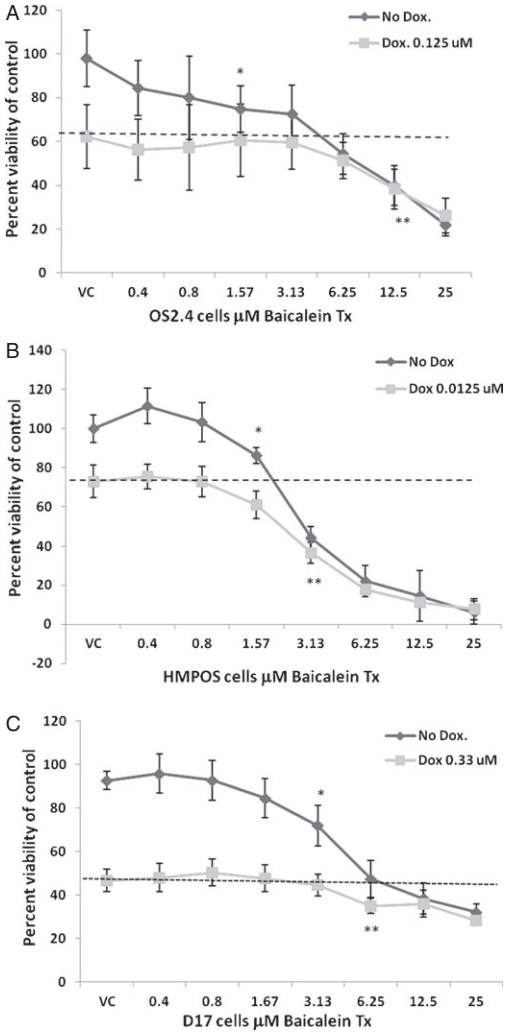

Figure 7. Doxorubicin/baicalein proliferation MTT assays. Cell lines (A) OS 2.4, (B) HMPOS and (C) D17 were examined at doses inducing a 30–60% decrease in cell proliferation with doxorubicin for 48 h with and without baicalein or vehicle controls (VC). The results of baicalein (black line) and baicalein with doxorubicin (grey line) remaining at or below dashed line indicate no survival advantage with the addition of baicalein to doxorubicin treatment. *Represents the first concentration, where a significant ($P < 0.05$) decrease in cell proliferation is observed with baicalein treatment. **Represents a significant ($P < 0.05$) decrease in cell proliferation for the baicalein with doxorubicin treatment line at this concentration and higher.

to date. When performing growth curve assays in lower serum, the antiproliferative activity was slightly similar in the range of 0.8–3 μM. Examination of anchorage-independent growth in soft agar is a potential measure of metastatic capability *in vitro*. Our results suggest that colony formation was inhibited with approximately 1–5 μM, similar to the growth curve cytostatic effects. A serum concentration of approximately 1 μM is potentially physiologically achievable based on pharmacokinetics in other monogastric species after oral dosing.[24–26] The pharmacokinetics in rats and humans suggest a serum half-life of approximately 2 h, whereas primate studies suggest a longer half-life of 4–6 h after oral dosing of approximately 150–500 mg kg^{-1} body weight.[26] The serum concentrations in these primate studies peaked at approximately 1–2 μM,[26] and although serum concentration may not reflect tissue concentrations, the findings in other monogastrics warrant further oral pharmacokinetic studies in dogs as well as xenografted osteosarcoma bearing nude mouse models to examine baicalein's potential for efficacy.

Previous studies examining the potential mechanisms by which baicalein exerts its negative effects on cell growth demonstrated alterations in the cell cycle causing stasis in $G_{1/0}$ with relationships to p53 and/or p21 status.[20,21] Examination of the cell cycle showed mild induction of apoptosis at 10 μM within 24 h in the OS 2.4 and HMPOS cell lines with no significant alterations in the cell cycle other than a mild increase in $G_{1/0}$ in the OS 2.4 cells when compared with control cells. Additionally, Western blot analysis of all three cell lines showed no appreciable p53 expression when compared with the A-72 cells, and that baicalein treatment did not alter its expression in all the cell lines examined. These data suggest that p53 upregulation or phosphorylation is not involved in osteosarcoma cell response to baicalein. This lack of p53 is not uncommon in canine osteosarcoma, which points towards other mechanism of apoptosis induction.[27]

Apoptosis involves controlled steps that lead to the loss of cell viability. These steps cause changes in the morphology of the dying cell, and include cytoplasmic condensation, DNA fragmentation and subsequent alterations in membrane phospholipids through the intrinsic and/or extrinsic pathways.[28–30] The extrinsic pathway is signalled via ligand binding of death receptors such as Fas/CD95 or TNF receptor 1, and the intrinsic pathway is activated by mitochondrial alterations. Both the intrinsic and extrinsic pathways activate aspartate-specific cysteine proteases, known as

caspases, which induce global proteolysis once activated resulting in apoptosis.[30] Time-course immunoblotting using the D17 and HMPOS cell lines using caspase 3 showed that 10 μM baicalein induced activation of caspase-3 (17-kDa fragment) within 36–48 h in both HMPOS and D17 cell lines providing evidence that cell death was initiated via apoptosis.

Upstream mechanisms were investigated by examining other known cell signalling pathways previously shown to be affected by baicalein including the phosphorylation status of extracellular regulated ERK, JNK and AKT, all of which are involved in cell proliferation and survival, but showed little alteration. Baicalein-induced alterations in mitochondrial permeability were also assessed through examination of Bcl-2 and Bax whose expression and balance within mitochondria play a role in mitochondrial-induced apoptosis. Bcl-2 and Bax are members of a family of proteins that have conserved BH domains. This family of proteins contains members that are either proapoptotic (Bax, Bak and Bok) or antiapoptotic (Bcl-2 family) and cell survival can depend partly on the activities of these proteins.[31,32] Apoptotic members of the Bcl-2 family compete with Bax to prevent oligomerization of the caspase cascade, thereby protecting the cell against programmed cell death.[33] Baicalein has been shown to induce the intrinsic pathway of apoptosis through alterations of common mitochondrial proapoptotic and antiapoptotic proteins, primarily through induction of Bax (proapoptotic) and decrease in Bcl-2 (antiapoptotic).[13–15,28,34,35] Our findings suggest that canine osteosarcoma cells undergo apoptosis through similar induction by decreasing expression of Bcl-2, whereas Bax expression remains relatively constant or modestly increased and precedes the activation of caspase-3. Additionally, MitoPT staining was performed on D17 and HMPOS cells at 24 and 36 h post-treatment with baicalein-treated cells transitioned from a punctated red mitochondrial staining to a more diffuse green fluorescence, suggesting a similar time frame for mitochondrial permeability and the downregulation of Bcl-2.

Flavones are traditionally thought of as antioxidants and previous studies have shown that baicalein has antiapoptotic effects on myocytes, which is thought to be partially due to baicalein's antioxidant effects.[36,37] Baicalein has also been shown to reduce plasma concentrations of TNF-α, a proinflammatory cytokine that has the potential to trigger apoptosis.[38] Therefore, because of these potential cell survival effects it is important to treat cells in combination with a common chemotherapeutic agent known to produce free radical damage. The cardiotoxic side effects associated with doxorubicin are due to free radical damage making it an ideal agent to examine the effects of baicalein during chemotherapeutic treatment of cells.[39] Despite the known antiapoptotic effects on myocardial cells and antioxidant effects, baicalein did not hinder doxorubicin's ability to decrease cell viability in all three osteosarcoma cell lines. Toxicologic probit analysis showed that IC_{50} of D17 and HMPOS cells with or without doxorubicin remained similar at around 10 and 4 μM, respectively. However, the OS 2.4 cells showed a very mild shift in IC_{50} from around 1 μM without doxorubicin to 5 μM, suggesting possible similar inhibitory mechanisms for both baicalein and doxorubicin in this cell line. Overall, there were no major shifts in IC_{50} or enhanced cell survival, suggesting that baicalein does not protect cells from doxorubicin's inhibitory effects.

In conclusion, the use of baicalein in these *in vitro* systems shows some utility in cytostatic and proapoptotic events. The slowing of growth and apoptosis seems to be initiated by alterations in mitochondrial membrane permeability through decreases in the Bcl-2 family of proteins. Although the concentrations needed ranged between 1 and 10 μM *in vitro*, this may still be within a physiologically achievable range; however, future pharmacokinetic studies are needed to evaluate its therapeutic potential. Oral delivery systems in a xenograft model of D17 and HMPOS cells are warranted to determine whether primary or metastatic growth is diminished with oral baicalein treatment. Baicalein will likely never have primary chemotherapeutic utility to treat canine osteosarcoma. However, baicalein and other flavones may provide some safe and mildly effective treatment options during remission, or for those not electing to undergo traditional treatment. Extensive work is needed to elucidate its potential utility in treating osteosarcoma.

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Evaluation of antioxidant capacity and inflammatory cytokine gene expression in horses fed silibinin complexed with phospholipid

Eileen S. Hackett, DVM, PhD; Khursheed R. Mama, DVM; David C. Twedt, DVM; Daniel L. Gustafson, PhD

Objective—To evaluate antioxidant capacity and inflammatory cytokine gene expression in horses fed silibinin complexed with phospholipid.

Animals—5 healthy horses.

Procedures—Horses consumed increasing orally administered doses of silibinin phospholipid during 4 nonconsecutive weeks (0 mg/kg, 6.5 mg/kg, 13 mg/kg, and 26 mg/kg of body weight, twice daily for 7 days each week). Dose-related changes in plasma antioxidant capacity, peripheral blood cell glutathione concentration and antioxidant enzyme activities, and blood cytokine gene expression were evaluated.

Results—Plasma antioxidant capacity increased throughout the study period with increasing dose. Red blood cell nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase I activity decreased significantly with increasing doses of silibinin phospholipid. No significant differences were identified in glutathione peroxidase activity, reduced glutathione or oxidized glutathione concentrations, or expression of tumor necrosis factor α, interleukin-1, or interleukin-2.

Conclusions and Clinical Relevance—Minor alterations in antioxidant capacity of healthy horses that consumed silibinin phospholipid occurred and suggest that further study in horses with liver disease is indicated. (*Am J Vet Res* 2013;74:1333–1339)

Hepatitis results in high morbidity and mortality rates in horses,[1,2] and investigation of new therapeutic agents is needed. Oxidative injury is a prominent mechanism of hepatic injury, and the positive effects of the milk thistle derivative silibinin on antioxidant capacity, as well as its direct oxidant-scavenging ability, are important features in its hepatoprotective actions.[3] The antioxidant properties of silibinin are dose dependent in mice.[4] However, the effects of silibinin administration on antioxidant capacity in horses are unknown. Multiple assays for enzymes and substrates can be performed to evaluate antioxidant capacity in vivo. Estimation of the plasma ORAC provides information on resistance to peroxyl radical injury[5] and has been used to estimate antioxidant capacity in horses.[6] The GPOX enzyme is a potent detoxifier of superoxide anions and hydrogen peroxide and in this manner limits oxidative injury.[7] Another critical enzyme in oxidant protection, NQO1, functions to catalyze the 2-electron reduction of quinones to hydroquinones in cells, limiting 1-electron reduction, which results in formation of reactive oxygen species.[8] Glutathione is the major intracellular reducing agent, functioning to protect against oxidative stress.[9] Shifting of GSH to GSSG indicates a shift of redox status toward oxidative stress.[10] Maximizing antioxidant capacity is protective against oxidative stress and injury from oxidative processes.

Amplification of inflammation caused by potent cytokines released by myeloid cells, such as TNFα and IL-1, promotes a cascade of events that results in sequestration of polymorphonuclear leukocytes in tissues and parenchymal dysfunction.[11] Inflammatory responses are

Abbreviations

AUC	Area under the curve
β-GUS	β-glucuronidase
DCPIP	2,6-dichlorophenolindophenol
GPOX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
IL	Interleukin
NADPH	Nicotinamide adenine dinucleotide phosphate
NQO1	Nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase I
ORAC	Oxygen radical absorbance capacity
PBMC	Peripheral blood mononuclear cells
RT	Reverse transcription
TNFα	Tumor necrosis factor α

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From the Department of Clinical Sciences, College of Veterinary Medicine and Biological Sciences, Colorado State University, Fort Collins, CO. 80523.
Address correspondence to Dr. Hackett (Eileen.Hackett@colostate.edu).

beneficial in recognition of foreign antigens and clearing infection. However, prolonged or overexuberant expression of inflammatory cytokines can be detrimental and has been linked to pathological changes associated with disease.[12] Hepatocellular injury may be caused by a combination of the primary oxidative effect and the secondary inflammatory response induced by damaged hepatocytes.[13] Tumor necrosis factor α is directly toxic to hepatocytes and induces apoptosis.[14] Interleukin-1β and IL-6 reduce hepatocyte protein synthesis, carbohydrate metabolism, and cytochrome P450–dependent detoxification.[15] Blood chemokine concentration has been associated with severity of hepatic disease in humans.[16] Derangement in the balance of pro- and anti-inflammatory serum cytokines is characteristic of alcoholic cirrhosis and is predictive of prognosis and mortality in humans.[17] Inhibiting cytokine release and subsequent inflammatory cell recruitment may limit organ damage. Silibinin protects against inflammation by limiting oxidative injury, inhibiting neutrophil migration, and regulating inflammatory mediators in rats.[11] Silibinin inhibits expression and synthesis of inflammatory cytokines TNFα, IL-1, and IL-2 in the presence of diseases and inflammatory stimuli in mice.[18,19]

The purpose of the study reported here was to evaluate antioxidant capacity and inflammatory cytokine gene expression in horses fed silibinin complexed with phospholipid. It was hypothesized that oral silibinin administration would increase antioxidant capacity in the blood of healthy horses, that these effects would be dose dependent, and that oral silibinin phospholipid administration would not alter gene expression of inflammatory cytokines in the blood of healthy horses because of the absence of preexisting disease or inflammation.

Materials and Methods

This study was performed in conjunction with an institutional animal care and use committee–approved phase II pharmacokinetic study.[20] Five horses owned by the Colorado State University Veterinary Teaching Hospital and acclimatized to their housing were used with permission, and environmental conditions were not changed. Horses were group housed in a paddock without access to grass. Horses received water ad libitum and Timothy grass hay once daily, providing for consumption of approximately 12 kg of hay/horse per day. All horses were geldings with a mean ± SD age of 13 ± 5 years (median, 14 years [range, 5 to 17 years]) and mean weight of 582 ± SD 65 kg (median, 615 kg [range, 472 to 625 kg]). There were 3 Quarter Horses, 1 Arabian, and 1 Andalusian. Horses were screened prior to inclusion in the study for evidence of gastrointestinal tract or liver dysfunction by use of physical examination and serum biochemical analyses and were selected only if they readily consumed the carrier diet (400 g of pelleted feed,[a] 50 g of wheat bran, and 150 mL of water [per meal]). Body weight was measured with a commercial scale, and signalment was recorded. Horses consumed each dose twice daily each day for 7 days during 4 administration periods, with progressively higher doses of silibinin phospholipid[b] administered during each period and with each period separated by a washout period (minimum of 2 weeks). During week 1, twice daily, horses consumed the carrier diet without silibinin phospholipid. During week 2, horses were fed the diet plus 20 mg of silibinin phospholipid/kg of carrier diet, resulting in a 6.5 mg/kg of body weight dose of silibinin. During week 3, horses were fed the diet plus 40 mg of silibinin phospholipid/kg of diet, resulting in a 13 mg/kg dose of silibinin. During week 4, horses were fed the diet plus 80 mg of silibinin phospholipid/kg of diet, resulting in a 26 mg/kg dose of silibinin. Mean ± SD total dose of silibinin phospholipid administered in feed in week 2 was 11.6 ± 1.3 g (median, 12.3 [range, 9.4 to 12.5 g]), in week 3 was 23.3 ± 2.6 g (median, 24.6 g [range, 18.9 to 25.0 g], and week 4 was 46.5 ± 5.2 g (median, 49.2 g [range, 37.8 to 50.0 g]). All 5 horses received identical treatments, except on day 7 of week 4 (highest-dose week), when the final meal supplemented with silibinin phospholipid was incompletely consumed (1 horse) or consumed slowly (1 horse).

Sample collection—Blood samples (total volume, 40 mL) were obtained from horses on day 1, prior to administration of unsupplemented diet or carrier diet mixed with silibinin phospholipid, and on day 7, 1 hour following the final meal of the study diet, each week. Blood for antioxidant analysis was collected directly into EDTA tubes. Plasma was immediately separated via centrifugation at 2,500 × *g* for 10 minutes, transferred to cryovials, submerged in liquid nitrogen until frozen, and stored at –80°C until analysis. Following plasma removal, the buffy coat layer was transferred to a 15-mL tube, and the pelleted RBCs were transferred to a cryovial, submerged in liquid nitrogen until frozen, and stored at –80°C until analysis. The buffy coat sample was brought to a final 6-mL sample volume with PBS solution, layered onto 4 mL of a mixture of nonionic, synthetic polymer of sucrose[c] and sodium diatrizoate,[d] and centrifuged at 800 × *g* for 30 minutes at 20°C. Following centrifugation, the fraction containing PBMCs was collected and washed twice with PBS solution. The PBMCs were counted with a hemacytometer and resuspended in PBS solution prior to freezing at –80°C until analysis. Blood for cytokine gene expression analysis was collected directly into commercially available evacuated tubes containing a proprietary additive to stabilize the in vivo gene transcription profile by reduction of in vitro RNA degradation,[e] maintained at 20°C for 60 minutes, and then frozen at –20°C until analysis as per the manufacturer's instructions.

Protein assay—Protein measurement in plasma, RBC, and PBMC samples was necessary to report protein-corrected values of glutathione and antioxidant enzymes. The RBC and PBMC samples required additional processing prior to analysis and were thawed on ice and diluted in 25mM Tris (pH, 7.4). Samples were then disrupted by sonication[f] in three 2-second bursts at 30% power on ice and centrifuged at 15,000 × *g*; the supernatant was collected for further analysis. Colorimetric measurement of protein was performed by use of bicinchoninic acid and standard curves of bovine serum albumin.[g] Samples were added to a 96-well microplate with 200 µL of working reagent containing bicinchoninic acid, and incubated for 30 minutes at 37°C.

Following incubation, absorbance was measured at 562 nm with a microplate reader.[h] Net absorbance was calculated by subtracting values of blank samples from values of bovine albumin standards and test sample replicates. Standard curves were graphed by plotting mean blank-corrected albumin standard values versus concentration and test samples estimated via linear regression. Protein concentration was reported in milligrams per milliliter.

ORAC—The ORAC of plasma was measured with a commercially available assay[i] as described by Ungvari et al.[21] The assay evaluated the ability of plasma samples to delay oxidation of a fluorescent probe by peroxyl radicals relative to known concentrations of a water-soluble vitamin E analog[22] and has been validated in horses and other species.[23] Plasma samples were thawed on ice, vortexed, and diluted 1:100. Fluorescein solution[j] and either plasma or standard curve samples were added to a 96-well microtiter plate and incubated for 30 minutes at 37°C. The plate was read immediately following addition of 2,2'-azobis(2-methylpropionamidine) hydrochloride.[k] Fluorescence was recorded every 84 seconds for 1 hour with a fluorescent microplate reader[h] with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Net AUC for plasma samples was calculated by subtracting blank sample AUC from test sample AUC and then compared with an antioxidant standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid[l] constructed by graphing the net AUC of vitamin E analog samples of known concentrations. Lower limit of quantitation was 2.5µM vitamin E analog. Linear regression was used to estimate the vitamin E analog equivalents of plasma samples on the basis of the vitamin E analog standard curve. Plasma ORAC was compared among horses that were administered different doses of silibinin phospholipid.

NQO1 activity—The NQO1 of PBMC and RBC samples was measured by use of the method described by Gustafson et al.[24] The PBMC and RBC lysates were thawed on ice. A 25mM Tris plus 0.7% bovine serum albumin solution was added to a methacrylate cuvette, followed by DCPIP[m] (40µM final solution) and nicotinamide adenine dinucleotide[n] (200µM final solution). Samples were evaluated in duplicate and mixed immediately prior to measurement of absorbance at 600 nm for 120 seconds via spectrophotometer,[o] with and without the addition of dicumarol (20µM final solution). The NQO1 activity was defined as the dicumarol-inhibited decrease in absorbance at 600 nm, or the difference in the change in optical density per minute between dicumarol-negative and dicumarol-positive samples. The NQO1 activity was converted to nmol of DCPIP reduced/min through calculations that used the extinction coefficient of DCPIP ($21 mM^{-1}cm^{-1}$). The NQO1 was normalized for protein content and expressed as nmol of DCPIP/min/mg.

GPOX activity—The GPOX activity of PBMC and RBC samples was measured by use of the method described by Gustafson et al.[25] A GPOX assay has been validated in horses.[6] The reaction mixture consisted of 2.59 mL of 50mM potassium phosphate buffer (pH, 7.0) with 1mM EDTA, 10 µL of 5mM sodium azide, 100 µL of 150mM GSH, 100 µL of 2.2mM hydrogen peroxide, 5 µL of glutathione reductase, and 100 µL of 8.4mM NADPH in a methacrylate cuvette. Test samples were added after a linear rate was established at 340 nm absorbance for 240 seconds in a spectrophotometer.[o] The GPOX activity was defined as the rate of NADPH oxidation in the presence of glutathione and glutathione reductase, or the difference in linear rates following sample addition. The GPOX activity was converted to pmol of NADPH reduced per minute through calculations that used the extinction coefficient of NADPH ($6.2 mM^{-1}cm^{-1}$). The GPOX was normalized for protein content and expressed as pmol of NADPH/min/g.

GSH and GSSG concentration—The GSH and GSSG concentration assays used in this study have been validated in horses[26] and were performed according to instructions from a commercially available assay.[p] The PBMC and RBC lysates were thawed on ice and precipitated with 5% sulfosalicylic acid.[q] The PBMC samples were diluted 2-fold or did not require dilution for analysis. The RBC samples were diluted 10- to 100-fold for analysis. Samples were added to a 96-well microtiter plate with a working solution containing 5,5′-dithiobis(2-nitrobenzoic acid)[r] and glutathione reductase.[s] Following 5 minutes of incubation at 20°C, NADPH solution[t] was added and the plate was read immediately. A microplate reader[h] recorded absorption of samples every 60 seconds for 5 minutes at 412 nm. The resulting slope was plotted from the change in absorbance at 412 nm/min. Standard curves of GSH[u] and GSSG[v] were analyzed, and linear regression of the change in absorbance at 412 nm/min was used to estimate the concentration of GSH and GSSG in test samples. Lower limit of quantitation of the assays was 0.5 ng/mL. Estimated concentration of GSH and GSSG was normalized for protein content and expressed in nanomoles per milligram.

RT-PCR assay—An RT-PCR assay was used to quantitate cytokine gene expression in blood samples. Samples were thawed, and total RNA was extracted by use of a commercial kit[w] and manufacturer's instructions. Samples were converted to cDNA via RT by use of 1.0 µg of RNA sample and RT master mix,[x] with incubation at 42°C for 15 minutes and 95°C for 5 minutes as described.[27] Equine-specific intron-spanning primer and probe sets were used.[28] Cytokines evaluated included TNFα, IL-1, and IL-2. Reaction mixtures composed of 5 µL of cDNA, 6.25 µL of nuclease-free water, 1.25 µL of 20X assay mix for the primer-probe set, and 12.5 µL of master mix[w] were incubated at 95°C for 10 minutes and underwent 40 cycles in a sequence detection system.[y] β–glucuronidase was used as the housekeeping gene. Changes in gene expression ($\Delta\Delta C_T$) were calculated by use of the following formula[29]:

$$\Delta\Delta C_T = ([\text{Cytokine threshold cycle} - \beta\text{-GUS threshold cycle}]_{\text{SILIBININ DOSE GROUP}} - [\text{mean cytokine threshold cycle} - \text{mean } \beta\text{-GUS threshold cycle}]_{\text{DAY 1 SAMPLE}}).$$

Results were reported as relative cytokine gene expression calculated by use of $2^{-\Delta\Delta CT}$, calibrated to samples from day 1 prior to administration of the blank diet for each individual gene.

Statistical analysis—Changes in plasma antioxidant capacity, glutathione, antioxidant enzymes in RBCs and PBMCs, and quantity of cytokine mRNA relative to silibinin dose, were analyzed by use of repeated measures ANOVA with Bonferroni multiple comparisons for post hoc pairwise comparisons. Values of $P < 0.05$ were considered significant.

Results

All 5 horses completed the study. Each 7-day period of twice-daily feeding of silibinin phospholipid was well tolerated.

ORAC—Plasma ORAC was measured for all time points (**Table 1**). Plasma samples delayed oxidation of the fluorescent probe to a greater extent than did blank samples. Plasma ORAC increased significantly (P = 0.005) throughout the study period.

Table 1—Plasma ORAC (mean ± SD [median {range}]) of horses orally administered various doses of silibinin phospholipid.

Silibinin dose (mg/kg)	Sample day	ORAC*
0	1	22.5 ± 1.8[a] (22.8 [20.2–24.8])
0	7	24.8 ± 1.8[a] (25.0 [22.2–27.7])
6.5	1	24.6 ± 1.6[a,b] (24.1 [22.9–26.9])
6.5	7	26.1 ± 1.6[b] (26.2 [24.0–27.7])
13	1	26.5 ± 0.7[b,c] (26.3 [25.7–27.3])
13	7	25.8 ± 0.8[b,c] (25.9 [24.8–26.7])
26	1	27.0 ± 0.7[c] (26.9 [24.2–27.3])
26	7	26.4 ± 1.3[c] (26.9 [24.2–27.3])

*Vitamin E analog equivalents per milliliter of plasma protein.
[a–c]Within a column, values with different superscript letters are significantly ($P < 0.05$) different.

NQO1 and GPOX activity

Differences in NQO1 activity of PBMC samples were not detected among horses fed different doses of silibinin phospholipid, (**Table 2**; P = 0.087). In RBC samples, NQO1 activity decreased significantly (P = 0.017) during the study period. Differences in GPOX activity of PBMC (P = 0.357) and RBC (P = 0.248) samples were not detected among dosage groups.

GSH and GSSG concentrations

Differences in GSH and GSSG concentration of PBMC and RBC samples were not detected among groups (Table 2). Differences in GSSG:GSH ratio were not detected in PBMC samples (P = 0.453). The GSSG to GSH ratio decreased with silibinin phospholipid dose in RBC samples, but this difference was not significant (P = 0.47).

RT-PCR assay

In 1 horse, the RT-PCR assay housekeeping gene was amplified too late, invalidating the cytokine data. Therefore, this horse was excluded and only 4 horses were included in the final analysis. Differences in TNFα, IL-1, and IL-2 expression were not detected among groups. Relative quantities of gene expression calibrated to the first sampling period, day 1 of the

Table 2—Mean ± SD (median [range]) values (corrected for sample protein concentration) of endogenous antioxidant enzyme activities and glutothione concentrations in PBMCs and RBCs of horses orally administered various doses of silibinin phospholipid.

Silibinin dose (mg/kg)	Sample day	Sample type	NQO1 activity (nmol of DCPIP/min/mg)	GPOX activity (pmol of NADPH/min/g)	GSH (nmol/mg)	GSSG (nmol/mg)	GSSG:GSH
0	1	PBMC	235 ± 211 (190.7 [77.8–591.5])	9.5 ± 3.9 (10.7 [5.4–13.6])	4.16 ± 2.97 (3.36 [1.36–8.79])	3.53 ± 1.60 (3.65 [1.09–5.38])	1.24 ± 1.14 (1.03 [0.36–3.19])
0	1	RBC	1.26 ± 0.86[a] (0.82 [0.46–2.52])	6.3 ± 4.1 (5.4 [3.1–12.2])	3.88 ± 1.34 (3.90 [2.58–5.81])	0.85 ± 0.18 (0.86 [0.56–1.00])	0.24 ± 0.08 (0.22 [0.15–0.37])
0	7	PBMC	534 ± 243 (552.3 [259.3–871.8])	16.6 ± 6.9 (15.7 [8.9–27.2])	5.50 ± 4.50 (5.14 [0.75–12.77])	4.36 ± 2.41 (4.31 [2.21–8.17])	1.30 ± 1.11 (0.93 [0.39–3.17])
0	7	RBC	0.55 ± 0.37[a,b] (0.47 [0.18–0.97])	11.1 ± 4.6 (7.2 [7.0–15.4])	2.74 ± 3.63 (1.10 [1.02–9.24])	0.28 ± 0.20 (0.19 [0.15–0.63])	0.15 ± 0.06 (0.16 [0.07–0.23])
6.5	1	PBMC	12.3 ± 8.2 (12.6 [3.5–22.4])	10.2 ± 4.0 (9.7 [6.3–16.7])	12.0 ± 5.73 (13.3 [2.44–16.58])	3.96 ± 2.55 (2.77 [1.56–6.86])	0.37 ± 0.19 (0.41 [0.15–0.64])
6.5	1	RBC	0.79 ± 0.50[a,b] (0.78 [0.20–1.50])	11.2 ± 5.8 (12.1 [5.5–18.9])	5.53 ± 2.25 (5.01 [3.48–9.13])	0.50 ± 0.40 (0.40 [0.16–1.13])	0.09 ± 0.08 (0.07 [0.05–0.23])
6.5	7	PBMC	101 ± 96 (53.2 [13.5–254.0])	8.3 ± 6.5 (6.4 [1.7–15.4])	16.9 ± 13.2 (19.67 [0.13–35.04])	14.9 ± 18.7 (7.12 [1.35–47.8])	11.4 ± 23.9 (1.36 [0.06–54.17])
6.5	7	RBC	0.48 ± 0.30[a,b] (0.51 [0.07–0.90])	12.4 ± 7.0 (13.3 [5.3–23.6])	5.37 ± 2.26 (5.56 [1.69–7.75])	0.78 ± 0.51 (0.63 [0.34–1.66])	0.19 ± 0.14 (0.14 [0.05–0.37])
13	1	PBMC	163 ± 185 (56.6 [4.2–404.9])	12.9 ± 4.9 (13.0 [20.2–7.4])	5.39 ± 5.19 (4.38 [0.18–13.2])	9.50 ± 10.2 (4.70 [1.36–47.8])	5.17 ± 6.69 (1.07 [0.40–15.9])
13	1	RBC	0.35 ± 0.15[b] (0.29 [0.25–0.61])	12.0 ± 1.0 (11.8 [11.2–13.6])	4.24 ± 1.88 (4.10 [1.99–6.52])	0.67 ± 0.27 (0.58 [0.26–0.94])	0.16 ± 0.05 (0.14 [0.10–0.23])
13	7	PBMC	185 ± 231 (71.5 [19.9–569.5])	12.4 ± 3.6 (11.1 [8.2–17.6])	10.2 ± 8.50 (8.19 [0.16–21.1])	4.54 ± 1.83 (3.98 [3.27–7.68])	4.53 ± 8.75 (0.55 [0.19–20.15])
13	7	RBC	0.37 ± 0.38[b] (0.13 [0.07–0.82])	10.8 ± 3.2 (9.9 [7.9–16.3])	3.52 ± 1.08 (3.32 [2.01–4.98])	0.62 ± 0.44 (0.52 [0.21–1.34])	0.17 ± 0.05 (0.17 [0.06–0.27])
26	1	PBMC	200 ± 144 (194.1 [30.9–264.1])	11.9 ± 3.9 (11.9 [6.3–16.8])	6.45 ± 5.48 (7.51 [0.10–12.10])	9.02 ± 9.11 (4.01 [3.59–24.8])	9.53 ± 15.8 (3.30 [0.30–37.44])
26	1	RBC	0.67 ± 0.57[b] (0.38 [0.17–1.43])	9.3 ± 2.2 (10.1 [6.9–11.8])	4.48 ± 0.84 (4.99 [3.31–5.31])	0.81 ± 0.44 (0.88 [0.15–1.29])	0.17 ± 0.08 (0.19 [0.03–0.24])
26	7	PBMC	572 ± 435 (368.2 [132.3–1231.6])	8.9 ± 4.6 (7.6 [3.9–16.2])	5.75 ± 5.84 (3.33 [0.24–15.43])	7.84 ± 4.12 (7.70 [3.74–14.54])	7.62 ± 13.4 (2.37 [0.24–31.6])
26	7	RBC	0.32 ± 0.09[b] (0.32 [0.23–0.47])	9.1 ± 4.3 (6.6 [6.1–16.1])	4.28 ± 1.25 (4.24 [2.50–5.94])	0.55 ± 0.27 (0.55 [0.20–0.88])	0.12 ± 0.04 (0.12 [0.08–0.17])

[a,b]Within a column and sample type, values with different superscript letters are significantly ($P < 0.05$) different.

Table 3—Blood cytokine gene expression (mean ± SD [median {range}]) of horses orally administered various doses of silibinin phospholipid.

Silibinin dose (mg/kg)	Sample day	TNFα	IL-1	IL-2
0	1	Referent	Referent	Referent
0	7	1.17 ± 0.52 (1.15 [0.56–1.84])	1.12 ± 0.21 (1.13 [0.87–1.36])	1.73 ± 1.51 (1.24 [0.51–3.94])
6.5	1	0.81 ± 0.36 (0.87 [0.33–1.18])	1.06 ± 0.19 (1.12 [0.79–1.21])	0.98 ± 0.31 (0.94 [0.66–1.37])
6.5	7	0.89 ± 0.56 (0.64 [0.56–1.72])	1.30 ± 0.51 (1.23 [0.74–1.97])	0.68 ± 0.30 (0.68 [0.36–1.02])
13	1	1.16 ± 0.49 (1.28 [0.50–1.56])	1.49 ± 1.27 (0.88 [0.79–3.39])	1.04 ± 0.28 (1.09 [0.68–1.29])
13	7	0.85 ± 0.41 (0.98 [0.25–1.18])	1.39 ± 0.20 (1.43 [1.12–1.60])	1.41 ± 0.62 (1.52 [0.66–1.96])
26	1	0.75 ± 0.37 (0.85 [0.23–1.05])	1.04 ± 0.31 (0.99 [0.77–1.40])	1.32 ± 0.20 (1.29 [1.14–1.57])
26	7	1.03 ± 0.64 (1.03 [0.26–1.82])	1.21 ± 0.45 (1.16 [0.73–1.81])	1.31 ± 0.50 (1.15 [0.89–2.04])

Gene expression values indicate relative quantity of gene calibrated to day 1 of the unsupplemented diet (0 mg/kg) week.

unsupplemented diet week, were determined (**Table 3**).

Discussion

The primary antioxidant effect identified in the healthy horses fed diets supplemented with silibinin in the present study was alteration in plasma ORAC. Temporal effects and those of the carrier diet may also have been sources of alterations in plasma ORAC in this study. Modest increases in plasma ORAC have also been associated with decreases in plasma lipid hydroperoxides in horses following exercise-induced stress.[6] In the present study, the GSSG:GSH ratio in RBC samples decreased with increased doses of silibinin phospholipid, although this decrease was not significant. Erythrocyte NQO1 activity decreased with increasing dose, but a significant change in PBMC NQO1 activity was not observed. The GPOX activity was not altered with silibinin administration, although silibinin has been reported to have positive effects on antioxidant capacity in blood cells and plasma, including restoration of antioxidant defenses in pathological disease states. Maintenance of GPOX activity is a primary mechanism of silibinin protection against RBC lipid peroxidation and damage secondary to oxidation.[30] Silibinin administration significantly increases RBC GPOX activity in humans undergoing hemodialysis[31] and ameliorates decreases in serum GPOX activity that occur secondary to experimental renal ischemia and reperfusion in rats.[32] Silibinin is also protective against decreases in GPOX activity and GSH concentration in human RBCs that occur secondary to peroxide exposure in vitro.[30] Silibinin restores PBMC GSH concentration in humans with β-thalassemia major, and improves PBMC proliferative responses.[9] Silibinin also improves the PBMC GSSG:GSH ratio in vitro following oxidant-induced damage.[10] In healthy cats, silibinin increases PBMC GSH concentration but does not alter the GSSG:GSH ratio.[33] Although ORAC has not been specifically measured following silibinin administration, protective effects on serum and plasma antioxidant capacity have been detected.[11,32] Information regarding silibinin effects on NQO1 activity is not available, although dose-dependent increases in NADPH2:quinone reductase have been reported in mice in vivo.[4]

In a study[34] of rats that were administered silibinin via the intraperitoneal route, increases in GSH concentration were tissue specific and occurred primarily in the liver and intestines. The authors attributed the tissue-specific effects to the basic pharmacokinetics of silibinin, which undergoes predominantly biliary excretion and is maintained in high local concentrations by enterohepatic circulation. It is presumed that the antioxidant protective effects of silibinin are similarly concentrated in the tissues of the liver and intestines. Therefore, it is expected that the changes evident in the blood of horses consuming silibinin would be greater in the liver, which is the target organ in horses with liver disease. Hepatic antioxidant effects of silibinin are well documented.[35–38] Less is known regarding the relative antioxidant effect on blood versus hepatic tissues. In experimental carbon tetrachloride-induced hepatitis, hepatic and RBC GSH were both measured and silibinin administration improved GSH concentration in both tissues, but to a greater degree in hepatic tissue.[39]

Much has been learned from in vitro and in vivo experiments with respect to the peripheral anti-inflammatory effects of silibinin administration. Silibinin reverses increases in serum TNFα, IL-1β, and IL-6 expression in rats with sepsis induced by cecal ligation and perforation.[11] A decrease in acute lung and brain injury accompanies this anti-inflammatory effect.[11] In rats with experimental nonalcoholic fatty liver disease, silibinin decreases plasma TNFα expression concurrent with improvements in liver inflammation and fatty infiltration evident via histologic examination.[40] In rats, silibinin significantly reduces serum TNFα and IL-1 expression associated with partial hepatectomy and the resultant inflammatory response.[41] Release of TNFα and cytotoxicosis secondary to toxic damage are decreased by silibinin administration in perfused livers and isolated Kupffer cells.[42] In canine hepatocytes, silibinin ameliorates the proinflammatory influence of IL-1β, including production of chemotactic cytokines, and reduces hepatocyte damage.[13] Silibinin also dimin-

ishes the proinflammatory influence of IL-1 in human hepatic stellate cells.[43]

Gene expression of the inflammatory cytokines TNFα, IL-1, and IL-2 did not change in the present study of healthy horses fed diets supplemented with silibinin phospholipid. In the absence of an active inflammatory stimulus, this was not surprising. However, the effects of silibinin on inflammatory cytokines have been detected in the blood of patients with naturally occurring diseases, especially those diseases in which oxidative injury is prominent. In chronic hepatitis C virus infection, silibinin administration inhibits TNFα production by PBMCs.[12] Production of TNFα by blood lymphocytes in humans with end-stage diabetic nephropathy is significantly reduced following silibinin administration.[44] Silibinin also reduces inflammatory cytokines and disease severity in humans with non alcoholic fatty liver disease.[45–47]

Results of the present study provide baseline data on the effects of silibinin in healthy horses. Minor changes were observed in antioxidant capacity, which was consistent with previous observations in healthy cats.[33] Because of the pharmacokinetics of silibinin,[3] despite low bioavailability, its antioxidant effects will likely be most prominent in the liver and intestinal tract of horses.

a. Equine Senior Horse Feed, Purina Mills, St Louis, Mo.
b. Siliphos, Indena Pharmaceuticals Inc, Milan, Italy.
c. Sigma Ficoll F4375, Sigma-Aldrich United States, St Louis, Mo.
d. Sigma Histopaque 1077, Sigma-Aldrich United States, St Louis, Mo.
e. PreAnalytix PAXgene blood RNA tube, Qiagen, Valencia, Calif.
f. Sonic Dismembrator, Fisher Scientific, Pittsburgh, Pa.
g. 23225 - BCA Protein Assay Kit, Thermo Scientific, Waltham, Mass.
h. Synergy HT, Bio-tek Instruments Inc, Winooski, Vt.
i. Cell Biolabs Inc, San Diego, Calif.
j. Cell Biolabs 234502, San Diego, Calif.
k. AAPH, Cell Biolabs 234503, San Diego, Calif.
l. Trolox, Cell Biolabs 234504, San Diego, Calif.
m. Sigma D1878, Sigma-Aldrich United States, St Louis, Mo.
n. NADH, Sigma N4505, Sigma-Aldrich United States, St Louis, Mo.
o. DU 800 UV/Vis, Beckman Coulter, Brea, Calif.
p. Sigma-Aldrich United States, St Louis, Mo.
q. Sigma S2130, Sigma-Aldrich United States, St Louis, Mo.
r. DTNB, Sigma D8130, Sigma-Aldrich United States, St Louis, Mo.
s. Sigma G3664, Sigma-Aldrich United States, St Louis, Mo.
t. Sigma N6505, Sigma-Aldrich United States, St Louis, Mo.
u. Sigma G4544, Sigma-Aldrich United States, St Louis, Mo.
v. Sigma G4376, Sigma-Aldrich United States, St Louis, Mo.
w. PreAnalytix PAXgene blood RNA extraction kit, Qiagen, Valencia, Calif.
x. Promega, Madison, Wis.
y. Applied Biosystems, Foster City, Calif.

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Appendix IX Abstracts of recent journal articles demonstrating breadth current of research

Note abstracts link to pubmed, by clicking on title.

IX-A Veterinary botanical medicine and Aquaculture

Valladão GM, Gallani SU, Ikefuti CV, da Cruz C, Levy-Pereira N, Rodrigues MV, Pilarski F. **Essential oils to control ichthyophthiriasis in pacu, Piaractus mesopotamicus (Holmberg): special emphasis on treatment with Melaleuca alternifolia.** J Fish Dis. 2016 Jan 18

In vitro effect of the Melaleuca alternifolia, Lavandula angustifolia and Mentha piperita essential oils (EOs) against Ichthyophthirius multifiliis and in vivo effect of M. alternifolia for treating ichthyophthiriasis in one of the most important South American fish, Piaractus mesopotamicus (Holmberg), were evaluated. The in vitro test consisted of three EOs, the results demonstrated that all tested EOs showed a cytotoxic effect against I. multifiliis compared to control groups ($P < 0.05$). The in vivo treatment for white spot disease was performed in a bath for 2 h day^{-1} for 5 days using the M. alternifolia EO (50 µL L^{-1}). In this study, 53.33% of the fish severely infected by I. multifiliis survived after the treatment with M. alternifolia (50 µL L^{-1}) and the parasitological analysis has shown an efficacy of nearly 100% in the skin and gills, while all the fish in the control group died. Furthermore, the potential positive effect of M. alternifolia EO against two emergent opportunistic bacteria in South America Edwardsiella tarda and Citrobacter freundii was discussed.

M Dos Santos W, S de Brito T, de A Prado S, G de Oliveira C, C De Paula A, C de Melo D, A P Ribeiro P. **Cinnamon (Cinnamomum sp.) inclusion in diets for Nile tilapia submitted to acute hypoxic stress.** Fish Shellfish Immunol. 2016 Jul;54:551-5.

The aim of this study was to evaluate the possible effects of diets supplemented with probiotics and different cinnamon levels (powder and essential oil) on immunological parameters of Nile tilapia after being subjected to acute stress by hypoxia. Three hundred and thirty juvenile male tilapia fish (66.08 ± 2.79 g) were distributed in 30 tanks of 100 L capacity (11/cage) with a water recirculation system. The animals were fed for 71 days with diets containing extruded cinnamon powder at different levels (0.5, 1, 1.5, 2%), cinnamon essential oil (0.05, 0.1, 0.15; 0.2%) and probiotics (0.4%), all in triplicate. At the end of the experiment, the fish (200.36 ± 19.88 g) of the different groups were subjected to stress by hypoxia. Hypoxia was achieved by capturing the animals with a net, keeping them out of the water for three minutes, and then sampling the blood 30 min after the procedure to determine the levels of cortisol, glucose, haematocrit, lysozyme, bactericidal index, total protein, and its fractions. The animals kept blood homeostasis after hypoxic stress. Diet supplementation with 0.5% cinnamon powder improved the fish immune response, since it resulted in an increase of 0.5% in γ-globulin level. Administration of 0.15% cinnamon essential oil resulted in an increase of α1 and α2-globulins, which may be reflected in increased lipid content of the carcass and the hepatosomatic index. More studies are necessary to better understand the effects of these additives for fish immunity.

Ghehdarijani MS, Hajimoradloo A, Ghorbani R, Roohi Z. **The effects of garlic-supplemented diets on skin mucosal immune responses, stress resistance and growth performance of the Caspian roach (Rutilus rutilus) fry.** Fish Shellfish Immunol. 2016 Feb;49:79-83.

This study was conducted to evaluate the effects of garlic supplementation on some skin mucus immune parameters, mucus antimicrobial activity and growth performance of the Caspian roach (Rutilus rutilus caspicus) fry. Fish (1 ± 0.07 g) were divided into four groups fed diets containing 0 (control), 5, 10 and 15 g kg(-1) garlic for 8 weeks. The results showed that there was a significant increase in weight gain and specific growth rate in those fish fed garlic diets compared with the control ($P < 0.05$). Condition factor was not significantly affected by garlic dosage. At the end of trial, the epidermal mucus protein level, alkaline phosphatase and antimicrobial activity against 2 g-negative bacteria (Escherichia coli and Serratia marcescens) and gram-positive bacteria (Streptococcus faecium and Micrococcus luteus) were

measured. Skin mucus alkaline phosphatase, protein levels and antimicrobial activity were increased following garlic administration, and the bacterial growth inhibition zones were significantly elevated in garlic-fed fish ($P < 0.05$). In salinity stress experiment, no differences were observed for survival rate among the experimental diets. No mortality was recorded during the feeding trial. These results indicated that dietary garlic beneficially affects the skin mucus immune parameters and growth performance of the Caspian roach fry.

Kareem ZH, Abdelhadi YM, Christianus A, Karim M, Romano N **Effects of some dietary crude plant extracts on the growth and gonadal maturity of Nile tilapia (Oreochromis niloticus) and their resistance to Streptococcus agalactiae infection**. Fish Physiol Biochem. 2016 Apr;42(2):757-69.

A 90-day feeding trial was conducted on the growth performance, feeding efficacy, body indices, various hematological and plasma biochemical parameters, and histopathological examination of the gonads from male and female Nile tilapia fingerlings when fed different crude plant extracts from Cinnamomum camphora, Euphorbia hirta, Azadirachta indica, or Carica papaya at 2 g kg(-1) compared to a control diet. This was followed by a 14-day challenge to Streptococcus agalactiae. All treatments were triplicated, and each treatment consisted of 30 fish. Results showed that C. papaya extracts were the most effective at delaying gonadal maturation to both male and female tilapia, as well as significantly increasing ($P < 0.05$) growth performance compared to the control treatment. Similarly, dietary C. camphora and E. hirta extracts also significantly improved growth, while no significant growth effect was detected between the A. indica and control treatments ($P > 0.05$). Further, crude body lipid was lower in the C. camphora, E. hirta and C. papaya treatments, but was only significantly lower for the E. hirta treatment compared to the control. Meanwhile, none of the hematological or biochemical parameters were significantly affected, although plasma ALT was significantly lower for tilapia fed A. indica compared to the control. After the 14-day bacterial challenge, tilapia fed C. camphora supplementation had significantly higher survival, compared to the control, but was not significantly higher than the other supplemented diets. Results indicate that dietary C. papaya extract can significantly promote growth and delay gonadal maturation to both male and female tilapia, while C. camphora was the most effective prophylactic to S. agalactiae and may be a cost-effective and eco-friendly alternative to antibiotics.

Kirubakaran CJ, Subramani PA, Michael RD. **Methanol extract of Nyctanthes arbortristis seeds enhances non-specific immune responses and protects Oreochromis mossambicus (Peters) against Aeromonas hydrophila infection.** Res Vet Sci. 2016 Apr;105:243-8.

Immunostimulation using medicinal plant extracts is a promising approach for prevention and control of diseases with reference to sustainable fish farming. Oreochromis mossambicus, dubbed as aquatic chicken is a cultured fish worldwide and a laboratory model organism. Aeromonas hydrophila is one of the major bacterial pathogens in fish farming that causes huge loss to aquaculture industries. In this study, we investigated the efficacy of methanol extract of Nyctanthes arbortristis seeds on disease resistance of O. mossambicus against live virulent A. hydrophila. We also investigated its effect on the non-specific immune parameters such as serum lysozyme, myeloperoxidase, antiprotease and specific immune parameters in terms of specific serum antibody titres assayed by bacterial agglutination test. Our studies indicate that intra-peritoneal administration of 20mg/kg methanol extract increases the Relative Percent Survival (RPS) of O. mossambicus challenged with LD80 of A. hydrophila. Further, both non-specific and specific immune parameters were enhanced by the methanol extract. Further experiments at molecular levels in the laboratory and also efficacy testing at field level are essential before applying this plant product in aquaculture industry.

Fridman S, Sinai T, Zilberg D. **Efficacy of garlic based treatments against monogenean parasites infecting the guppy (Poecilia reticulata (Peters)**. Vet Parasitol. 2014 Jun 16;203(1-2):51-8.

Monogenean infections of commercially farmed fishes are responsible for significant economic losses. Garlic (Allium sativum) is a well-known spice which also possesses anti-microbial and anti-

parasitical properties. The current work aimed to test the efficacy of garlic-based treatments against infection with monogenean sp. in the guppy (Poecilia reticulata). Clipped sections of tail fins of guppies heavily infected with Gyrodactylus turnbulli were exposed to aqueous garlic extract (7.5 to 30 mL L(-1)) and visually observed under a dissecting microscope. Results revealed that exposure to garlic caused detachment of parasite and cessation of movement indicating death. A positive correlation was seen between garlic concentration and time to detachment and death of parasites, which, at the highest concentration of 30 mL L(-1), occurred at 4.1 and 8.6 min, respectively. Bathing in aqueous garlic extract (7.5 and 12.5 mL L(-1)) was tested in guppies infected with G. turnbulli. Prior acute toxicity tests revealed the maximum tolerance levels of guppies to garlic extract to be 12.5 mL L(-1) for 1h. Bathing of infected fish in garlic extract (7.5 and 12.5 mL L(-1)) significantly ($p<0.05$) reduced infection prevalence and intensity as compared to the control. Oral treatments using dry garlic powder-supplemented diet were tested on guppies infected with G. turnbulli and Dactylogyrus sp. Fish were fed with food containing 10% and 20% dry garlic powder for 14 days. Groups fed with garlic supplemented diets showed significantly reduced ($p<0.05$) mean prevalence and mean intensity of parasites as compared to the control. Dietary application of garlic did not appear to affect palatability. Fresh crushed garlic was added at a level of 1 gL(-1) and applied as an indefinite bath for 14 days. This treatment was seen to significantly reduce ($p<0.05$) parasite prevalence and mean intensity as compared to the control. Histopathology revealed elevated muscular dystrophy in the 20% garlic-fed group, as compared to control. These findings demonstrate the potential of garlic as a natural alternative to currently used chemical treatments for monogenean sp. infection in the guppy.

Schelkle B, Snellgrove D, Cable J. **In vitro and in vivo efficacy of garlic compounds against Gyrodactylus turnbulli infecting the guppy (Poecilia reticulata)**. Vet Parasitol. 2013 Nov 15;198(1-2):96-101

Traditional compounds used to treat fish diseases in aquaculture and the ornamental fish industry (such as formalin and malachite green) can be more toxic to the hosts than their parasites. With the reviviscence in the use of herbal products, various botanicals have been heralded as cures for particular pathogens, but the efficacy of these compounds for parasitic worms is questionable. Here, we tested a range of garlic (Allium sativum) products against a major aquarium pathogen, Gyrodactylus turnbulli, infecting the guppy (Poecilia reticulata). All garlic products significantly reduced parasite mean survival time in vitro, from 13 h to <1 h. In fully randomised trials, the number of parasites was also significantly reduced on infected fish exposed to garlic from different sources. Two garlic treatments (minced and granule forms) reduced worm burdens by 66% and 75% after three doses, whereas Chinese freeze-dried garlic and allyl disulphide were 95% effective after a single application. In fact, Chinese freeze dried garlic was equally effective as Levamisole, a licensed livestock dewormer that is highly effective against G. turnbulli but not routinely prescribed for use in fish; hence, garlic may be a potential alternative treatment for gyrodactylosis.

Militz TA, Southgate PC, Carton AG, Hutson KS. **Efficacy of garlic (Allium sativum) extract applied as a therapeutic immersion treatment for Neobenedenia sp. management in aquaculture.** J Fish Dis. 2014 May;37(5):451-61

Garlic, Allium sativum L., extract administered as a therapeutic bath was shown to have antiparasitic properties towards Neobenedenia sp. (MacCallum) (Platyhelminthes: Monogenea) infecting farmed barramundi, Lates calcarifer (Bloch). The effect of garlic extract (active component allicin) immersion

on Neobenedenia sp. egg development, hatching success, oncomiracidia (larvae) longevity, infection success and juvenile Neobenedenia survival was examined and compared with freshwater and formalin immersion. Garlic extract was found to significantly impede hatching success (5% ± 5%) and oncomiracidia longevity (<2 h) at allicin concentrations of 15.2 µL L(-1) , while eggs in the seawater control had >95% hatching success and mean oncomiracidia longevity of 37 ± 3 h. At much lower allicin concentrations (0.76 and 1.52 µL L(-1)), garlic extract also significantly reduced Neobenedenia infection success of L. calcarifer to 25% ± 4% and 11% ± 4%, respectively, compared with 55% ± 7% in the seawater control. Juvenile Neobenedenia attached to host fish proved to be highly resistant to allicin with 96% surviving 1-h immersion in 10 mL L(-1) (15.2 µL L(-1) allicin) of garlic extract. Allicin-containing garlic extracts show potential for development as a therapy to manage monogenean infections in intensive aquaculture with the greatest impact at the egg and larval stages.

Abd El-Galil MA, Aboelhadid SM. **Trials for the control of trichodinosis and gyrodactylosis in hatchery reared Oreochromis niloticus fries by using garlic.** Vet Parasitol. 2012 Apr 30;185(2-4):57-63.

The present work was designed to study the prevalence of trichodinosis and gyrodactylosis in Oreochromis niloticus fries, and to test the therapeutic efficacy and preventive efficacy of garlic oil and crushed garlic cloves. Trichodinosis and gyrodactylosis are ectoparasitic diseases that affect most warm freshwater fish, especially fries and fingerlings. In a private O. niloticus fish hatchery, the prevalence of trichodinosis in 5-, 15- and 30-day-old-fries was 37%, 23% and 40.5%, respectively. The highest infection intensity was detected in 30-day-old-fries. The gyrodactylosis was reported only in combination with trichodinosis. In addition, we found that its prevalence in 5-, 15- and 30-day-old-fries was 17%, 19.5% and 29%, respectively. Mortality rate of fry in the first month of life was 53% as a result of injury to these two types of parasites. The garlic oil and crushed garlic cloves were tested in both in vitro and earthen ponds of the hatchery. Using 2-, 2.5- and 3-ppt (parts per thousand) garlic oil for 4h in vitro water bath treatment resulted in 100% recovery, while 1 and 1.5 ppt garlic oil, respectively, needed 24 and 16 h to treat the infected fries. The treatment by 3 ppt garlic oil as a water bath for 1h treated the two diseases in 55% in 7 days from application in the hatchery earthen pond. In the mean time, 300 mg L(-1) crushed garlic cloves as an indefinite bath in the hatchery earthen pond eliminated 68% of the diseases. The same protocol for preventing the two diseases resulted in obtaining 65% and 75% of parasite free fries, for garlic oil and crushed garlic cloves, respectively, compared to 53% of the control fries.

Levy G, Zilberg D, Paladini G, Fridman S**. Efficacy of ginger-based treatments against infection with Gyrodactylus turnbulli in the guppy (Poecilia reticulata (Peters)).** Vet Parasitol. 2015 Apr 30;209(3-4):235-41.

Monogenean infections of commercially farmed fishes are responsible for significant economic losses and existing chemical therapeutants, often stressful to the fish, pose associated risks. As part of a recent trend to move towards the use of alternative, plant-based remedies for commonly occurring aquaculture-related diseases, the efficiency of ginger (Zingiber officinale) was investigated against the monogenean parasite Gyrodactylus turnbulli in the guppy. In vitro trials revealed the clear anti-parasitic effects of ginger. Ethanolic and aqueous extracts, prepared from freeze dried ginger, were tested. An increase in extract concentration was associated with reduced time to parasite immobilisation, with ethanolic extract being more efficient; at 75 and 200ppt aqueous ginger extract parasites died at 65.6±2.8 and 1.8±0.2min, respectively, whereas at 5 and 40ppt ethanolic extract parasites died at 26.1±0.7 and 4.9±0.3min, respectively. Bathing G. turnbulli-infected fish in ethanolic ginger extract (i.e. 5 and 7.5ppt for 90 and 30min, respectively) significantly reduced infection prevalence and intensity when compared to the water and ethanol controls. The higher concentration (i.e. 7.5ppt) proved as equally effective as Praziquantel, the conventionally used chemical treatment for gyrodactylosis, with the fish appearing to be completely cleared of the infection in both cases. Oral treatments of G. turnbulli-infected guppies with diets supplemented with 10 and 20% ginger powder proved to be ineffective in decreasing parasite load. These findings demonstrate that immersion in ginger extract offers an effective, alternative treatment against monogenean infection in fish.

Gabriel NN, Qiang J, He J, Ma XY, Kpundeh MD, Xu P. **Dietary Aloe vera supplementation on growth performance, some haemato-biochemical parameters and disease resistance against Streptococcus iniae in tilapia (GIFT).** Fish Shellfish Immunol. 2015 Jun;44(2):504-14.

This study investigated effects of dietary Aloe vera on growth performance, some haemato-biochemical parameters and disease resistance against Streptococcus iniae in tilapia (GIFT). Five groups were designed including a basal diet (control) and 100% A. vera powder incorporated in fish feed at 0.5% 1%, 2%, and 4%/kg feed, which were administered for 8 weeks. Fish fed 0.5%, 1%, and 2% A. vera supplemented diet significantly improved ($p < 0.05$) weight gain, absolute growth rate and specific growth rate. Feed intake significantly increased in fish fed with A. vera diet at 1% and 2%/kg feed. Feed efficiency ratio, feed conversion ratio, and hepatosomatic index were significantly enhanced in 4% A. vera supplemented fish over unsupplemented ones ($p < 0.05$). Several haemato-biochemical indices were examined before and after fish were challenged with S. iniae pathogen containing 7.7 × 10(6) CFU cells mL(-1). A. vera supplemented fish showed a significant increase ($p < 0.05$) in red blood cells, hematocrits (Hb), hemoglobin (Hb), white blood cells (WBC), neutrophils, monocytes, eosinophils, serum total protein, glucose and cortisol after challenge when compared to unsupplemented ones. Meanwhile, 4% A. vera supplemented fish showed a decrease ($p < 0.05$) in RBC, Hb, Ht, WBC, and mean corpuscular hemoglobin (MCH) after challenge compared to unsupplemented ones and other supplemented ones. In addition, lower mean corpuscular volume values (MCV) ($p < 0.05$) were observed in fish fed with A. vera diet at 2% and 4% A. vera/kg feed than those fed unsupplemented diet. Unchallenged fish fed 0.5%, 1%, and 2% A. vera showed significantly higher values ($p < 0.05$) of mean corpuscular hemoglobin concentration (MCHC) than those fed unsupplemented diet and 4% A. vera supplemented diet. There was a significant increase ($p < 0.05$) in the neutrophil to lymphocyte ratio (N/L) within experimental groups after challenge; N/L ratio in A. vera unsupplemented fish and those supplemented with A. vera diet at 1%/kg feed increased significantly ($p < 0.05$) throughout challenge period; while those fed 4% A. vera supplemented diet maintained higher values at all experimental stages among groups. There was a significant correlation ($p < 0.05$, $r = 0.53$) between N/L ratio and glucose concentration, 96 h after challenge. Aloe had no significant effect ($p > 0.05$) on the survival of the fish when compared to the control; no mortality was recorded in challenge trial. Overall, our results indicated that dietary aloe supplementation could improve growth, feed utilization, and haemato-biochemical parameters of cultured tilapia.

Zhou J, Li C, Wang L, Ji H, Zhu T. **Hepatoprotective effects of a Chinese herbal formulation, Yingchen decoction, on olaquindox-induced hepatopancreas injury in Jian carp (Cyprinus carpio var. Jian).** Fish Physiol Biochem. 2015 Feb;41(1):153-63.

In order to identify effective hepatoprotective herbs for clinical application in fish farming, 200 mg/kg olaquindox (OLA) was added to a basal diet (group 1, control) to form OLA diet (group 2), then 1.35, 2.7 and 5.4 % (w/w) of a Chinese herbal formulation, Yingchen decoction (YCD), were added to the OLA diet to form three additional diets for groups 3, 4 and 5, respectively. A total of 375 juvenile Jian carp (Cyprinus carpio var. Jian) (52.12 ± 2.95 g/tail) were divided into five groups (triplicates per group) and fed the five diets mentioned above, respectively, for 6 weeks. At the termination of feeding experiment, serum biochemical indexes, viability of hepatocytes and the hepatopancreas microstructure for each group were detected and observed. The results showed that serum ALT and AST in group 2 were significantly higher than the control ($P < 0.05$). Plasma membranes hepatocyte nuclei in group 2 were found to be mostly indistinct, compared to group 1, and gradually recovered with the increasing supplementation of YCD in group 3, 4 and 5. The viability of isolated hepatocytes in group 2 was the lowest and gradually recovered with the increasing supplementation of YCD in group 3, 4 and 5. The results suggest that YCD protected the Jian carp hepatopancreas against injury from OLA, and that 5.4 % YCD would be the optimum dosage in a Jian carp diet.

Tang J, Cai J, Liu R, Wang J, Lu Y, Wu Z, Jian J. **Immunostimulatory effects of artificial feed supplemented with a Chinese herbal mixture on Oreochromis niloticus against Aeromonas hydrophila.** Fish Shellfish Immunol. 2014 Aug;39(2):401-6.

The effects of a Chinese herbal mixture (CHM) composed of astragalus, angelica, hawthorn, Licorice root and honeysuckle on immune responses and disease resistant of Nile tilapia (Oreochromis niloticus GIFT strain) were investigated in present study. Fish were fed diets containing 0 (control), 0.5%, 1.0%, 1.5% or 2.0% CHM (w/w) for 4 weeks. And series of immune parameters including lysozyme, cytokine genes TNF-α and IL-1β,

superoxide dismutase (SOD), peroxidase (POD), malondialdehyde (MDA) were measured during test period. After four weeks of feeding, fish were infected with Aeromonas hydrophila and mortalities were recorded. Results of this study showed that feeding Nile tilapia with CHM-supplementation diet stimulated lysozyme activity, SOD activity and POD activity in serum, induced TNF-α and IL-1β mRNA expression in head kidney and spleen, but decreased serum MDA content. All CHM-supplemental groups showed reduced mortalities following A. hydrophila infection compared with the group fed the control diet. These results suggested that this CHM can be applied as a tilapia feed supplement to elevate fish immunity and disease resistance against A. hydrophila

Fu Y, Zhang Q, Xu DH, Xia H, Cai X, Wang B, Liang J. **Parasiticidal effects of Morus alba root bark extracts against Ichthyophthirius multifiliis infecting grass carp.** Dis Aquat Organ. 2014 Feb 19;108(2):129-36

Ichthyophthirius multifiliis (Ich), an important fish parasite, can cause significant losses in aquaculture. To find efficacious drugs to control Ich, the root bark of white mulberry Morus alba was evaluated for its antiprotozoal activity. Bark was powdered and extracted with 1 of 5 organic solvents: petroleum ether, chloroform, ethyl acetate, acetone, or methanol. The extracts were concentrated, dissolved in 0.1% (v/v) DMSO, and used for anti-Ich trials. Acetone and ethyl acetate extracts significantly reduced the survival of Ich tomonts and theronts. In vitro, acetone extract at 25 mg l-1 killed all non-encysted tomonts, at 50 mg l-1 eradicated all encysted tomonts, and at 8 mg l-1 caused mortality of all theronts. Ethyl acetate extract at 50 mg l-1 eliminated all non-encysted tomonts, at 100 mg l-1 killed all encysted tomonts and terminated tomont reproduction, and at 8 mg l-1 killed all theronts. Low concentrations (2 and 4 mg l-1) of acetone and ethyl acetate extracts could not kill all theronts after 4 h exposure, but a significant decrease in theront infectivity was observed following 30 min of pretreatment with the extracts. The 96 h LC(50) values of acetone and ethyl acetate extracts to grass carp were 79.46 and 361.05 mg l-1, i.e. much higher than effective doses for killing Ich theronts (8 mg l-1 for both extracts) and non-encysted tomonts (12.5 and 25 mg l-1, respectively). Thus M. alba extract may be a potential new, safe, and efficacious drug to control Ich

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IX-B Veterinary botanical medicine and Small Animals

Chou HI, Chen KS, Wang HC, Lee WM. Effects of cranberry extract on prevention of urinary tract infection in dogs and on adhesion of Escherichia coli to Madin-Darby canine kidney cells. Am J Vet Res. 2016 Apr;77(4):421-7.

To determine effects of cranberry extract on development of urinary tract infection (UTI) in dogs and on adherence of Escherichia coli to Madin-Darby canine kidney (MDCK) cells. ANIMALS 12 client-owned dogs (in vivo experiment) and 6 client-owned dogs (in vitro experiment). 12 dogs with a history of recurrent UTI received an antimicrobial (n = 6) or cranberry extract (6) orally for 6 months. Dogs were monitored for a UTI. For the in vitro experiment, cranberry extract was orally administered to 6 dogs for 60 days. Voided urine samples were collected from each dog before and 30 and 60 days after onset of extract administration. Urine was evaluated by use of a bacteriostasis assay. An antiadhesion assay and microscopic examination were used to determine inhibition of bacterial adherence to MDCK cells. None of the 12 dogs developed a UTI. The bacteriostasis assay revealed no zone of inhibition for any urine samples. Bacterial adhesion was significantly reduced after culture with urine samples obtained at 30 and 60 days, compared with results for urine samples obtained before extract administration. Microscopic examination revealed that bacterial adherence to MDCK cells was significantly reduced after culture with urine samples obtained at 30 and 60 days, compared with results after culture with urine samples obtained before extract administration. Oral administration of cranberry extract prevented development of a UTI and prevented E coli adherence to MDCK cells, which may indicate it has benefit for preventing UTIs in dogs.

Nardoni S, Costanzo AG, Mugnaini L, Pisseri F, Rocchigiani G, Papini R, Mancianti F. An open-field study comparing an essential oil-based shampoo with miconazole/chlorhexidine for hair coat disinfection in cats with spontaneous microsporiasis. J Feline Med Surg. 2016 Jan 18

The goal of the present study was to compare the antifungal efficacy of an essential oil (EO) shampoo proven to be effective against Microsporum canis with miconazole/chlorhexidine for topical hair coat disinfection in cats treated concurrently with oral itraconazole. Cats received treatment with oral itraconazole (Itrafungol) at a dose of 5 mg/kg/day pulse administration for 1 week, every 2 weeks for at least 6 weeks and were washed twice a week with a neutral shampoo with added EOs of Thymus serpyllum (2%), Origanum vulgare and Rosmarinus officinalis (5% each) for the period of systemic treatment. This protocol was compared with a conventional treatment (oral itraconazole + 2% miconazole/2% chlorhexidine shampoo). The treatment was well tolerated and adverse effects were not recorded. All cats were clinically negative at week 11. With respect to animals with extensive lesions, the speed of resolution was higher in cats with focal lesions. The animals showing diffuse lesions required more than a course of treatment to achieve a mycological cure. There was no significant difference between the number of weeks to obtain mycological cure for cats treated with EOs and animals treated conventionally. The treatment appeared to be effective and well appreciated by the owners. The use of shampoo with the added EOs of T serpyllum, O vulgare and R officinalis would seem an interesting, natural alternative to conventional topical treatment.

Blaskovic M, Rosenkrantz W, Neuber A, Sauter-Louis C, Mueller RS. The effect of a spot-on formulation containing polyunsaturated fatty acids and essential oils on dogs with atopic dermatitis. Vet J. 2014 Jan;199(1):39-43

Recent studies have shown that immunological aberrations and epidermal barrier defects could be important in the pathogenesis of canine atopic dermatitis (CAD) and that oral polyunsaturated fatty acids (PUFAs) might influence the epidermal barrier. The aim of this study was to evaluate the effects of a spot-on formulation containing PUFAs and essential oils on pruritus and lesions caused by CAD. Forty-eight privately owned dogs of

different breeds, ages and genders diagnosed with atopic dermatitis were included in a randomized, double-blinded, placebo-controlled, multicentre clinical trial. Dogs were treated with a spot-on formulation containing PUFAs and essential oils or placebo on the dorsal neck once weekly for 8weeks. Before and after the study, CAD extent and severity index-03 (CADESI-03) and pruritus scores were determined by veterinarians and owners, respectively. There was significantly more improvement in CADESI-03 and pruritus scores in the treatment group than in the placebo group (P=0.011 and P=0.036, respectively). Additionally, more dogs improved by at least 50% in CADESI-03 and pruritus scores in the treatment group than in the placebo group (P=0.008 and P=0.070, respectively). No adverse reactions were observed. The topical preparation containing PUFAs and essential oils was a safe treatment and beneficial in ameliorating the clinical signs of CAD.

Moreau M, Lussier B, Pelletier JP, Martel-Pelletier J, Bédard C, Gauvin D, Troncy E. A medicinal herb-based natural health product improves the condition of a canine natural osteoarthritis model: a randomized placebo-controlled trial. Res Vet Sci. 2014 Dec;97(3):574-81.

An oral herb-based natural health product (NHP) was evaluated in the canine natural osteoarthritis model. At baseline, the peak vertical force (PVF, primary endpoint) and case-specific outcome measure of disability (CSOM) were recorded in privately-owned dogs. Dogs (16/group) were randomized to receive NHP formulations or a negative control. The PVF was measured at week (W) 4 and W8. Daily locomotor activity was recorded using accelerometer. The CSOMs were assessed bi-weekly by the owner. The NHP-treated dogs (n = 13) had higher PVF at W4 (p = 0.020) and W8 (p <0.001) when compared to baseline. The changes at W8 were higher than control dogs (n = 14, p <0.027) and consistent with Cohen's d effect size of 0.7 (95% confidence interval: 0.0-1.5). The NHP-treated dogs had higher locomotor activity at W8 (p = 0.025) when compared to baseline. No significant change was observed for the CSOM. The NHP improved the clinical signs of osteoarthritis in this model.

Szweda M, Szarek J, Dublan K, Męcik-Kronenberg T, Kiełbowicz Z, Bigoszewski M. Effect of mucoprotective plant-derived therapies on damage to colonic mucosa caused by carprofen and robenacoxib administered to healthy dogs for 21 days. Vet Q. 2014;34(4):185-93.

The hypothesis was that Non-steroidal anti-inflammatory drugs (NSAIDs) may cause gastrointestinal damage in dogs. To determine the extent to which lansoprazole, liquorice extract, and a herbal solution exhibit protective effects on colonic mucosa when administered to dogs concurrently with the NSAIDs carprofen or robenacoxib , thirty-five healthy beagle dogs (15 male and 20 female) aged 13-14 weeks and weighing 4.3-5.5 kg at the beginning of the experiment were included. Endoscopy and biopsy of the caudal gastrointestinal tract were performed pretreatment and on the last day of a 21-day treatment period with (1) oral carprofen; (2) carprofen and the proton-pump inhibitor lansoprazole; (3) carprofen, liquorice extract, and a herbal solution that contained extracts of thyme, icelandic lichen, hyssop, and saponariae root; (4) robenacoxib; (5) robenacoxib and lansoprazole; (6) robenacoxib, liquorice extract, and herbal solution; or (7) an empty gelatin capsule. Statistical analyses were performed with the Kruskal-Wallis, Cochran's Q, and chi-squared test with p < 0.05 considered significant. Both carprofen and robenacoxib tested damaged the colonic mucosa with most severe microscopic lesions following administration of robenacoxib with lansoprazole. The risk of histopathological lesions in the colon increased most rapidly in robenacoxib with lansoprazole (absolute risk increase -0.85) similar to robenacoxib only (-0.75), whereas the best result was recorded following the plant remedies together with carprofen (-0.15) and the plant remedies together with robenacoxib (-0.2). TConcurrent administration of liquorice extract and an herbal solution with robenacoxib was associated with decreased severity of the NSAID-induced mucosal lesions.

Torkan S, Khamesipour F, Katsande S. Evaluating the effect of oral administration of Echinacea hydroethanolic extract on the immune system in dog. Auton Autacoid Pharmacol. 2015 Jul;35(1-2):9-13.

This study was designed to evaluate the effects of oral administration of Echinacea hydroethanolic extract on the dog's immune system. The study was performed on 14 dogs that were referred to the veterinary clinic. These dogs were randomly allocated to two equal treatment groups. The first group received 1 ml of 5% Echinacea hydroethanolic extract two times a day for 2 months, and the second group received a placebo (water). To do haematology and immunology tests, the dogs were bled on days 0, 30 and 60. Blood tests, including packed cell volume (PCV), haemoglobin (Hb), red blood cell count (RBC), white blood cell count

(WBC), counting neutrophils (Nut), lymphocytes (Lym), monocytes (Mon), eosinophils (Eos), basophils (Baso) and B cell, were performed. Furthermore, safety factor IgM and per cent of phagocytosis and phagocyte were measured from the blood sample. The results showed that in the group which received Echinacea PCV, Hb, RBC count, WBC count, Lym, Nut, the per cent of phagocytosis and IgM significantly increased ($P < 0.05$). Moreover, positive effects of Echinacea plant on the immune system were observed. There was a significant change in HTC, RBC, Hb over time in the group that received Echinacea and the per cent of phagocytosis and IgM ($P < 0.05$). The study establishes that these extracts might have appreciable immunostimulatory activity. However, further studies are required to confirm these findings.

Szweda M, Szarek J, Lew M, Szarek-Bęska A, Gulda D. Can liquorice extract and herbal solution prevent colonic mucosa damage caused by robenacoxib in dogs? Pol J Vet Sci. 2015;18(4):793-8.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in animals, especially in dogs, to manage pain due to inflammatory disease. This study investigated whether plant drugs can prevent mucosal injury induced by robenacoxib. We used fifteen healthy beagle dogs (7 male and 8 female) aged 4 months, weighing 4.2-5.1 kg at the beginning of the study. Endoscopy and biopsy of the colon were performed before and on the 21 day treatment with robenacoxib (1), robenacoxib, herbal solution with liquorice extract (2), placebo - an empty capsule (3). There were 5 animals in each group. The greatest microscopic damage in the colon was observed in animals which received robenacoxib. Plant drug administration reduced the severity of lesions in the colon when administered with robenacoxib (ARI = - 0.15). Conclusion: concurrent administration of liquorice extract and plant solution with robenacoxib was associated with significant decreased severity of the robenacoxib-induced colonic mucosal lesions.

Al-Mohizea AM, Ahad A, El-Maghraby GM, Al-Jenoobi FI, AlKharfy KM, Al-Suwayeh SA. Effects of Nigella sativa, Lepidium sativum and Trigonella foenum-graecum on sildenafil disposition in beagle dogs. Eur J Drug Metab Pharmacokinet. 2015 Jun;40(2):219-24.

The present study was conducted to investigate the effects of some commonly used herbs namely Nigella sativa, Lepidium sativum and Trigonella foenum-graecum on the pharmacokinetics of sildenafil in beagle dogs. The study design involved four treatments in a non-balanced crossover design. Sildenafil was given one tablet 100 mg orally to each dog and blood samples were obtained. After a suitable washout period, animals were commenced on a specific herb treatment for 1 week. Blood samples were withdrawn at different time intervals and sildenafil was analyzed by HPLC method. Oral administration of Nigella sativa resulted in reduction of $AUC_{0-\infty}$, C max and t 1/2 as compared to the control. Treatment of Lepidium sativum resulted in a significant reduction in the C max and AUC. There were no significant differences between the rests of the pharmacokinetic parameters relative to those of the control. For Trigonella foenum-graecum, the effects were similar to those obtained in case of Lepidium sativum. It was concluded that concurrent use of investigated herbs alters the pharmacokinetics of sildenafil. Co-administration of investigated herbs should be cautious since their concomitant use might result in decrease in sildenafil bioavailability.

van Dooren I, Faouzi Mel A, Foubert K, Theunis M, Pieters L, Cherrah Y, Apers S. Cholesterol lowering effect in the gall bladder of dogs by a standardized infusion of Herniaria hirsuta L. J Ethnopharmacol. 2015 Jul 1;169:69-75.

To investigate the efficacy of a standardized infusion of Herniaria hirsuta against choleltihiasis, and evaluation of its genotoxicity. An in vivo experiment to evaluate the cholesterol lowering effect of a infusion of H. hirsuta in the gall bladder of dogs was carried out. Dogs were divided into 3 groups i.e. control dogs (CG), dogs treated with ursodeoxycholic acid (UDCA) (2×7.35mg/kg body weight/day) and dogs treated with the standardized infusion (HG) (2×48.5mg/kg body weight/day). Dogs were fed a fatty diet during 120 days after which a diet without additional fat was introduced till day 180. Treatment started 30 days after introduction of the fatty diet and lasted till the end of the experiment. A bile and blood sample of each dog was collected every 30 days, after which the concentration of cholesterol was determined. An Ames test was performed according to the OECD-guidelines. Conclusion: Prolonged use of this standardized H. hirsuta extract resulted in a cholesterol-lowering effect in the bile of dogs. Since this pharmacological effect prevents the formation of gallstones and can contribute to solving existing gallstones, a standardized infusion of H. hirsuta may have a positive effect in the treatment of gallstones in human patients.

Hanzlicek AS, Roof CJ, Sanderson MW, Grauer GF. The Effect of Chinese rhubarb, Rheum officinale, with and without benazepril on the progression of naturally occurring chronic kidney disease in cats. J Vet Intern Med. 2014 Jul-Aug;28(4):1221-8.

Renal fibrosis is common in progressive kidney disease. Transforming growth factors β (TGF-β) are important mediators of all types of fibrosis, including renal fibrosis. Chinese rhubarb has been shown to have antifibrotic properties in part because of inhibition of TGF-β and has slowed the progression of kidney disease in rodent models. The hypothesis is that administration of a Chinese rhubarb supplement will slow the progression of chronic kidney disease (CKD) in cats and the concurrent administration of Chinese rhubarb and benazepril will be more effective than either alone. Cats with naturally Twenty-nine client-owned occurring IRIS Stage 2 or early Stage 3 CKD and without comorbidity such as cancer, urinary tract obstruction, urinary tract infection, poorly controlled hyperthyroidism, or systemic hypertension were enrolled in the study. A randomized, positive-controlled, prospective study was performed. Cats received Chinese rhubarb, benazepril, or both in addition to standard treatment for CKD. Repeated measures ANOVA was used to assess changes in serum creatinine concentration, body weight, hematocrit, urine protein: urine creatinine ratio (UPC), and systemic arterial blood pressure over time between and within treatment groups over an average of 22 months.

No significant differences were detected in serum creatinine concentration, body weight, hematocrit, UPC, and systemic arterial pressure over time between or within treatment groups.

This study failed to detect a significant difference in the progression of CKD in cats treated with Chinese rhubarb, benazepril, or both. Further study in specific subsets of cats with CKD is warranted.

Wirth KA, Kow K, Salute ME, Bacon NJ, Milner RJ. In vitro effects of Yunnan Baiyao on canine hemangiosarcoma cell lines. Vet Comp Oncol. 2014 Jun 29.

Yunnan Baiyao is a Chinese herbal medicine that has been utilized for its anti-inflammatory, haemostatic, wound healing and pain relieving properties in people. It has been utilized in the veterinary profession to control bleeding in dogs with hemangiosarcoma (HSA) and has been anecdotally reported to prolong survival times in dogs with this neoplasm. This study evaluated the in vitro activity of Yunnan Baiyao against three canine HSA cell lines after treatment with increasing concentrations of Yunnan Baiyao (50, 100, 200, 400, 600 and 800 μg mL^{-1}) at 24, 48 and 72 h. Mean half maximum inhibitory concentration (IC_{50}) at 72 h for DEN, Fitz, SB was 369.9, 275.9 and 325.3 μg mL^{-1} , respectively. Caspase-3/7 activity increased in correlation with the IC_{50} in each cell line which was confirmed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, APO-BRDU Kit; BD Biosciences, San Jose, CA, USA) assay. VEGF in cell supernatant was also quantified. Overall, the study found that Yunnan Baiyao causes dose and time dependent HSA cell death through initiation of caspase-mediated apoptosis, which supports future studies involving Yunnan Baiyao.

Low SB, Peak RM, Smithson CW, Perrone J, Gaddis B, Kontogiorgos E. Evaluation of a topical gel containing a novel combination of essential oils and antioxidants for reducing oral malodor in dogs. Am J Vet Res. 2014 Jul;75(7):653-7

The objective of this study was to evaluate the effectiveness of a topically applied gel containing essential oils (menthol and thymol) and polyphenolic antioxidants (phloretin and ferulic acid) for reducing halitosis in dogs. A blinded crossover clinical trial was conducted. 20 Dogs received a dental cleaning and examination (periodontal examination including periodontal probing and assessments of plaque, calculus, and gingivitis). Owners then applied a gel (active or placebo) to oral soft tissues twice daily for a 4-week period. Teeth of the dogs were cleaned again, and owners applied the other gel for a 4-week period. Clinicians scored halitosis immediately after the initial cleaning and at 4 and 8 weeks, and owners scored halitosis weekly.Halitosis assessment by clinicians revealed that both groups had improvement in halitosis scores. Two dogs were removed because of owner noncompliance. In the active-to-placebo group (n = 9), halitosis was significantly reduced during application of the active gel but increased during application of the placebo. Seven of 9 owners reported increased halitosis when treatment was changed from the active gel to the placebo. In the placebo-to-active group (n = 9), halitosis decreased during application of the placebo and continued to decrease during application of the active gel. Seven of 9 owners reported a decrease in halitosis with the active gel.An oral topically applied gel with essential oils and polyphenolic antioxidants applied daily after an initial professional dental cleaning decreased oral malodor in dogs.

Breuer E, Efferth T. Treatment of Iron-Loaded Veterinary Sarcoma by Artemisia annua. Nat Prod Bioprospect. 2014 Apr;4(2):113-8.

Artemisinin, a constituent of Artemisia annua L., is a well-known antimalarial drug. Artemisinin-type drugs also inhibit cancer growth in vitro and in vivo. Herbal extracts of A. annua inhibit the growth of cancer cell lines. Here, we report on the use of capsules containing powder of Herba Artemisiae annuae to treat pet sarcoma. The surgical tumor removal as standard treatment was supplemented by adjuvant therapy with A. annua. One cat and one dog with fibrosarcoma survived 40 and 37 months, respectively, without tumor relapse. Two other dogs suffering from fibrosarcoma and hemangioendothelial sarcoma also showed complete remission and are still alive after 39 and 26 months, respectively. A. annua was well tolerated without noticeable side effects. These four cases indicate that A. annua may be a promising herbal drug for cancer therapy.

Helmerick EC, Loftus JP, Wakshlag JJ. The effects of baicalein on canine osteosarcoma cell proliferation and death. Vet Comp Oncol. 2014 Dec;12(4):299-309.

Flavonoids are a group of modified triphenolic compounds from plants with medicinal properties. Baicalein, a specific flavone primarily isolated from plant roots (Scutellaria baicalensis), is commonly used in Eastern medicine for its anti-inflammatory and antineoplastic properties. Previous research shows greater efficacy for baicalein than most flavonoids; however, there has been little work examining their effects on sarcoma cells, let alone canine cells. Three canine osteosarcoma cell lines (HMPOS, D17 and OS 2.4) were treated with baicalein to examine cell viability, cell cycle kinetics, anchorage-independent growth and apoptosis. Results showed that osteosarcoma cells were sensitive to baicalein at concentrations from approximately 1 to 25 µM. Modest cell cycle changes were observed in one cell line. Baicalein was effective in inducing apoptosis and did not prevent doxorubicin cell proliferation inhibition in all the cell lines. The mechanism for induction of apoptosis has not been fully elucidated; however, changes in mitochondrial permeability supersede the apoptotic response.

Furukawa N, Manabe N, Kase Y, Hattori T, Imamura H, Kusunoki H, Haruma K. Intragastric infusion of rikkunshito (kampo) induces proximal stomach relaxation in conscious dogs. Auton Neurosci. 2013 Dec;179(1-2):14-22. (Note: This is Liu Jun Zi Tang used by veterinary herbalists for many gastrointestinal conditions.)

Abnormal proximal gastric relaxation is one of the causes of functional dyspepsia. The purpose of this study is to use a barostat in conscious dogs to determine the effects of rikkunshito, which is considered to have beneficial effects on functional dyspepsia, on the proximal stomach. Eight beagles were used. A gastrocutaneous fistula and force transducers were surgically implanted in the middle corpus and gastric antrum and duodenum, respectively. After a recovery period, a plastic bag was inserted through the gastrocutaneous fistula and the proximal stomach was distended using a barostat. First, four dogs were used to investigate the pressure-volume relation in the fasted and postprandial phases. Second, the stomachs of four different dogs were continuously distended at minimal distending pressure +2 mmHg, and 5 min later were infused with warmed liquid rikkunshito (2 g/20 mL) or water through the gastrocutaneous fistula. Finally, changes in the proximal gastric volume and gastrointestinal motility were observed. The proximal stomach was significantly more pliable in the postprandial phase than in the fasted phase. The proximal gastric volume increased immediately after liquid infusion under constant pressure in both phases and duodenal motility was accelerated. The effect of rikkunshito was significantly greater and lasted longer than that of water. No significant difference between the effects during the fasted or postprandial phase and no change in the gastric antrum motility were observed when rikkunshito was infused. These results indicate that rikkunshito accelerates duodenal motility and relaxes the proximal stomach.

Ogbu SO, Agwu KK, Asuzu IU. Effect of Gongronema latifolium on gastric emptying in healthy dogs. World J Gastroenterol. 2013 Feb 14;19(6):897-902.

The aim of this study was to investigate sonographically the effect of Gonogronema latifolium (G. latifolium) on gastric emptying of semi-solid meals in healthy dogs.In a randomized, placebo-controlled experiment,

twenty-five clinically healthy dogs were randomly allotted into five groups of five dogs in each group. The placebo group served as the control, and the low, moderate and high dose groups ingested the methanolic leaf extract of G. latifolium in capsules at 100 mg/kg, 250 mg/kg and 500 mg/kg, respectively, while the prokinetic group ingested 0.5 mg/kg capsules of metoclopramide. After a 12-h fast, each group ingested its treatment capsules 30 min before the administration of a test meal. Measurements of gastric emptying and blood glucose levels were obtained 30 min before and immediately after the ingestion of the test meal and thereafter every 15 min for 4 h. This was followed by further measurements every 30 min for another 2 h. The gastric emptying times of the placebo, low dose, moderate dose, high dose and prokinetic dose groups were 127.0 ± 8.2 min, 135.5 ± 3.7 min, 155.5 ± 3.9 min, 198.0 ± 5.3 min and 59.0 ± 2.5 min, respectively. Gastric emptying times of the moderate and high dose groups were significantly slower than in the placebo control group (155.5 ± 3.9 min, 198.0 ± 5.3 min vs 127.0 ± 8.2 min, P = 0.000). No significant difference in gastric emptying between the low dose and placebo control groups was noted (135.5 ± 3.7 min vs 127.0 ± 8.2 min, P = 0.072). Gastric emptying of the prokinetic group was significantly faster than that of the control group (59.0 ± 2.5 min vs 127.0 ± 8.2 min, P = 0.000). The hypoglycaemic effect of G. latifolium and gastric emptying were inversely related (r = -0.95, P = 0.000).

Glardon, Pache, Magnenat, Pin, Parvis. Viscum album L. (Iscador) in the cat: tolerance, adverse reactions and indications. Schweiz Arch Tierheilkd. 2014 Aug;156(8):381-8.

In this retrospective study, the tolerance to subcutaneus mistletoe injections (Viscum album L.), adverse reactions and possible indications have been evaluated in feline patients of a small animal clinic. Among the 22 cats treated between 2008 and 2013, 4 did not accept the injections done by the owner, 7 showed slight short time adverse reactions, that disappeared spontaneously. No long term (more than 70 days) adverse reaction directly related to the Viscum album treatment could be identified. This study shows that Iscador(®) can be injected subcutaneously without a risk of worsening of the clinical signs or exacerbation of tumors. The antitumoral, but also immune-modulating and anti-inflammatory properties offer interesting treatment opportunities for dermatologic, odonto-stomatologic or allergic patients.

Hanzlicek AS, Roof CJ, Sanderson MW, Grauer GF. The Effect of Chinese rhubarb, Rheum officinale, with and without benazepril on the progression of naturally occurring chronic kidney disease in cats. J Vet Intern Med. 2014 Jul-Aug;28(4):1221-8.

Renal fibrosis is common in progressive kidney disease. Transforming growth factors β (TGF-β) are important mediators of all types of fibrosis, including renal fibrosis. Chinese rhubarb has been shown to have antifibrotic properties in part because of inhibition of TGF-β and has slowed the progression of kidney disease in rodent models. The hypothesis was that the administration of a Chinese rhubarb supplement will slow the progression of chronic kidney disease (CKD) in cats and the concurrent administration of Chinese rhubarb and benazepril will be more effective than either alone. Twenty-nine client-owned cats with naturally occurring IRIS Stage 2 or early Stage 3 CKD and without comorbidity such as cancer, urinary tract obstruction, urinary tract infection, poorly controlled hyperthyroidism, or systemic hypertension were enrolled in the study. A randomized, positive-controlled, prospective study was performed. Cats received Chinese rhubarb, benazepril, or both in addition to standard treatment for CKD. Repeated measures ANOVA was used to assess changes in serum creatinine concentration, body weight, hematocrit, urine protein: urine creatinine ratio (UPC), and systemic arterial blood pressure over time between and within treatment groups over an average of 22 months. No significant differences were detected in serum creatinine concentration, body weight, hematocrit, UPC, and systemic arterial pressure over time between or within treatment groups. This study failed to detect a significant difference in the progression of CKD in cats treated with Chinese rhubarb, benazepril, or both. Further study in specific subsets of cats with CKD is warranted.

Yanai M, Mochiki E, Ogawa A, Morita H etal Intragastric administration of rikkunshito stimulates upper gastrointestinal motility and gastric emptying in conscious dogs. Gastroenterol. 2013 May;48(5):611-9.

Traditional Japanese medicine, known as Kampo medicine, consists of mixtures of several medicinal herbs widely used to treat upper gastrointestinal disorders in Japan. Rikkunshito, one of these medicines, has not been evaluated with respect to its influence on gastrointestinal motor activity. We investigated the effect of rikkunshito on upper gastrointestinal motility and plasma ghrelin concentrations in conscious dogs. Contractile

response to intragastric administration of rikkunshito was studied via surgically implanted force transducers. A powdered extract of rikkunshito (1.3, 2.7, and 4.0 g) dissolved in water was administered into the stomachs of normal and vagotomized dogs before feeding and gastric emptying was evaluated. Several inhibitors of gastrointestinal motility (atropine, hexamethonium, and ondansetron) were injected intravenously before intragastric administration of rikkunshito. Plasma acylated ghrelin levels after intragastric administration of rikkunshito were measured. In a fasting state, intragastric administration of rikkunshito induced phasic contractions in the duodenum and jejunum in normal dogs. Rikkunshito-induced contractions were inhibited by atropine, hexamethonium and ondansetron. In vagotomized dogs, rikkunshito induced phasic contractions, similar to normal dogs. Gastric emptying was accelerated by intragastric administration of rikkunshito in a dose-dependent manner. The plasma acylated ghrelin level 150 min after intragastric administration of 4.0 g of rikkunshito was significantly higher than the control value. Intragastric administration of rikkunshito stimulates gastrointestinal contractions in the interdigestive state through cholinergic neurons and 5-HT type 3 receptors. Moreover, rikkunshito increases plasma acylated ghrelin levels. Rikkunshito may alleviate gastrointestinal disorders through its prokinetic effects.

Mugnaini L, Nardoni S, Pinto L, Pistelli L, Leonardi M, Pisseri F, Mancianti F. In vitro and in vivo antifungal activity of some essential oils against feline isolates of Microsporum canis. J Mycol Med. 2012 Jun;22(2):179-84

The treatment of dermatophytoses due to Microsporum canis is cumbersome and relapses can occur. Volatile essential oils (EOs) obtained from plants would seem to represent suitable tools to contrast mycoses both in human and animals. The anti-M. canis activity of some EOs chemically characterized was evaluated both in vitro and in vivo. Eleven feline isolates of M. canis were tested by microdilution against EOs extracted from Thymus serpillum, Origanum vulgare, Rosmarinus officinalis, Illicium verum and Citrus limon. A mixture composed by 5% O. vulgare, 5% R. officinalis and 2% T. serpillum, in sweet almond oil was administered to seven infected, symptomatic cats. T. serpillum and O. vulgare showed the lowest MICs, followed by I. verum, R. officinalis and C. limon. The assay performed on mixture showed that antimycotic activity of each component was enhanced. Four out of seven treated cats recovered both clinically and culturally. T. serpillum and O. vulgare EOs showed a strong antifungal activity. Preliminary data suggest a possible application in managing feline microsporiasis. Considering the potential zoonotic impact of this infection, the use of alternative antimycotic compounds would be of aid to limit the risk of environmental spreading of arthrospores.

Fukunaga K, Orito K. Time-course effects of St John's wort on the pharmacokinetics of cyclosporine in dogs: interactions between herbal extracts and drugs. J Vet Pharmacol Ther. 2012 Oct;35(5):446-51.

To clarify the interaction between St John's wort (SJW) and cyclosporine (CsA) in dogs, the pharmacokinetics of CsA before and during the repeated administration of SJW were analyzed. In the SJW group, SJW (300 mg) was given orally to four dogs every 24 h for 14 days. A single dose of CsA (5 mg/kg) was given orally 7 days before and 7 and 14 days after the initiation of the repeated administration of SJW. In the Control group, a single dose of CsA (5 mg/kg) was given orally to four other dogs in accordance with that in the SJW group. Blood samples from both groups were collected, and whole-blood concentrations of CsA were determined using high-performance liquid chromatography with UV detection. The maximum whole-blood concentration and AUC(0-∞) of the SJW group were significantly lower and the CL(tot) /F and V(d) /F were significantly higher than those in the Control group 7 and 14 days after the initiation of repeated SJW. Thus, repeated administrations of SJW affect the pharmacokinetic profiles of CsA in dogs. Further studies are necessary to elucidate the mechanisms of interaction between SJW and CsA in dogs.

Head E, Murphey HL, Dowling AL etal. A combination cocktail improves spatial attention in a canine model of human aging and Alzheimer's disease. J Alzheimers Dis. 2012;32(4):1029-42.

Alzheimer's disease (AD) involves multiple pathological processes in the brain, including increased inflammation and oxidative damage, as well as the accumulation of amyloid-β (Aβ) plaques. We hypothesized that a combinatorial therapeutic approach to target these multiple pathways may provide cognitive and neuropathological benefits for AD patients. To test this hypothesis, we used a canine model of human aging and AD. Aged dogs naturally develop learning and memory impairments, human-type Aβ deposits, and oxidative damage in the brain. Thus, 9 aged beagles (98-115 months) were treated with a medical food cocktail

containing (1) an extract of turmeric containing 95% curcuminoids; (2) an extract of green tea containing 50% epigallocatechingallate; (3) N-acetyl cysteine; (4) R-alpha lipoic acid; and (5) an extract of black pepper containing 95% piperine. Nine similarly aged dogs served as placebo-treated controls. After 3 months of treatment, 13 dogs completed a variable distance landmark task used as a measure of spatial attention. As compared to placebo-treated animals, dogs receiving the medical food cocktail had significantly lower error scores ($t11 = 4.3$, $p = 0.001$) and were more accurate across all distances ($F(1,9) = 20.7$, $p = 0.001$), suggesting an overall improvement in spatial attention. Measures of visual discrimination learning, executive function and spatial memory, and levels of brain and cerebrospinal fluid Aβ were unaffected by the cocktail. Our results indicate that this medical food cocktail may be beneficial for improving spatial attention and motivation deficits associated with impaired cognition in aging and AD.

Skorupski KA, Hammond GM, Irish AM, Kent MS, Guerrero TA, Rodriguez CO, Griffin DW. Prospective randomized clinical trial assessing the efficacy of Denamarin for prevention of CCNU-induced hepatopathy in tumor-bearing dogs. J Vet Intern Med. 2011 Jul-Aug;25(4):838-45.

Increases in liver enzymes occur in up to 86% of dogs receiving CCNU and can result in treatment delay or early discontinuation of treatment. Denamarin contains S-adenosylmethionine and silybin, both of which have been investigated as treatments for various liver diseases. Dogs on CCNU receiving Denamarin have lower alanine aminotransferase (ALT) activity than dogs not receiving Denamarin. Dogs on Denamarin are less likely to require treatment delay because of hepatopathy and are more likely to complete their prescribed course of CCNU. Dogs with lymphoma, mast cell tumor, or histiocytic sarcoma that were prescribed CCNU with or without corticosteroids and with normal ALT activity were eligible for enrolment. Dogs were prospectively randomized to receive either concurrent Denamarin during CCNU chemotherapy or to receive CCNU alone. Liver-specific laboratory tests were run before each dose of CCNU. Increased liver enzyme activity occurred in 84% of dogs receiving CCNU alone and in 68% of dogs on concurrent Denamarin. Dogs receiving CCNU alone had significantly greater increases in ALT, aspartate aminotransferase, alkaline phosphatase, and bilirubin and a significantly greater decrease in serum cholesterol concentrations than dogs receiving concurrent Denamarin. Dogs receiving CCNU alone were significantly more likely to have treatment delayed or discontinued because of increased ALT activity.Increased liver enzyme activity occurs commonly in dogs receiving CCNU chemotherapy. These results support the use of concurrent Denamarin to minimize increased liver enzyme activity in dogs receiving CCNU chemotherapy. Denamarin treatment also increases the likelihood of dogs completing a prescribed CCNU course.

Schmidt V, McEwan N, Volk A, Helps J, Morrell K, Nuttall T. The glucocorticoid sparing efficacy of Phytopica in the management of canine atopic dermatitis. Vet Dermatol. 2010 Feb;21(1):96-105

This double-blind randomized placebo-controlled trial indicates that Phytopica can be an effective glucocorticoid sparing agent in canine atopic dermatitis (AD). Twenty-two dogs with perennial AD [Canine Atopic Dermatitis with Severity Index (CADESI-03) >or= 60] were given 200 mg/kg Phytopica or an identical placebo in food once daily for 56 days. All dogs were initially given 0.4 mg/kg methyl-prednisolone once daily, which was then adjusted according to the daily pruritus score (0-100 mm visual analogue scale). The cumulative dose and pruritus score were lower in the Phytopica than the placebo group. There were statistically significant time and treatment effects for the methyl-prednisolone dose and pruritus score, but there were no significant differences between the Phytopica and placebo groups in the proportion of dogs that achieved a > 50% reduction in dose or pruritus scores at day 56; the mean CADESI-03 scores at days 0, 28 and 56; the numbers achieving >50% reduction in CADESI-03 at days 28 and 56; or in the owners' global efficacy score at days 28 and 56. Adverse events included diarrhoea (three Phytopica and one placebo treated dog), polyuria/polydipsia (three dogs in each group), and polyphagia, intermittent anorexia and panting (one dog each in the placebo group). None of these by themselves required withdrawal of treatment.

Marsella R, Messinger L, Zabel S, Rosychuck R, Griffin C, Cronin PO, Belofsky G, Lindemann J, Stull D. A randomized, double-blind, placebo-controlled study to evaluate the effect of EFF1001, an Actinidia arguta (hardy kiwi) preparation, on CADESI score and pruritus in dogs with mild to moderate atopic dermatitis. Vet Dermatol. 2010 Feb;21(1):50-7.

Canine atopic dermatitis (AD) is common and new therapies are beneficial. This multicentric, randomized, double-blind, placebo-controlled study tested the efficacy of Actinidia arguta (hardy kiwi) (EFF1001) in dogs with mild/moderate AD. The study was divided into two stages. Stage 1 lasted 6 weeks. In the first 2 weeks prednisolone [days 1-3: 0.2 mg/kg twice daily (BID), days 4-14: 0.2 mg/kg every other day (EOD)] was administered. Responsive dogs were placed on prednisolone 0.2 mg/kg EOD + assigned test article [either placebo or EFF1001 (30 mg/kg)] once daily for 4 weeks. Stage 1 responders were advanced to stage 2, which involved 4 weeks of just EFF1001. Clinicians scored lesions using Canine Atopic Dermatitis Extent and Severity Index (CADESI) and owners scored pruritus using a Pruritus Visual Analogue Scale. Seventy-seven dogs were enrolled, 76 were randomized on day 14, and 57 (57/76 = 75%) completed stage 1 (27 in EFF1001 and 30 in placebo). At the end of stage 1, 35 of 57 dogs (35/57 = 61%) responded (18 in EFF1001 and 17 in placebo) and advanced to stage 2. At completion of stage 1, CADESI scores did not significantly differ between groups while pruritus decreased in EFF1001 group and approached significance. At completion of stage 2, 19 dogs (19/35 = 54%) responded (15/19 = 79% had received EFF1001 and 4/19 = 21% placebo in stage 1). After completing stage 2, dogs placed on EFF1001 throughout the study were 3.5 times more likely to either maintain or improve scores than those that started it in stage 2. It is concluded that EFF1001 is beneficial adjunctive therapy after prolonged use.

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IX-C Veterinary Botanical Medicine and Cattle including Dairy

Wang J, Liu M, Wu Y etal Medicinal herbs as a potential strategy to decrease methane production by rumen microbiota: a systematic evaluation with a focus on Perilla frutescens seed extract. Appl Microbiol Biotechnol. 2016 Sep 22.

Mitigation of the methane (CH_4) emission from ruminants is needed to decrease the environmental impact of ruminant animal production. Different plant materials and chemicals have been tested, but few are both effective and practical. Medicinal herbs contain biological compounds and antimicrobials that may be effective in lowering the CH_4 production. However, few studies have systematically evaluated medicinal herbs for their effect on CH_4 production or on the rumen microbiota. In this study, extracts from 100 medicinal herbs were assessed for their ability to decrease CH_4 production by rumen microbiota in vitro. The extracts of 12 herbs effectively lowered the CH_4 production, with the extract of Perilla frutescens seeds being the most effective. The major components of P. frutescens seed extract were identified, and the effects of the extract on the fermentation characteristics and populations of rumen methanogens, fungi, protozoa, and select bacteria were also assessed. The decreased CH_4 production induced by the P. frutescens seed extract was accompanied by an increased abundance of Ruminobacter, Selenomonas, Succinivibrio, Shuttleworthis, Pseudobutyrivbrio, Anaerovibrio, and Roseomonas and a decreased abundance of Methanobrevibacter millerae. The abundance of Pedobacter, Anaeroplasma, Paludibacter, Ruminococcus, and unclassified Lachnospiraceae was positively correlated with the CH_4 production, with no effects on volatile fatty acids. This study suggests that medicinal herbs may be used to mitigate the CH_4 emission from ruminants.

Xu W, Guan R, Lu Y et al Therapeutic effect of polysaccharide fraction of Atractylodis macrocephalae Koidz. in bovine subclinical mastitis. BMC Veterinary Research (2015) 11:165

Mastitis is considered the most significant and persistent disease in dairy cows, bringing about large economic losses. Subclinical mastitis brings about major cost implications, for it is difficult to detect due to absence of any visible indications and can persist in the mammary tissue throughout lactation. Immunomodulators have been widely used to reduce intramammary infections by modulating bovine mammary gland. Atractylodis macrocephalae Koidz. polysaccharides (RAMP), extracted from herbal medicine, has been used widely especially for its immunomodulatory function for many years. The objective of this study was to estimate an oil emulsified Atractylodis macrocephalae Koidz. polysaccharides (RAMP-O) as a potential therapeutic agent to treat subclinical mastitis by subcutaneous injection of RAMP-O in the area of supramammary lymph node in lactating cows via analysis of SCC, IMIs and injection of RAMP-O in the area of supramammary lymph node significantly reduced milk SCC and NAGase activity compared with control. The quarters with bacterial infection were also progressively reduced in RAMP-O treated cows and only 9 quarters were found to have bacterial infection, while no obvious change was found in the control group. Subcutaneous injection of RAMP-O in the area of supramammary lymph node had therapeutic value in the treatment of bovine subclinical mastitis by reducing SCC, NAGase and IMIs in milk. Considering both the therapeutic effect and the cost of RAMP-O, 32 mg per dose was found most suitable to reduce milk SCC and NAGase. Therefore, RAMP-O deserves further study for its use in treatment of bovine mastitis.

Baravalle C, Silvestrini P, Cadoche MC, Beccaria C, Andreotti CS, Renna MS, Pereyra EA, Ortega HH, Calvinho LF, Dallard BE. Intramammary infusion of Panax ginseng extract in bovine mammary gland at cessation of milking induces changes in the expression of toll-like receptors, MyD88 and NF-kB during early involution. Res Vet Sci. 2015 Jun;100:52-60.

The purposes of this study were to explore TLR2 and TLR4 participation and MyD88 and NF-κB activation in bovine mammary glands (BMG) treated with Panax ginseng (PG) at involution and verify the effect of PG in the cytokine expression. Quarters were infused at the end of lactation with PG solution (3 mg/ml), placebo or kept as uninoculated controls. Cows were slaughtered at 7 d after cessation of milking and mammary tissue

samples were taken. A significant increase of TLR2, TLR4, MyD88, NF-κB, IL-1β, IL-6 and TGF-β1 mRNA expression was observed in PG-treated quarters. Immunostaining of TLR2 and TLR4 was significantly higher in PG mammary tissues. The percentages of immunopositive cells for NF-κB-p65 were significantly higher in PG-treated quarters. The BMG responded to PG extract components possibly by TLR2 and TLR4 signaling pathway. These results provide an insight into potential mechanisms by which PG stimulates innate immunity during BMG involution.

Mordmuang A, Shankar S, Chethanond U, Voravuthikunchai SP. Effects of *Rhodomyrtus tomentosa* Leaf Extract on Staphylococcal Adhesion and Invasion in Bovine Udder Epidermal Tissue Model. Nutrients. 2015 Oct 15;7(10):8503-17.

Bovine mastitis is one of the most important infectious diseases in dairy herds, and staphylococci are the most important etiologic agents of this disease. Antibiotics and chemical agents used in livestock for prevention and cure of the disease can accumulate in milk and give rise to food safety concerns. Rhodomyrtus tomentosa leaf extract was studied as an alternative approach to reduce the bacterial infections. The ethanolic extract of this plant demonstrated antibacterial activity with minimum inhibitory concentration (MIC) values as low as 16-64 µg/mL against staphylococcal isolates. In addition, the extract had an effect on the bacterial cell surface properties by increasing its hydrophobicity in a concentration dependent manner. To further extend the antibacterial efficacy, silver nanoparticles synthesized with the extract, a pure rhodomyrtone, and liposomal encapsulated rhodomyrtone were applied and their inhibitory effects on bacterial adhesion and invasion were determined by ex vivo study in a bovine udder epidermal tissue model. These agents exerted remarkable antibacterial activity against staphylococci and decreased the adhesion of the bacterial cells to the tissues. These results supported that R. tomentosa ethanolic extract could be applied as an alternative agent for bovine udder care in dairy farms.

Chen X, Shang F, Meng Y, Li L, Cui Y, Zhang M, Qi K, Xue T. Ethanol extract of Sanguisorba officinalis L. inhibits biofilm formation of methicillin-resistant Staphylococcus aureus in an ica-dependent manner. J Dairy Sci. 2015 Dec;98(12):8486-91

Methicillin-resistant Staphylococcus aureus (MRSA) is an important nosocomial pathogen that shows resistance to many antibiotics and is usually associated with serious infections. Having the ability for biofilm formation increases resistance to antibiotics. Sanguisorba officinalis L. is a perennial plant that is distributed in the northern districts of China and has been used as a traditional Chinese medicine. In this study, the effect of S. officinalis on MRSA strain SA3 isolated from a dairy cow with mastitis was evaluated by testing the growth and biofilm formation ability of MRSA cultured with or without ethanol extracts of S. officinalis. The results showed that the ethanol extract of S. officinalis strongly inhibited the biofilm formation of MRSA. With a confocal laser scanning microscope system, we observed that the biofilm structure of the test group with the addition of S. officinalis appeared looser and had less biomass compared with the control group without S. officinalis. Furthermore, we found that the transcript levels of the icaADBC operon remarkably decreased upon addition of the ethanol extract of S. officinalis, indicating that S. officinalis inhibits biofilm formation of MRSA in an ica-dependent manner.

Mordmuang A, Voravuthikunchai SP. Rhodomyrtus tomentosa (Aiton) Hassk. leaf extract: An alternative approach for the treatment of staphylococcal bovine mastitis. Res Vet Sci. 2015 Oct;102:242-6.

Antibiotic residues in dairy products as well as emergence of antimicrobial resistance in foodborne pathogens have been recognized as global public health concerns. The present work was aimed to study a potent antibacterial extract from natural product as an alternative treatment for staphylococcal bovine mastitis. Staphylococcal isolates (n=44) were isolated from milk samples freshly squeezed from individual cows. All staphylococcal isolates were resistant to ampicillin, ciprofloxacin, erythromycin, gentamicin, penicillin, except vancomycin. Rhodomyrtus tomentosa leaf ethanolic extract was accessed for its antibacterial activity and anti-inflammatory potential. The extract exhibited profound antibacterial activity against all of staphylococcal isolates with MIC and MBC values ranged from 16-64 µg/ml and 64->128 µg/ml, respectively. Moreover, the extract also exerted anti-protein denaturation and human red blood cell membrane stabilizing activity. The

results support the use of R. tomentosa extract that could be applied to cure bovine mastitis and to reduce inflammatory injury caused by the bacterial infections.

Zhou X, Yang C, Li Y, Liu X, Wang Y. Potential of berberine to enhance antimicrobial activity of commonly used antibiotics for dairy cow mastitis caused by multiple drug-resistant Staphylococcus epidermidis infection. Genet Mol Res. 2015 Aug 19;14(3):9683-92.

Berberine is a plant alkaloid with antimicrobial activity against a variety of microorganisms. In this study, the antimicrobial properties of berberine against multi-drug resistant field isolates of Staphylococcus epidermidis were investigated using berberine alone or in combination with a commonly used antibiotics in veterinary clinics, including penicillin, lincomycin, and amoxicillin. The results indicated that the minimum inhibitory concentrations of berberine, penicillin, lincomycin, and amoxicillin against field S. epidermidis isolates were 2-512, 0.8-213, 0.4-1024, and 0.4-256 mg/mL, respectively. Furthermore, the synergistic effects of antimicrobial activity against these multi-drug resistant isolates were observed when the berberine was combined with penicillin, lincomycin, or amoxicillin; no antagonistic effect of the combination was detected in any of the clinical isolates. These observations were further confirmed using a time-killing assay, in which a combination of 2 agents yielded a greater than 2.03-2.44 log10 decrease in colony-forming unit/mL compared with each agent alone. These findings suggest that berberine is a promising compound for preventing and treating multi-drug resistant S. epidermidis infected mastitis in dairy cows either alone or in combination with other commonly used antibiotics, such as penicillin, lincomycin, and amoxicillin.

Budri PE, Silva NC, Bonsaglia EC etal Effect of essential oils of Syzygium aromaticum and Cinnamomum zeylanicum and their major components on biofilm production in Staphylococcus aureus strains isolated from milk of cows with mastitis. J Dairy Sci. 2015 Sep;98(9):5899-904.

Bovine mastitis is an inflammation of the mammary glands of cows and causes significant economic losses in dairy cattle. Staphylococcus aureus is one of the microorganisms most commonly isolated. Novel agents are required in agricultural industries to prevent the development of mastitis. The production of biofilm by Staph. aureus facilitates the adhesion of bacteria to solid surfaces and contributes to the transmission and maintenance of these bacteria. The effect of the essential oils of Syzygium aromaticum (clove; EOSA) and Cinnamomum zeylanicum (cinnamon; EOCZ) and their major components, eugenol and cinnamaldehyde, on Staph. aureus biofilm formation on different surfaces was investigated. The results showed a significant inhibition of biofilm production by EOSA on polystyrene and stainless steel surfaces (69.4 and 63.6%, respectively). However, its major component, eugenol, was less effective on polystyrene and stainless steel (52.8 and 19.6%, respectively). Both EOCZ and its major component, cinnamaldehyde, significantly reduced biofilm formation on polystyrene (74.7 and 69.6%, respectively) and on stainless steel surfaces (45.3 and 44.9%, respectively). These findings suggest that EOSA, EOCZ, and cinnamaldehyde may be considered for applications such as sanitization in the food industry.

Cui D, Wang S, Wang L, Wang H, Liu Y Prophylactic strategy with herbal remedy to reduce puerperal metritis risk in dairy cows: A randomized clinical trial LivestockScience181(2015)231–235

Puerperal metritis is an important disorder usually within 21 days postpartum in dairy cattle that occurs within 21 days postpartum, and herbal remedies are believed to be beneficial for post partum female livestock. Sheng HuaTang is a prime example of herbal formula used as a therapeutic aid in prevention or control of post partum disease for centuries in China. In the present study, we were to evaluate the efficacy of Sheng HuaTang as a prophylactic strategy for lowering puerperal metritis risks and improving reproductive performance in dairy cows under field conditions. A total of 311 clinically healthy cows were randomly allocated to the intervention group or the control group 2–4 h after delivery. Treated cows (n=158) received Sheng HuaTang with an oral dose of 0.36g crude herb/kg bw once daily for three consecutive days, whereas the controls (n=153) received no treatment. The logistic regression and survival analysis were used to analyse the incidence of puerperal metritis and reproduction parameters of cows between the two groups, respectively. The results showed that there was a significant reduction in the incidence of puerperal metritis (12.1%vs.33.3%, P=0.01, odd ratio [OR] 2.392) between Sheng Hua Tang group and the control group. The calving-to-first-service

interval (68.9717.7daysvs.80.5726.6 days, Po0.05) and service per conception(1.7vs.2.1, Po0.01) were lower in cows in Sheng HuaTang group than the controls. Additionally, Sheng HuaTang treatment effectively elevated the first AI conception proportion(61.1%vs.51.3%, Po0.05) and proportion of cows that were pregnant at 305 days in milk (89.8%vs.82.0%, Po0.01)compared with that of controls. The present results would support efforts to the use of Sheng HuaTang immediately after delivery as a prophylactic strategy for lowering puerperal metritis risk and improving the overall reproductive efficiency of dairy herds under these study circumstances. Thus, Sheng HuaTang treatment could represent an effective prophylactic strategy for bovine post partum care.

Jamra N[1], Das G[2], Singh P[1], Haque M[1]. Anthelmintic efficacy of crude neem (Azadirachta indica) leaf powder against bovine strongylosis. J Parasit Dis. 2015 Dec;39(4):786-8.

The present study was conducted to evaluate the anthelmintic efficacy of crude neem (Azadirachta indica) leaf powder against strongyle infections in cattle. Based on copro-examination, 30 cattle positive for strongyle infection with at least 250 [eggs per gram (EPG) of faeces] were selected and grouped as A, B and C (10 animals/group). Group A and B were treated respectively with fendendazole and neem leaf powder @ 5 and 500 mg/kg body weight, whereas Group C served as infected untreated control. Faecal sample from each animal of these groups was examined on day 0, 7, 14 and 28 post treatments and EPG was determined. The result showed significant decrease ($p < 0.05$) in EPG in Group A and B after day 7 post treatment but there was no significant variation in terms of EPG in control group. Thus it can be concluded that crude neem leaf powder has anthelmintic property and it can further be studied to isolate the active component to produce herbal anthelminthics.

Ravva SV[1], Korn A[2]. Effect of Neem (Azadirachta indica) on the Survival of Escherichia coli O157:H7 in Dairy Manure. Int J Environ Res Public Health. 2015 Jul 10;12(7):7794-803.

Escherichia coli O157:H7 (EcO157) shed in cattle manure can survive for extended periods of time and intervention strategies to control this pathogen at the source are critical as produce crops are often grown in proximity to animal raising operations. This study evaluated whether neem (Azadirachta indica), known for its antimicrobial and insecticidal properties, can be used to amend manure to control EcO157. The influence of neem materials (leaf, bark, and oil) on the survival of an apple juice outbreak strain of EcO157 in dairy manure was monitored. Neem leaf and bark supplements eliminated the pathogen in less than 10 d with a D-value (days for 90% elimination) of 1.3 d. In contrast, nearly 4 log CFU EcO157/g remained after 10 d in neem-free manure control. The ethyl acetate extractable fraction of neem leaves was inhibitory to the growth of EcO157 in LB broth. Azadirachtin, a neem product with insect antifeedant properties, failed to inhibit EcO157. Application of inexpensive neem supplements to control pathogens in manure and possibly in produce fields may be an option for controlling the transfer of foodborne pathogens from farm to fork.

Mullen KA, Lee AR, Lyman RL, Mason SE, Washburn SP, Anderson KL. Short communication: an in vitro assessment of the antibacterial activity of plant-derived oils. J Dairy Sci. 2014 Sep;97(9):5587-91

Nonantibiotic treatments for mastitis are needed in organic dairy herds. Plant-derived oils may be useful but efficacy and potential mechanisms of action of such oils in mastitis therapy have not been well documented. The objective of the current study was to evaluate the antibacterial activity of the plant-derived oil components of Phyto-Mast (Bovinity Health LLC, Narvon, PA), an herbal intramammary product, against 3 mastitis-causing pathogens: Staphylococcus aureus, Staphylococcus chromogenes, and Streptococcus uberis. Plant-derived oils evaluated were Thymus vulgaris (thyme), Gaultheria procumbens (wintergreen), Glycyrrhiza uralensis (Chinese licorice), Angelica sinensis, and Angelica dahurica. Broth dilution testing according to standard protocol was performed using ultrapasteurized whole milk instead of broth. Controls included milk only (negative control), milk + bacteria (positive control), and milk + bacteria + penicillin-streptomycin (antibiotic control, at 1 and 5% concentrations). Essential oil of thyme was tested by itself and not in combination with other oils because of its known antibacterial activity. The other plant-derived oils were tested alone and in combination for a total of 15 treatments, each replicated 3 times and tested at 0.5, 1, 2, and 4% to simulate concentrations potentially achievable in the milk within the pre-dry-off udder quarter. Thyme oil at concentrations ≥2% completely inhibited bacterial growth in all replications. Other plant-derived

oils tested alone or in various combinations were not consistently antibacterial and did not show typical dose-response effects. Only thyme essential oil had consistent antibacterial activity against the 3 mastitis-causing organisms tested in vitro. Further evaluation of physiological effects of thyme oil in various preparations on mammary tissue is recommended to determine potential suitability for mastitis therapy.

Mullen KA, Anderson KL, Washburn SP. Effect of 2 herbal intra-mammary products on milk quantity and quality compared with conventional and no dry cow therapy. J Dairy Sci. 2014;97(6):3509-22.

Dry cow therapy, administered at the end of lactation, is aimed at eliminating current and preventing future intramammary (IMM) bacterial infections and typically involves intramammary administration of antibiotics. Certified organic dairies in the United States are restricted from using antibiotics and must consider an alternative therapy or no dry cow therapy. The current study compared 2 herbal products to conventional dry cow therapy and no treatment for a total of 5 treatments over 2 trials. Trial 1 was conducted over 3 yr on 1 research farm and trial 2 included 4 commercial farms plus the research herd over 2 yr. Treatments included (1) a conventional IMM antibiotic and internal teat sealant (penicillin-dihydrostreptomycin and bismuth subnitrate; CON); (2) an herbal IMM product purported to act as a teat sealant (Cinnatube, New AgriTech Enterprises, Locke, NY; CIN); (3) an herbal IMM product (Phyto-Mast, Bovinity Health LLC, Narvon, PA; P-M); (4) Phyto-Mast and Cinnatube (PC); or (5) no dry cow therapy (NT). Each treatment group was balanced by breed, lactation number, due date, herd, and year. However, the CON treatment was used only in the research herd because of the intent to avoid antibiotic usage on the other 4 farms. Comparisons among treatments included the difference between pre- and posttreatment 305-d mature equivalent milk production (trial 1), somatic cell score change from dry-off to freshening at the cow and quarter levels (trials 1 and 2), and milk microbiology change over the dry period (trial 2). We detected no significant differences among treatments for milk yield differences between the lactation following treatment and the lactation preceding treatment. Changes in somatic cell score from one lactation to the next also did not differ significantly among treatments in either trial. Cure rates were not significantly different among treatments; only 19.6% of all quarters were infected at dry off. The proportion of quarters with new infections at 3 to 5d postcalving did not significantly differ among treatments, except between CIN and NT. Percentages (least squares means ± standard error) of quarters with new infections were 24 ± 21% for CON, 15 ± 7% for CIN, 30 ± 10% for P-M, 32 ± 11% for PC, and 35 ± 11% for NT. The efficacy of the herbal products was similar to that of conventional therapy, and the herbal products had no apparent adverse effects.

Cui D, Li J, Wang X, Xie J, Zhang K, Wang X, Zhang J, Wang L, Qin Z, Yang Z. Efficacy of herbal tincture as treatment option for retained placenta in dairy cows. Anim Reprod Sci. 2014 Feb;145(1-2):23-8.

Retained placenta remains therapeutic challenge in cattle. Certain traditional medicines are believed to be able to alleviate retained placenta condition and improve overall fertility in cows. The aim of the present study was to evaluate the efficacy of an herbal tincture for treatment of retained placenta. The herbal tincture was extracted from a combination of Herba Leonuri, Angelicae Sinensis Radix, Flos Carthami, Myrrha and Rhizoma Cyperi by percolation with 70% ethanol to a concentration of 0.5g crude herb/ml. Cows diagnosed with retained placenta (n=48) were randomly divided into one of two treatment groups (A and B), with animals in group A (n=26) receiving herbal tincture orally, and cows in group B (n=22) receiving oxytetracycline infusion into the uterus. Eighty six cows with no clinically visible pathological conditions, given birth alone and with no retained placenta diagnosis were included into control group (C). Retained placenta was expelled within 72h following initial treatment in 19 cows in group A, yet no cows in group B were recorded to expel placenta in the same time. The median number of days to first service (70.0 vs. 102.5 days; $P<0.05$) and median number of days open (76.0 vs. 134.0 days; $P<0.01$) were lower in group A than in group B. Percentage of cows pregnant within 100 days postpartum was the highest for animals in group A compared to controls (61.5% vs. 39.5%, $P<0.05$), and for animals in group B (61.5% vs. 22.7%; $P<0.01$). Herbal tincture used in the present study might facilitate expulsion of retained placenta and improve subsequent fertility, thus could present effective treatment option for retained placenta in cows.

Pinedo P, Karreman H, Bothe H, Velez J, Risco C. Efficacy of a botanical preparation for the intramammary treatment of clinical mastitis on an organic dairy farm. Can Vet J. 2013 May;54(5):479-84.

The objective was to evaluate the efficacy of a botanical product (PHYTO-MAST(®)) for the intra-mammary treatment of clinical mastitis (CM) in dairy cows managed in an organic system. The study involved 194 naturally occurring cases of clinical mastitis. Treatment was applied every 12 hours for 3 days and cows were evaluated for clinical cure starting on day 4. Outcomes of interest consisted of mastitis resolution at day 4, time to resolution, somatic cell score (SCS) after recovery, and bacteriological cure at 14 and 28 d after treatment. There was no significant effect on clinical mastitis resolution at day 4 for treatment compared with the control group. However, there was a faster recovery for the treatment group compared to the control group with median intervals from end of treatment to recovery of 4.6 d and 6.5 d, respectively. There was no effect on the probability of a SCS < 4 (200 000 SC/mL) after treatment. No significant effects were found for treatment on bacteriological cure at days 14 and 28.

Andreotti R, Garcia MV, Cunha RC, Barros JC. Protective action of *Tagetes minuta* (Asteraceae) essential oil in the control of Rhipicephalus microplus (Canestrini, 1887) (Acari: Ixodidae) in a cattle pen trial. Vet Parasitol. 2013 Oct 18;197(1-2):341-5.

The Rhipicephalus microplus tick is globally regarded as the most economically important ectoparasite of livestock, and the evolution of resistance to commercial acaricides among cattle tick populations is of great concern. The essential oil derived from Tagetes minuta may be efficacious against cattle tick infestation, and the results of a cattle pen trial using this essential oil for the control of ticks are reported here. The chemical composition of the essential oil was determined by GC-MS and NMR spectroscopy analyses, which revealed the presence of four major components in the essential oil. These components represent more than 70% of the essential oil: limonene (6.96%), β-ocimene (5.11%), dihydrotagetone (54.10%) and tagetone (6.73%). The results of the cattle pen trial indicated significant differences among the average values of the analyzed biological parameters, including the number of ticks, the average weight of the ticks, the average egg weight per engorged female and larval viability. Treatment with the T. minuta essential oil prepared in this study promoted significant effects on all biological indicators analyzed. Based on the biological indicators, the essential oil showed 99.98% efficacy compared to the control group when used at a 20% concentration. The results obtained in this study suggest that the T. minuta essential oil is a potential R. microplus tick control agent and may be used to mitigate the economic losses caused by tick infestation.

Cui D, Wang X, Wang L, Wang X, Zhang J, Qin Z, Li J, Yang Z. The administration of Sheng Hua Tang immediately after delivery to reduce the incidence of retained placenta in Holstein dairy cows. Theriogenology. 2014 Mar 15;81(5):645-50

Sheng Hua Tang, a classical herbal formula consisting of Radix Angelicae sinensis, Ligustici rhizoma, Semen persicae, Zingiberis rhizoma, and Radix glycyrrhizae, is known to be beneficial in alleviating postpartum diseases and facilitating a return to normal reproductive function. This study investigated whether the administration of Sheng Hua Tang within 2 to 4 hours after delivery was effective as a preventive treatment for reducing the risk of retained placenta in Holstein dairy cows. A total of 357 cows, each of which had delivered its calf spontaneously, were randomly allocated to one of two groups. In the treatment group, the cows (n = 175) received Sheng Hua Tang with an oral dose of 0.36 g crude herb per kg·body weight once daily for three consecutive days. The controls (n = 182) received no treatment. The placental retention proportion was 4.0% and 17.0% within 12 hours after delivery in the treated and control animals, respectively ($P < 0.01$). We found decreases in the calving-to-first-service interval (73.2 ± 25.1 vs. 81.9 ± 32.8 days; $P < 0.01$), calving-to-conception interval (93.4 ± 38.8 vs. 114.6 ± 42.9 days; $P < 0.01$), and service per conception (1.5 ± 0.8 vs. 1.9 ± 1.0 days; $P < 0.01$) in the treatment group compared with the control group. The first artificial insemination conception proportion was higher in the treatment group than in the control group (60.4% vs. 41.1%; $P = 0.01$). Moreover, the between-group difference in the proportion of cows that were pregnant within 180 days postpartum approached statistical significance (88.2% vs. 80.6%; $P = 0.07$). Sheng Hua Tang showed beneficial effects in reducing the incidence of retained placenta and improving subsequent reproductive performance in cows. This preventive treatment strategy would be effective in improving the management of puerperal health. The potential benefits of Sheng Hua Tang warrant further investigation to determine whether this preventive treatment strategy can be endorsed as a general preventive approach in postpartum cows.

Taga I, Lan CQ, Altosaar I. Plant essential oils and mastitis disease: their potential inhibitory effects on pro-inflammatory cytokine production in response to bacteria related inflammation.Nat Prod Commun. 2012 May;7(5):675-82. Review.

This paper highlights the role of plant volatile organic compounds, found in essential oils, for the treatment of bacteria related inflammation. This report is focused on tea tree oil, particularly its main compound terpinen-4-ol. Analysis of the published literature shows that many essential oils have significant antibacterial, antifungal and anti-inflammatory effects. Some of their major components, such as terpinen-4-ol, act by inhibiting pro-inflammatory cytokine expression while stimulating production of anti-inflammatory cytokines. Such observations may be exploited to encourage biotherapy against mastitis. The use of synthetic antibiotics is being increasingly discouraged because their presence in dairy milk may have potential downstream effects on population health and the agri-food chain. In the context of inflammation and related mammalian responses, understanding the interplay between volatile organic compounds, especially terpinen-4-ol, and cytokines during bacteria related inflammation should clarify their mode of action to control mastitis.

Baravalle C, Dallard BE, Cadoche MC, Pereyra EA, Neder VE, Ortega HH, Calvinho LF.Proinflammatory cytokines and CD14 expression in mammary tissue of cows following intramammary inoculation of Panax ginseng at drying off. Vet Immunol Immunopathol. 2011 Nov 15;144(1-2):52-60.

The lack of efficacy of conventional strategies for the maintenance of healthy udders in domestic cattle has prompted studies on the use of immunomodulators or biological response modifiers (BRM) for this purpose. These compounds are agents that modify the host's response to pathogens leading to beneficial effects on disease outcome. The objective of this study was to evaluate the effects of a single intramammary infusion of Panax ginseng (GS) extract on the amount of pro-inflammatory cytokines and the number of monocytes/macrophages present in bovine mammary tissues at drying off. Eight mammary quarters from six nonpregnant cows in late lactation were infused with 10 mL of GS (3mg/mL), six quarters were treated with 10 mL of placebo (vehicle alone) and six quarters were maintained as uninoculated controls. The analyses of tumor necrosis factor-alpha (TNF-α) by immunohistochemistry revealed that the production of this proinflammatory cytokine significantly increased ($P<0.05$) in the inoculated mammary glands of cows following BRM inoculation, whereas the interleukin-1 alpha (IL-1α) and IL-6 staining area was not affected by BRM treatment. The number of monocytes/macrophages detected with CD14 antibody was significantly higher ($P<0.05$) in BRM-treated quarters than in placebo and uninoculated control quarters. These results indicated an immunomodulator potential of the BRM used. The beneficial effect of the extract could be used as alternative therapy in the control of mastitis at drying off, either alone or in conjunction with dry cow antibiotic therapy.

Ghodrati H etal. Effect of Intramammary Injection of *Nigella Sativa* on Somatic Cell Count and *Staphylococcus Aureus* Count in Holstein Cows with *S. aureus* Subclinical Mastitis American Journal of Animal and Veterinary Sciences 6 (1): 31-34, 2011

The seeds of *Nigella sativa* Linn. (Ranunculaceae) known as black seed or black cumin, are used in herbal medicine all over the world for the treatment and prevention of a number of diseases and conditions that include asthma, diarrhea and dyslipidaemia.
In this study the effect of intramammary injection of *Nigella Sativa* Extract (NSE) in paraffin on quarter milk, quality and Somatic Cell Count (SCC) and the shedding pattern of *Staphylococcus aureus* were investigated. Thirty Holstein cows, naturally infected with *S. aureus* subclinical mastitis, were subjected to treatment with the NSE at a dose of 10 mL in paraffin (200 mg mL−1) per day for 3 days, or with 10 mL paraffin as control. The injection areas were checked for adverse reactions. The daily milk production was measured before and after treatment. Intramammary injection of NSE caused a
remarkable healing. Quarter milk samples were collected for bacteriological analysis and Somatic Cell Counts (SCC). The bacterial count moderately decreased in the treatment group. After the end of the treatment, the numbers of *S. aureus*-infected quarters and milk SCC tended to decrease in the NSEtreated
cows. These clarifications were significantly higher one week post-treatment than pretreatment. Similar changes were not observed in the control group.

The results of the present study showed that the NSE has potential as a therapeutic agent for *S. aureus* infection causing subclinical mastitis of dairy cows and may contribute to the cow's recovery from mastitis. In conclusion, the results indicate that *Nigella sativa* might act as an antibacterial *in vivo* in dairy cows.

McPhee CS, Anderson KL, Yeatts JL, Mason SE, Barlow BM, Baynes RE. Milk and plasma disposition of thymol following intramammary administration of a phytoceutical mastitis treatment. J Dairy Sci. 2011 Apr;94(4):1738-43.

Despite the recent growth of the organic dairy industry, organic producers and veterinarians have limited information when choosing mastitis treatments for animals in organic dairy production. Organic producers commonly administer homeopathic or other plant-based products without having research evaluating the efficacy of these products and using estimated or no withholding times to treat mastitis and other health problems in their herds. In this pilot study, we attempted to identify several active ingredients of Phyto-Mast (Penn Dutch Cow Care, Narvon, PA), a plant-based mastitis treatment used on organic dairy farms, and to quantify the product residue in milk and plasma after intramammary administration. We developed an assay to quantify thymol (one of the active ingredients in Phyto-Mast) in milk and plasma using gas chromatography and mass spectrometry (GC-MS). Thymol is a volatile aromatic compound with antiinflammatory properties. As a model for dairy cows, 5 healthy, lactating alpine dairy goats were given 5 mL of Phyto-Mast per udder half. For 10 d following treatment, we analyzed blood and milk samples for thymol residues using GC-MS. The GC-MS assay was very sensitive for thymol detection, to a concentration of 0.01 µg/mL in plasma. Using thymol as a marker, Phyto-Mast was detectable and quantifiable in plasma beginning with the 15-min posttreatment sample, but was no longer detectable in the 4-h posttreatment sample. Thymol residues were only detected in the 12-h posttreatment milk sample. An inflammatory response was not evident in the udder following phytoceutical administration. Although this study provides information about the elimination of thymol, the product contains several other active chemicals, which may have different pharmacokinetic behaviors. Further analysis and additional study animals will help to determine a milk withholding time for Phyto-Mast. Given the recent growth of the organic dairy industry, understanding the pharmacokinetics of therapeutics used in organic production and developing accurate withholding recommendations will help to ensure milk safety.

L. Pan et al. Effects of Radix Bupleuri extract supplementation on lactationperformance and rumen fermentation in heat-stressed lactating Holstein cows Animal Feed Science and Technology *187 (2014) 1– 8*

Radix Bupleuri extract (RBE) has been shown to mitigate negative effects of high ambient temperature. This experiment was conducted to investigate effects of RBE supplementation on lactation performance and rumen fermentation in Holstein cows under heatstress. Forty Holstein cows (75 ± 15 d in milk, 37.5 ± 1.8 kg of milk/d, and 1.7 ± 0.4 parity) were randomly assigned to one of four groups (n = 10). One of four treatment diets, assigned randomly to one of four groups, consisted of RBE supplementation at 0, 0.25,0.5 or 1.0 g/kg of the basal diet (concentrate and roughage) based on dry matter (DM).Cows were housed in a tie-stall barn and were individually fed the treatment diets. The experiment lasted for 10 wk in hot summer. During the experiment, average ambient temperatures and temperature-humidity indexes (THI) were respectively 27.5 ± 1.5, 29.8 ± 1.9and 28.1 ± 1.7◦C, and 78.2 ± 2.7, 79.8 ± 3.3 and 78.3 ± 3.4 at 0600, 1400 and 2200 h. Aver-age respiration rates (RR) with RBE at 0.25, 0.50 and 1.0 g/kg were 65.6, 60.3 and 67.4, respectively, vs. 71.4 (breaths/min) for the control ($P < 0.01$). Average rectal temperatures (RT) were 39.1, 39.0 and 39.1 vs. 39.3◦C for the control ($P < 0.01$). Moreover, cows supplemented with RBE increased dry matter intake (DMI, 22.8, 21.6 and 22.1 vs. 20.9 kg/d)($P < 0.05$) and milk production (34.2, 33.4 and 32.4 vs. 31.6 kg/d) ($P < 0.01$) compared with control. Percentages of milk protein and fat were similar among groups, while milk protein yield increased with increasing level of RBE (0.97, 0.95 and 0.92 vs. 0.89 kg/d for the control)($P < 0.01$). Milk fat yield also increased with RBE (1.13, 1.12 and 1.09 vs. 1.02 kg/d for the control) ($P < 0.05$). There was no treatment effect on diet apparent digestibility or volatile fatty acid (VFA) concentration among groups. Overall, supplemental RBE at 0.25 or 0.5 g/kg could mitigate the negative effects of heat stress on production in lactating Holstein cows.

Qiao G etal Effects of supplemental Chinese herbs on growth performance, blood antioxidant function and immunity status in Holstein dairy heifers fed high fibre diet Italian Journal of Animal Science 2013; volume 12:e20

Two experiments were carried out to investigate the effects of supplemental Chinese herbs, *Fructus Ligustri Lucidi* (FLL), *Radix Astragali* (RA) and *Radix Codonopsis* (RC) on growth performance, blood antioxidant and immune function in Holstein dairy heifers fed high fibre diet. Experiment 1 indicated that the supplementation of the three herbs had no effect on dry matter intake. FLL Supplementation increased heifers average daily gain (ADG), final body weight and feed efficiency. Experiment 2 indicated that FLL supplementation improved the blood antioxidant function with higher concentration of superoxide dismutase (SOD) and lower concentration of malondialdehyde (MDA), and improved immune function with lower concentrations of prostaglandin E2 (PGE2) and immunoreactive fibronectin (IFN-). Addition of FLL increased apparent digestibility of diet's dry matter and organic matter than the other groups. It was demonstrated that FLL supplementation improved nutrient digestion, feed efficiency, blood antioxidant function, immune and growth performance for Holstein dairy heifers.

Hashemzadeh-Cigari F, Khorvash M, Ghorbani GR etal. Effects of supplementation with a phytobiotics-rich herbal mixture on performance, udder health, and metabolic status of Holstein cows with various levels of milk somatic cell counts. J Dairy Sci. 2014 Dec;97(12):7487-97.

This study evaluated the effects of dietary supplementation of a novel phytobiotics-rich herbal mixture (PRHM) on feed intake, performance, udder health, ruminal fermentation, and plasma metabolites in cows with moderate or high somatic cell counts (SCC) in the milk. Twenty-four Holstein dairy cows (117 ± 26 d in milk and 46.3 ± 4.7 kg of milk/d at the start of the experiment) were blocked by parity and days in milk and split into 2 groups, based on SCC in the milk; 12 cows were with moderate SCC (260,000<SCC <500,000 cells/mL), whereas 12 other cows had high levels of SCC (>500,000 cells/mL) in the milk. Within each SCC group, cows were blocked by milk yield and parity, and were randomly assigned to 2 different feeding regimens. Half of the cows in each SCC group (n=6) were supplemented with PRHM (185 g/cow per day, providing 12.4 g of phenolic compounds per day), and the other half (n=6) were not supplemented in their diets. The experiment lasted 36 d, whereby the first 24 d were used for adaptation to the diets and the last 12 d for sampling. Data showed that supplementation of PRHM decreased somatic cell score in the milk, indicating improved udder health of cows with high initial SCC, but not in cows with moderate SCC. Also, cows supplemented with PRHM consumed more feed DM, produced greater amounts of milk, and showed an improvement of feed utilization efficiency. However, these cows also lost more back-fat thickness during the experiment. Supplementation of PRHM increased fat- and energy-corrected milk yields in cows with high initial SCC, but not in cows with moderate SCC. Supplementation of PRHM decreased milk fat content, whereas other milk components were not affected by PRHM feeding. The PRHM supplementation decreased the acetate-to-propionate ratio in the rumen fluid, but increased β-hydroxybutyrate and cholesterol concentration in the plasma, irrespective of the initial SCC level in the milk. Other plasma metabolites and liver enzymes were not affected by PRHM supplementation. Apparent nutrient digestibility did not differ among treatments. Overall, supplementation of PRHM seems to be an effective strategy to enhance performance and lower SCC, particularly in cows having high SCC levels in the milk. Further research is warranted to evaluate long-term effects of PRHM supplementation, especially with regard to metabolic health status and reproduction.

Schäfer M, Sharp P, Brooks VJ, Xu J, Cai J, Keuler NS, Peek SF, Godbee RG, Schultz RD, Darien BJ. Enhanced bactericidal activity against Escherichia coli in calves fed Morinda citrifolia (Noni) puree. J Vet Intern Med. 2008 Mar-Apr;22(2):499-502.

Although adequate colostrum intake and properly used antibiotics can provide much protection for the bovine neonate, increased antibiotic scrutiny and consumer demand for organic products have prompted investigations of natural immunomodulators for enhancing calf health. One plant-based immunomodulator, Morinda citrifolia (noni) fruit, is a well-recognized natural product that has a broad range of immunomodulatory effects. The hypothesis was that Neonatal calves fed noni puree would demonstrate whole blood phagocytic capacity in Gram-negative and Gram-positive in vitro assays. Blood samples were taken from 18 neonatal Holstein bull calves. Calves were divided into 2 groups: Group 1 comprised control calves, whereas Group 2 received 30 mL of noni puree twice a day in milk replacer. Day 0 blood samples were obtained between 36 and 48 hours of age before the first feeding of puree. Ethylenediaminetetraacetic acid anticoagulated blood was collected from each calf on days 0, 3, 7, and 14. Bactericidal assays were performed to estimate the percentage killing of Escherichia coli and Staphylococcus epidermidis. Blood samples from noni puree-fed calves displayed significantly more E. coli bacterial killing than did controls on day 14, and although

differences were not significant on days 0, 3, and 7, bacterial killing progressively increased over time. There was no significant difference between the groups for S. epidermidis killing. The immunomodulatory effect of noni puree may prove valuable in the future as production animal antibiotic use becomes more restricted. Additional clinical trials are warranted to investigate the clinical application of noni puree in promoting calf health.

Garcia M, Elsasser TH, Biswas D, Moyes KM.The effect of citrus-derived oil on bovine blood neutrophil function and gene expression in vitro.J Dairy Sci. 2015 Feb;98(2):918-26.

Fratini F, Casella S, Leonardi M, Pisseri F, Ebani VV, Pistelli L, Pistelli L. Antibacterial activity of essential oils, their blends and mixtures of their main constituents against some strains supporting livestock mastitis. Fitoterapia. 2014 Jul;96:1-7.

Fonseca AP, Estrela FT, Moraes TS, Carneiro LJ, Bastos JK, dos Santos RA, Ambrósio SR, Martins CH, Veneziani RC. In vitro antimicrobial activity of plant-derived diterpenes against bovine mastitis bacteria. Molecules. 2013 Jul 4;18(7):7865-72.

Kaithwas G, Mukerjee A, Kumar P, Majumdar DK. Linum usitatissimum (linseed/flaxseed) fixed oil: antimicrobial activity and efficacy in bovine mastitis. Inflammopharmacology. 2011 Feb;19(1):45-52.

Mukherjee R, De UK, Ram GC.Evaluation of mammary gland immunity and therapeutic potential of Tinospora cordifolia against bovine subclinical mastitis. Trop Anim Health Prod. 2010 Apr;42(4):645-51.

Ananda Baskaran S, Kazmer GW, Hinckley L, Andrew SM, Venkitanarayanan K. Antibacterial effect of plant-derived antimicrobials on major bacterial mastitis pathogens in vitro. J Dairy Sci. 2009 Apr;92(4):1423-9.

IX-D Veterinary Botanical Medicine and Equids

Ellse L, Sands B, Burden FA, Wall R. Essential oils in the management of the donkey louse, Bovicola ocellatus. Equine Vet J. 2016 May;48(3):285-9.

Chewing lice are widespread and clinically compromising parasites of livestock and equids. Their management is complicated by growing levels of resistance to commonly applied insecticides. Hence, the development of novel approaches to their control is of major clinical interest. The objectives of the study were to assess the effects of incorporating the essential oils of tea tree and lavender into a grooming programme for populations of donkeys with natural infestations of Bovicola ocellatus in the UK and Ireland when louse populations were at their winter seasonal peak. The study design was an in vivo field trial.Suspensions of 5% (v/v) tea tree or lavender oil or an excipient only control were groomed into the coats of winter-housed donkeys (n = 198) on 2 occasions, 2 weeks apart. Louse counts were conducted before each application and 2 weeks later.After 2 applications, the groups groomed with lavender or tea tree oil suspensions had a significant reduction in louse intensity, with a mean decline in louse abundance of 78% (95% confidence interval 76-80%). Louse numbers in the groups groomed with excipient only either did not change or increased significantly. Donkey hair length had no effect on the decline in louse numbers.These results demonstrate that the inclusion of essential oil suspensions during grooming can be used to manage louse populations successfully.

Peachey LE, Pinchbeck GL, Matthews JB, etal. An evidence-based approach to the evaluation of ethnoveterinary medicines against strongyle nematodes of equids. Vet Parasitol. 2015 May 30;210(1-2):40-52.

Cyathostomins are the most important gastrointestinal nematode infecting equids. Their effective control is currently under threat due to widespread resistance to the broad spectrum anthelmintics licenced for use in equids. In response to similar resistance issues in other helminths, there has been increasing interest in alternative control strategies, such as bioactive plant compounds derived from traditional ethnoveterinary treatments. This study used an evidence-based approach to evaluate the potential use of plant extracts from the UK and Ethiopia to treat cyathostomins. Plants were shortlisted based on findings from a literature review and additionally, in Ethiopia, the results of a participatory rural appraisal (PRA) in the Oromia region of the country. Systematic selection criteria were applied to both groups to identify five Ethiopian and four UK plants for in vitro screening. These included Acacia nilotica (L.) Delile, Cucumis prophetarum L., Rumex abyssinicus Jacq., Vernonia amygdalina Delile. and Withania somnifera (L.) Dunal from Ethiopia and Allium sativum L. (garlic), Artemisia absinthium L., Chenopodium album L. and Zingiber officinale Roscoe. (ginger) from the UK. Plant material was collected, dried and milled prior to hydro-alcoholic extraction. Crude extracts were dissolved in distilled water (dH2O) and dimethyl sulfoxide (DMSO), serially diluted and screened for anthelmintic activity in the larval migration inhibition test (LMIT) and the egg hatch test (EHT). Repeated measures ANOVA was used to identify extracts that had a significant effect on larval migration and/or egg hatch, compared to non-treated controls. The median effective concentration (EC-50) for each extract was calculated using PROBIT analysis. Of the Ethiopian extracts A. nilotica, R. abyssinicus and C. prophetarum showed significant anthelmintic activity. Their lowest EC-50 values were 0.18 (confidence interval (CI): 0.1-0.3), 1.1 (CI 0.2-2.2) and 1.1 (CI 0.9-1.4)mg/ml, respectively. All four UK extracts, A. sativum, C. album, Z. officinale and A. absinthium, showed significant anthelmintic activity. Their lowest EC-50 values were 1.1 (CI 0.9-1.3), 2.3 (CI 1.9-2.7) and 0.3 (CI 0.2-0.4)mg/ml, respectively. Extract of A. absinthium had a relatively low efficacy and the data did not accurately fit a PROBIT model for the dose response relationship, thus an EC-50 value was not calculated. Differences in efficacy for each extract were noted, dependent on the assay and solvent used, highlighting the need for a systematic approach to the evaluation of bioactive plant compounds. This study has identified bioactive plant extracts from the UK and Ethiopia which have potential as anthelmintic forages or feed supplements in equids.

Reisinger N, Schaumberger S, Nagl V, Hessenberger S, Schatzmayr G. Milk thistle extract and silymarin inhibit lipopolysaccharide induced lamellar separation of hoof explants in vitro. Toxins (Basel). 2014 Oct 6;6(10):2962-74.

The pathogenesis of laminitis is not completely identified and the role of endotoxins (lipopolysaccharides, LPS) in this process remains unclear. Phytogenic substances, like milk thistle (MT) and silymarin, are known for their anti-inflammatory and antioxidant properties and might therefore have the potential to counteract endotoxin induced effects on the hoof lamellar tissue. The aim of our study was to investigate the influence of endotoxins on lamellar tissue integrity and to test if MT and silymarin are capable of inhibiting LPS-induced effects in an in vitro/ex vivo model. In preliminary tests, LPS neutralization efficiency of these phytogenics was determined in an in vitro neutralization assay. Furthermore, tissue explants gained from hooves of slaughter horses were tested for lamellar separation after incubation with different concentrations of LPS. By combined incubation of explants with LPS and either Polymyxin B (PMB; positive control), MT or silymarin, the influence of these substances on LPS-induced effects was assessed. In the in vitro neutralization assay, MT and silymarin reduced LPS concentrations by 64% and 75%, respectively, in comparison PMB reduced 98% of the LPS concentration. In hoof explants, LPS led to a concentration dependent separation. Accordantly, separation force was significantly decreased by 10 µg/mL LPS. PMB, MT and silymarin could significantly improve tissue integrity of explants incubated with 10 µg/mL LPS. This study showed that LPS had a negative influence on the structure of hoof explants in vitro. MT and silymarin reduced endotoxin activity and inhibited LPS-induced effects on the lamellar tissue. Hence, MT and silymarin might be used to support the prevention of laminitis and should be further evaluated for this application.

Wilford S, Woodward E, Dunkel B. Owners' perception of the efficacy of Newmarket bloodroot ointment in treating equine sarcoids. Can Vet J. 2014 Jul;55(7):683-6.

A retrospective questionnaire-based survey was used to determine the perceived efficacy of Newmarket bloodroot ointment in treating equine sarcoids. In 49 horses with 74 sarcoids, 64 sarcoids responded either completely (n = 49) or partially (n = 15) while 10 did not respond or worsened. Sarcoids < 2 cm responded better to treatment (P < 0.001) than did larger sarcoids.

Cecchini S[1], Paciolla M[1], Caputo AR[2], Bavoso A[1]. Antioxidant Potential of the Polyherbal Formulation "ImmuPlus": A Nutritional Supplement for Horses. Vet Med Int. 2014;2014:434239. doi: 10.1155/2014/434239. Epub 2014 May 4.

In order to counteract harmful effects of oxidative stress due to pathological conditions or physical exercise, horses are often administered dietary supplements having supposed high antioxidant activities. The aim of the present study was to identify the in vitro antioxidant potential of "ImmuPlus", a polyherbal formulation (Global Herbs LTD, Chichester, West Sussex, Great Britain), containing three medicinal plants (Withania somnifera, Tinospora cordifolia, and Emblica officinalis), known in Ayurveda for their use in human disease treatment. Extracts obtained by different solvents (water, methanol, ethanol, acetone, and hexane) were tested for total antioxidant capacity, total reducing power, scavenging activity against DPPH radical, and total polyphenol and flavonoid contents. Our results showed that, except as regards hexane, all the used solvents are able to extract compounds having high antioxidant activity, even when compared to ascorbic acid. Regression analysis showed significant correlations between antioxidant properties and polyphenol/flavonoid contents, indicating the latter, known for their beneficial effects on health of human and animal beings, as major components responsible for the strong antioxidant capacities. Moreover, obtained results suggest the effective role of the polyherbal mixture as good source of antioxidants in horses.

Ellse L, Burden FA, Wall R. Control of the chewing louse Bovicola (Werneckiella) ocellatus in donkeys, using essential oils. Med Vet Entomol. 2013 Dec;27(4):408-13

Infestations by lice can be a significant clinical and welfare issue in the management of large animals. The limited range of commercial pediculicides available and the development of resistance have led to the need to explore alternative louse management approaches. The results of in vitro and in vivo trials undertaken to control populations of the donkey chewing louse, Bovicola ocellatus (Piaget) (Phthiraptera: Trichodectidae)

using the essential oils of tea tree (Melaleuca alternifolia) and lavender (Lavandula angustifolia) are reported here. Results of contact and vapour bioassays showed that 5% (v/v) tea tree and lavender oils resulted in > 80% louse mortality after 2 h of exposure. On farms, separate groups of 10 donkeys sprayed with 5% (v/v) tea tree and lavender oil as part of their usual grooming regime showed significant reductions in louse numbers compared with a control group (0.2% polysorbate 80 in water). These findings indicate that tea tree and lavender essential oils can provide clinically useful levels of control of B. ocellatus when used as part of a grooming routine and suggest that with further development could form the basis of an easy to apply and valuable component of a louse management programme for donkeys.

Talbot WA, Pinchbeck GL, Knottenbelt DC, Graham H, McKane SA. A randomised, blinded, crossover study to assess the efficacy of a feed supplement in alleviating the clinical signs of headshaking in 32 horses. Equine Vet J. 2013 May;45(3):293-7

Feed supplements are commonly used by owners to alleviate headshaking; however, randomised, controlled trials are required to assess their efficacy. The object of the study was to determine the efficacy of a feed supplement for alleviation of the clinical signs of headshaking using a randomised, blinded, placebo-controlled trial. Using a crossover design, 44 horses previously diagnosed with chronic idiopathic headshaking received both the supplement and a matching placebo per os for 28 days with a washout period between of 14 days. Video recordings were taken at rest and exercise prior to the study and at the end of both periods of treatment. The degree of headshaking was assessed in a blinded, randomised manner by 2 veterinary surgeons. At the same time points, owners completed a questionnaire to assess the severity of headshaking signs. A Wilcoxon signed rank test was used to compare the scores while on supplement and placebo. Using the video assessments, there was no significant difference between scores while on supplement compared with placebo (P = 0.7). Using the questionnaire responses, there was no significant difference between scores for any activity when the placebo and the supplement were compared with each other. However, owners reported significant improvement during all activities for both placebo and supplement compared with pretreatment scores. The supplement offered no benefit over a placebo in alleviating the clinical signs of headshaking. There appeared to be a significant proxy placebo effect when the outcome was based on subjective owner perception of clinical signs. This study demonstrated no beneficial effect of this supplement on the clinical signs of headshaking. The study did show a significant placebo effect, thereby highlighting the necessity of properly conducted, randomised controlled trials, with blinding, to assess true treatment effects in trials in animals.

Hackett ES, Mama KR, Twedt DC, Gustafson DL. Evaluation of antioxidant capacity and inflammatory cytokine gene expression in horses fed silibinin complexed with phospholipid. Am J Vet Res. 2013 Oct;74(10):1333-9.

To evaluate antioxidant capacity and inflammatory cytokine gene expression in horses fed silibinin complexed with phospholipid. 5 healthy horses were orally administered increasing doses of silibinin phospholipid during 4 nonconsecutive weeks (0 mg/kg, 6.5 mg/kg, 13 mg/kg, and 26 mg/kg of body weight, twice daily for 7 days each week). Dose-related changes in plasma antioxidant capacity, peripheral blood cell glutathione concentration and antioxidant enzyme activities, and blood cytokine gene expression were evaluated. Plasma antioxidant capacity increased throughout the study period with increasing dose. Red blood cell nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase I activity decreased significantly with increasing doses of silibinin phospholipid. No significant differences were identified in glutathione peroxidase activity, reduced glutathione or oxidized glutathione concentrations, or expression of tumor necrosis factor α, interleukin-1, or interleukin-2. Minor alterations in antioxidant capacity of healthy horses that consumed silibinin phospholipid occurred and suggest that further study in horses with liver disease is indicated.

Payne SE, Kotze AC, Durmic Z, Vercoe PE. Australian plants show anthelmintic activity toward equine cyathostomins in vitro. Vet Parasitol. 2013 Sep 1;196(1-2):153-60.

Anthelmintic resistance in gastrointestinal parasites of horses is an increasing problem, particularly in cyathostomins, and there is a need to find alternative means for the control of these parasites. We screened crude extracts from 37 species of Australian native plants for their anthelmintic activity in vitro against cyathostomin larvae (development from egg to third larval stage), with the aim of identifying those species that may be suitable for incorporation into sustainable parasite management programs. Water extracts from

seven species, namely Acacia baileyana, Acacia melanoxylon, Acacia podalyriifolia, Alectryon oleifolius, Duboisia hopwoodii, Eucalyptus gomphocephala and Santalum spicatum completely inhibited larval development (100% inhibition compared to the control), while another 10 species caused 90% inhibition at the initial screening concentration of 1400 µg of extractable solids/mL. The seven most potent extracts produced IC50 values (concentration of extract which resulted in a 50% inhibition of development) in the range 30.9-196 µg/mL. Fourteen extracts were incubated with polyvinylpolypyrrolidone (PVPP) before the assays, which removed the anthelmintic activity from 12 of these extracts, indicating that tannins were likely to be the bioactive compound responsible for the effect, while in two species, i.e. A. melanoxylon and D. hopwoodii, compounds other than tannins were likely to be responsible for their anthelmintic action. Our results suggest that a number of Australian native plants have significant anthelmintic activity against cyathostomin larval development in vitro. There is potential for these plants to be used as part of sustainable parasite control programs in horses, although more research is needed to identify the compounds responsible for the anthelmintic effects and confirm their activity in vivo.

Pearson W, Fletcher RS, Kott LS. Oral rosmarinic acid-enhanced Mentha spicata modulates synovial fluid biomarkers of inflammation in horses challenged with intra-articular LPS. J Vet Pharmacol Ther. 2012 Oct;35(5):495-502.

A biological extract of high-rosmarinic acid mint (HRAM) has previously demonstrated inhibitory effects on lipopolysaccharide (LPS)-induced prostaglandin E(2) (PGE(2)), nitric oxide (NO) and glycosaminoglycan (GAG) release in vitro. This study was undertaken to determine whether HRAM added to feed produces similar effects in horses challenged with intra-articular LPS. Eight horses received HRAM (0 or 28.1 ± 1.3 g/day; n = 4 per group) in their feed for 24 days in a blinded manner. On day 21, all horses received an intra-articular injection of LPS (0.3 ng) into their left or right intercarpal joint. Synovial fluid (SF) samples were taken on postinjection day (PID)-21 (i.e. prior to commencement of supplementation), PID0, PID0.25, PID0.5, PID1 and PID3 and analysed for PGE(2), GAG, NO, protein and total nucleated cells counts. Blood biochemistry and haematology screens were conducted at PID-21, PID0, PID1 and PID3. There was a significant reduction in LPS-induced PGE(2) and GAG in SF in horses supplemented with HRAM compared with controls and a tendency to increase complement recognition protein accumulation in synovial fluid of HRAM horses. Plasma from HRAM horses had reduced total white blood cells, segmented neutrophils (compared with baseline concentrations) and lymphocytes (compared with controls), and increased SF nucleated cell count (compared with baseline concentrations and controls). It is concluded that HRAM offered as part of the feed alter biomarkers of inflammation in SF of LPS-challenged horses. Larger studies that seek to clarify effects of HRAM on synovial fluid cell counts and possible role of HRAM-induced interference with complement signalling are warranted.

Hackett ES, Mama KR, Twedt DC, Gustafson DL . Pharmacokinetics and safety of silibinin in horses. Am J Vet Res. 2013 Oct;74(10):1327-32.

To determine the oral bioavailability, single and multidose pharmacokinetics, and safety of silibinin, a milk thistle derivative, in healthy horses, 9 healthy horses were initially administered silibinin IV and silibinin phospholipid orally in feed and via nasogastric tube. Five horses then consumed increasing orally administered doses of silibinin phospholipid during 4 nonconsecutive weeks (0 mg/kg, 6.5 mg/kg, 13 mg/kg, and 26 mg/kg of body weight, twice daily for 7 days each week). Bioavailability of orally administered silibinin phospholipid was 0.6% PO in feed and 2.9% via nasogastric tube. During the multidose phase, silibinin had nonlinear pharmacokinetics. Despite this, silibinin did not accumulate when given twice daily for 7 days at the evaluated doses. Dose-limiting toxicosis was not observed. Silibinin phospholipid was safe, although poorly bio-available, in horses. Further study is indicated in horses with hepatic disease.

Shmalberg J, Xie H. Acupuncture and Chinese herbal medicine for treating horses. Compend Contin Educ Vet. 2011 May;33(5):E1-11.

Acupuncture exerts diffuse analgesic effects through the release of endogenous opioids and other locally and centrally acting mediators. Successful therapeutic interventions for various musculoskeletal conditions in horses are well documented, and acupuncture may significantly enhance performance. The use of acupuncture is specifically supported in treating nonsurgical gastrointestinal disorders, in which specific techniques can alter motility and contribute to visceral analgesia. This article describes the use of acupuncture

and Chinese herbal medicine for equine reproductive management and for treating respiratory disease. A careful review of available data and ongoing efforts to enhance unbiased research should continue to guide practitioners of evidence-based medicine in refining the most useful applications of acupuncture and Chinese herbal medicine.

Tinworth KD, Harris PA, Sillence MN, Noble GK. Potential treatments for insulin resistance in the horse: a comparative multi-species review. Vet J. 2010 Dec;186(3):282-91.

Insulin resistance and hyperinsulinaemia increase the risk of laminitis and horse owners and veterinarians should attempt to enhance insulin sensitivity in at-risk groups. In obese animals this may be achieved, in part, by promoting weight loss and increasing exercise, but such intervention may not be appropriate in non-obese insulin-resistant animals, or where exercise is contra-indicated for clinical reasons. An alternative approach to controlling insulin sensitivity in obese and non-obese horses may be the use of certain herbal compounds that have shown promise in humans and laboratory animals, although little is known of the effects of these compounds in horses. The herbs can be grouped according to their primary mechanism of action, including activators of the peroxisome proliferator-activated receptors, anti-obesity compounds, anti-oxidants, compounds that slow carbohydrate absorption, insulin receptor activators and stimulators of glucose uptake, with some herbs active in more than one pathway. Certain herbs have been prioritised for this review according to the quality and quantity of published studies, the reported (or extrapolated) safety profile, as well as potential for efficacy, all of which will hopefully motivate further research in this field.

Brindley MA, Widrlechner MP, McCoy JA, Murphy P, Hauck C, Rizshsky L, Nikolau B, Maury W. Inhibition of lentivirus replication by aqueous extracts of Prunella vulgaris. Virol J. 2009 Jan 20;6:8.

Various members of the mint family have been used historically in Chinese and Native American medicine. Many of these same family members, including Prunella vulgaris, have been reported to have anti-viral activities. To further characterize the anti-lentiviral activities of P. vulgaris, water and ethanol extractions were tested for their ability to inhibit equine infectious anaemia virus (EIAV) replication. Aqueous extracts contained more anti-viral activity than did ethanol extracts, displaying potent anti-lentiviral activity against virus in cell lines as well as in primary cell cultures with little to no cellular cytotoxicity. Time-of-addition studies demonstrated that the extracts were effective when added during the first four h of the viral life cycle, suggesting that the botanical constituents were targeting the virion itself or early entry events. Further analysis revealed that the extracts did not destroy EIAV virion integrity, but prevented viral particles from binding to the surface of permissive cells. Modest levels of anti-EIAV activity were also detected when the cells were treated with the extracts prior to infection, indicating that anti-EIAV botanical constituents could interact with both viral particles and permissive cells to interfere with infectivity. Size fractionation of the extract demonstrated that eight of the nine fractions generated from aqueous extracts displayed anti-viral activity. Separation of ethanol soluble and insoluble compounds in the eight active fractions revealed that ethanol-soluble constituents were responsible for the anti-viral activity in one fraction whereas ethanol-insoluble constituents were important for the anti-viral activity in two of the other fractions. In three of the five fractions that lost activity upon sub-fractionation, anti-viral activity was restored upon reconstitution of the fractions, indicating that synergistic anti-viral activity is present in several of the fractions. Our findings indicate that multiple Prunella constituents have profound anti-viral activity against EIAV, providing additional evidence of the broad anti-viral abilities of these extracts. The ability of the aqueous extracts to prevent entry of viral particles into permissive cells suggests that these extracts may function as promising microbicides against lentiviruses.

Christen-Clottu O, Klocke P, Burger D, Straub R, Gerber V. Treatment of clinically diagnosed equine sarcoid with a mistletoe extract (Viscum album austriacus).J Vet Intern Med. 2010 Nov-Dec;24(6):1483-9.

Equine sarcoids (ES) are common, difficult to treat, and have high recurrence rates. Viscum album extracts (VAE) are used in human cancer treatment. The hypothesis is that therapy with VAE (Iscador P) is effective in the treatment of ES. Fifty-three horses (444 ES); 42 were treated with VAE or placebo as monotherapy; 11 were treated with VAE or placebo after selective excision of ES. A prospective, randomised, blinded, clinical trial was carried out. Horses were randomly assigned to treatment (VAE; n=32) or control group (Placebo; n=21). One milliliter of VAE (Iscador P) in increasing concentrations from 0.1 to 20 mg/mL or physiological

NaCl solution was given SC 3 times a week over 105 days. Number, localization, and type of the ES were documented over 12 months. A subset of 163 clinically diagnosed equine sarcoid (CDES) lesions (95 VAE, 68 Placebo) was evaluated in detail, considering clinical findings and tumor volume. No undesired adverse effects were observed except for mild edema at the injection site in 5 of 32 horses (16%). Complete or partial regression was observed in 13 horses of the VAE group (41%) and in 3 of the control horses (14%; P<.05). After VAE treatment, 48 of 95 CDES (67%) showed an improvement compared with 17 of 68 CDES in the control group (40%; P<.01). Twenty-seven CDES had disappeared completely in the VAE group (38%) compared with 9 CDES in the control group (13% NS). VAE (Iscador P) represents a safe and effective treatment for CDES.

Sabini MC, Escobar FM, Tonn CE, Zanon SM, Contigiani MS, Sabini LI. Evaluation of antiviral activity of aqueous extracts from Achyrocline satureioides against Western equine encephalitis virus. Nat Prod Res. 2012;26(5):405-15.

Achyrocline satureioides (Asteraceae) is a medicinal plant traditionally used in Argentina for the treatment of intestinal infections and various digestive disorders. Its infusion is widely utilised for respiratory problems and viral infections. The objective of this study was to investigate cytotoxicity, virucidal and antiviral properties of the cold aqueous extract (CAE) and hot aqueous extract (HAE) of this plant against Western equine encephalitis virus (WEEV). Cytotoxicity in Vero cells was evaluated by maximum non-cytotoxic concentration (MNCC), neutral red (NR) uptake and MTT reduction methods. To study the antiviral activity of aqueous extracts, plaque reduction assay was performed after pre-treatment of host cells, adsorption, penetration and post-penetration of the virus. Extracellular virus inactivation was also analysed by the same method. Extracts showed strong inhibitory activity after virus penetration with selective index values of 32 (NR) and 63.3 (MTT) for the CAE, and 16.2 (NR) and 24.3 (MTT) for the HAE. Both extracts exhibited virucidal action with lower efficacy than their antiviral properties. The present results demonstrate that aqueous extracts of A. satureioides are active against WEEV. Further studies are needed in order to identify which compounds could be responsible for this effect, and how they exert antiviral action.

Pisseri F, Bertoli A, Nardoni S, Pinto L, Pistelli L, Guidi G, Mancianti F. Antifungal activity of tea tree oil from Melaleuca alternifolia against Trichophyton equinum: an in vivo assay. Phytomedicine. 2009 Nov;16(11):1056-8.

Dermatophytes are a group of keratinophilic and keratinolytic molds, some of which are responsible for ringworm. Among them Trichophyton equinum, which mostly infects equids, can cause extensive outbreaks in stud farms. The conventional treatment of equine trichophytosis is topic, based upon medicated shampoos to reduce the spread of infection among the animals. Nevertheless the popularity of phytotherapy is at an all-time peak, and the interest for natural alternatives or complements to conventional drug therapy is challenging both in human and veterinary field. Among herbal remedia Tea Tree Oil (TTO) shows a wide range of antimicrobial activities. A randomized open clinical trial was carried out on 60 thoroughbred breeding horses affected by equine ringworm. The animals were randomly divided into 2 groups of 30 subjects. Diagnostic criteria were the presence of clinical signs and positive T. equinum culture. Specificity control using TTO mixture in 5 not dermatophyte affected animals was achieved also. The antimycotic activity against T. equinum of a mixture containing 25% TTO in sweet almond oil, was evaluated in vivo treating 30 subjects, the others were administered enilconazole 2% solution. The animals of both groups were topically treated twice a day for 15 days with a 25% mixture of TTO diluted in sweet almond oil and every 3 days, four times with enilconazole rinses, respectively. The clinical and mycological outcome were evaluated at day 30 from the start of the treatments. Data analysis was performed by chi square test. All the treated animals showed complete clinical and aetiological healing. Part of control subjects also, showed an improvement and none of them exacerbate the lesions. This therapeutic protocol appears to be effective and versatile, being applicable immediately after physical examination, prior to have the laboratory response. It could be an alternative for practitioners interested in herbal medicines, contributing to fulfill the gap existing between in vitro and clinical studies.

Pearson W. Concurrent use of veterinary drugs and herbal medicines in racing standardbreds. Can Vet J. 2009 Dec;50(12):1283-5.

Standardbred trainers from 1 racetrack and 7 off-track training facilities were surveyed to determine the most common drugs, and prevalence of concurrent herb administration. Furosemide (on-track) and anti-inflammatory drugs (off-track) were the most common drugs administered. Among horses on-track, 9.8% received herbs compared with 13.8% off-track horses; 67% and 58% of these horses, respectively, received concurrent drugs.

Williams CA, Lamprecht ED. Some commonly fed herbs and other functional foods in equine nutrition: a review. Vet J. 2008 Oct;178(1):21-31.

Most herbs and functional foods have not been scientifically tested; this is especially true for the horse. This paper reviews some of the literature pertinent to herbal supplementation in horses and other species. Common supplements like Echinacea, garlic, ginger, ginseng, and yucca are not regulated, and few studies have investigated safe, efficacious doses. Ginseng has been found to exert an inhibitory effect on pro-inflammatory cytokines and cyclooxygenase-2 expression. Equine studies have tested the anti-inflammatory effects of a single dose of ginger, post-exercise. Echinacea has been reported to have anti-inflammatory and antioxidant properties. Yucca contains steroid-like saponins, which produce anti-inflammatory, antioxidant, and anti-spasmodic effects. However, some herbs have drug-like actions that interact with dietary components and may contain prohibited substances like salicylates, digitalis, heroin, cocaine and marijuana. Horses fed garlic at >0.2g/kg per day developed Heinz body anaemia. Drug-herb interactions are common and caution needs to be taken when implementing 'natural product' usage.

Colas C, Popot MA, Garcia P, Bonnaire Y, Bouchonnet S. Analysis of iridoids from Harpagophytum and eleutherosides from Eleutherococcus senticosus in horse urine. Biomed Chromatogr. 2008 Aug;22(8):912-7

LC/ESI-MS n methods have been previously set up to detect the administration of (i) Harpagophytum and (ii) preparations containing a plant capable of anti-stress properties: Eleutherococcus senticosus. Harpagoside has been found to be the main indicator of Harpagophytum administration in the horse. These methods have been applied to a large number of horse urine samples of various origins. Regarding the detection of Harpagophytum administration, harpagoside, harpagide and 8-para-coumaroyl harpagide were detected together in only one sample out of 317. Eleutheroside E was found to be the main indicator of Eleutherococcus senticosus administration. It was detected in post-administration samples collected from two horses having received a feed supplement containing Eleutherococcus senticosus for several days. Out of the 382 samples tested, eleutheroside E was found in an unexpected large number of urine samples (39%) of various origins and its presence cannot be only due to the sole use of herbal dietary supplements.

Pearson W, Orth MW, Lindinger MI. Differential anti-inflammatory and chondroprotective effects of simulated digests of indomethacin and an herbal composite (Mobility) in a cartilage explant model of articular inflammation. J Vet Pharmacol Ther. 2007 Dec;30(6):523-33.

Herbs are an increasingly popular treatment option for horses with cartilage inflammation, despite a relative paucity of research demonstrating efficacy. The research objective was to evaluate the differential anti-inflammatory and chondroprotective efficacy of a simulated digest of indomethacin and a commercially available herbal product in a cartilage model of osteoarthritis. Cartilage explant was integrated with simulated digestion of indomethacin and the herbal product in order to account, at least in part, for the actions of major digestive enzymes and pH. The resulting digests were ultrafiltrated (50 kDa), to account for absorption from the GI tract and movement into the cartilage matrix. We hypothesized that (i) a simulated digest of indomethacin would block interleukin 1 beta-(IL-1) dependent formation of prostaglandin E2 (PGE2) and nitric oxide (NO) without protecting cartilage against IL-1-induced glycosaminoglycan (GAG) release, and (ii) the herbal product would reduce PGE2 and NO in IL-1-stimulated explants, and inhibit release of GAG, in IL-1-stimulated explants. Results showed that indomethacin is an effective anti-inflammatory, evidenced by strong

inhibition of IL-1-induced PGE2 and NO from cartilage explants. However, indomethacin provided no protection against IL-1-induced GAG release. Simulated digest of the herbal extract significantly inhibited IL-1-induced NO production and GAG release, while having a slight increase in PGE2. These data provide evidence for the anti-inflammatory effect of indomethacin on IL-1-stimulated cartilage explants, and the herbal product Mobility may be a useful adjunct in arthritis because of its chondroprotective properties in IL-1-stimulated cartilage.

Pearson W, Charch A, Brewer D, Clarke AF. Pilot study investigating the ability of an herbal composite to alleviate clinical signs of respiratory dysfunction in horses with recurrent airway obstruction. Can J Vet Res. 2007 Apr;71(2):145-51.

Recurrent airway obstruction (RAO), known previously as chronic obstructive pulmonary disease (COPD), is a debilitating respiratory condition that significantly contributes to lost training days and illness in racehorses. Herbs are becoming increasingly popular for the prophylaxis or treatment of the clinical signs of RAO despite a paucity of research on efficacy and safety. We evaluated the ability of an herbal composite containing garlic, white horehound, boneset, aniseed, fennel, licorice, thyme, and hyssop to reduce the clinical signs of RAO, hypothesizing that the product would safely reduce signs and would improve the inflammatory cell profile within the lungs. The composite was fed to 6 horses with symptomatic RAO for 21 d in a crossover manner. Ventigraphs were used to record respiratory rate and intrapleural pressure; the proportion of inflammatory cells in fluid aspirated from the trachea was determined. Blood biochemical and hematologic screening was conducted to identify possible adverse effects. Treatment with the composite did not result in statistically significant changes in any of the parameters evaluated. A trend to a decrease in respiratory rate ($P = 0.1$) and an increase in the proportion of macrophages ($P = 0.1$) was observed in the horses receiving the herbal composite compared with placebo. These data indicate a potential for the herbal composite to safely reduce the elevated respiratory rate in horses with RAO. Future research with a greater number of horses is warranted to further characterize the effect of this product on horses with RAO.

Anour R, Leinker S, van den Hoven R. Improvement of the lung function of horses with heaves by treatment with a botanical preparation for 14 days. Vet Rec. 2005 Dec 3;157(23):733-6

O'Neill W, McKee S, Clarke AF. Immunological and haematinic consequences of feeding a standardised Echinacea (Echinacea angustifolia) extract to healthy horses. Equine Vet J. 2002 May;34(3):222-7.

Pearson W, Boermans HJ, Bettger WJ, McBride BW, Lindinger MI. Association of maximum voluntary dietary intake of freeze-dried garlic with Heinz body anemia in horses. Am J Vet Res. 2005 Mar;66(3):457-65.

Jatzlau A, Abdel-Ghaffar F, Gliem G, Mehlhorn H. Nature helps: food addition of micronized coconut and onion reduced worm load in horses and sheep and increased body weight in sheep. Parasitol Res. 2014 Jan;113(1):305-10.

Al-Quraishy S, Abdel-Ghaffar F, Al-Rasheid KA, Mehlhorn J, Mehlhorn H.Observations on effects of a neem seed extract (MiteStop®) on biting lice (mallophages) and bloodsucking insects parasitizing horses. Parasitol Res. 2012 Jan;110(1):335-9.

Smarsh DN, Liburt N, Streltsova J, McKeever K, Williams CA. Oxidative stress and antioxidant status in intensely exercising horses administered nutraceutical extracts. Equine Vet J Suppl. 2010 Nov;(38):317-22

IX-E Veterinary Botanical Medicine and Sheep and Goats

Cobellis G, Yu Z, Forte C, Acuti G, Trabalza-Marinucci M. Dietary supplementation of Rosmarinus officinalis L. leaves in sheep affects the abundance of rumen methanogens and other microbial populations.J Anim Sci Biotechnol. 2016 Apr 27;7:27.

Rumen microbiome has a great influence on ruminant health and productivity. Different plant extracts have been tested for their ability to modulate the rumen microbiome to improve feed digestion and fermentation. Among the evaluated plant extracts, essential oils, tannins, and saponins appeared to have positive effects on rumen protein metabolism, volatile fatty acids production, and methane and ammonia production. The objective of this study was to evaluate the effect of rosemary (Rosmarinus officinalis L.) leaves and essential oils on rumen microbial populations. Four ruminally cannulated sheep were used in a 4×4 Latin square design fed (21 d/period): 1) a control diet composed of alfalfa hay and concentrate pellet (CTR), 2) CTR supplemented with 7 g/d/sheep of rosemary essential oil adsorbed on an inert support (EO), 3) CTR with 10 g/d/sheep of dried and ground rosemary leaves (RL), and 4) CTR with 10 g/d of dried and ground rosemary leaves pelleted into concentrate (RL pellet). Abundance of total bacteria, archaea, protozoa, and some select bacterial species or groups was quantified using qPCR, while the community of bacteria and archaea was profiled using denaturing gradient gel electrophoresis. No difference in abundance was noted for total bacteria, protozoa, or Ruminococcus flavefaciens between the control and the treatments, but the rosemary leaves, either in loose form or in pellet, decreased the abundance of archaea and the genus Prevotella ($P < 0.001$). The rosemary leaves in loose form also decreased ($P < 0.001$) the abundance of Ruminococcus albus and Clostridium aminophilum, while the EO increased ($P < 0.001$) the abundance of Fibrobacter succinogenes. The community of bacteria and archaea was not affected by any of the supplements. Being able to affect the abundance of several groups of rumen microbes that are known to be involved in degradation of protein and fiber and production of methane and ammonia, rosemary leaves may be used to modulate rumen microbiome and its function.

Smeti S, Joy M, Hajji H, Alabart JL, Muñoz F, Mahouachi M, Atti N. Effects of Rosmarinus officinalis L. essential oils supplementation on digestion, colostrum production of dairy ewes and lamb mortality and growth. Anim Sci J. 2015 Jul;86(7):679-88.

The aim of this study was to evaluate the effect of rosemary essential oils (REO) and the forage nature on ewes' performances, immune response and lambs' growth and mortality. Forty-eight dairy ewes (Sicilo-Sarde) were fed oat-hay or oat-silage supplemented with 400 g of concentrate during pregnancy and 600 g during postpartum. The experimental concentrate contained the same mixture as the control (barley, soybean meal and mineral vitamin supplement) more 0.6 g/kg of REO. Two groups were obtained with each forage (Hay groups: H-C and H-REO; Silage groups: S-C and S-REO). REO increased the dry matter (DM) intake, the nitrogen intake and retention being higher with the silage groups ($P < 0.05$). REO increased solid non-fat ($P = 0.004$) and fat contents of colostrum which was higher with hay ($P = 0.002$). REO decreased lamb mortality ($P < 0.05$) which averaged 21% for control groups and 6% for H-REO, while no mortality was recorded with S-REO. REO dietary supply improved forage intake and tended to ameliorate colostrum production; it could be a natural additive to improve ewes' performances.

Mugnaini L, Nardoni S, Pistelli L, Leonardi M, Giuliotti L, Benvenuti MN, Pisseri F, Mancianti F. A herbal antifungal formulation of Thymus serpillum, Origanum vulgare and Rosmarinus officinalis for treating ovine dermatophytosis due to Trichophyton mentagrophytes. Mycoses. 2013 May;56(3):333-7.

A number of herbal products with anti-inflammatory, antiseptic and antimycotic properties are available for dermatological usage. The successful treatment of 13 sheep affected by ringworm due to Trichophyton mentagrophytes with a mixture consisting of essential oils (EOs) of Thymus serpillum 2%, Origanum vulgare 5% and Rosmarinus officinalis 5% in sweet almond (Prunus dulcis) oil. The effectiveness of EOs and of the major

components of the mixture (thymol, carvacrol, 1,8 cineole, α-pinene, p-cymene, γ-terpinene) against the fungal clinical isolate was evaluated by a microdilution test. Thirteen animals were topically administered with the mixture twice daily for 15 days. The other sheep were administered with a conventional treatment (seven animals) or left untreated (two animals). Minimum inhibitory concentration (MIC) values were 0.1% for T. serpillum, 0.5% for O. vulgare, 2.5% for I. verum and 5% for both R. officinalis and C. limon. Thymol and carvacrol showed MICs of 0.125% and 0.0625%. A clinical and aetiological cure was obtained at the end of each treatment regimen in only the treated animals. Specific antimycotic drugs licenced for food-producing sheep are not available within the European Community. The mixture tested here appeared to be a versatile tool for limiting fungal growth.

James PJ, Callander JT. Dipping and jetting with tea tree (Melaleuca alternifolia) oil formulations control lice (Bovicola ovis) on sheep. Vet Parasitol. 2012 Oct 26;189(2-4):338-43.

The in vivo pediculicidal effectiveness of 1% and 2% formulations of tea tree (Melaleuca alternifolia) oil (TTO) against sheep chewing lice (Bovicola ovis) was tested in two pen studies. Immersion dipping of sheep shorn two weeks before treatment in both 1% and 2% formulations reduced lice to non detectable levels. No lice were found on any of the treated sheep despite careful inspection of at least 40 fleece partings per animal at 2, 6, 12 and 20 weeks after treatment. In the untreated sheep louse numbers increased from a mean (± SE) of 2.4 (± 0.7) per 10 cm fleece part at 2 weeks to 12.3 (± 4.2) per part at 20 weeks. Treatment of sheep with 6 months wool by jetting (high pressure spraying into the fleece) reduced louse numbers by 94% in comparison to controls at two weeks after treatment with both 1% and 2% TTO formulations. At 6 and 12 weeks after treatment reductions were 94% and 91% respectively with the 1% formulation and 78% and 84% respectively with the 2% formulation. TTO treatment also appeared to reduce wool damage in infested sheep. Laboratory studies indicated that tea tree oil 'stripped' from solution with a progressive reduction in concentration as well as volume as more wool was dipped, indicating that reinforcement of active ingredient would be required to maintain effectiveness when large numbers of sheep are treated. The results of these studies suggest significant potential for the development of ovine lousicides incorporating TTO.

Hawken PA, Fiol C, Blache D. Genetic differences in temperament determine whether lavender oil alleviates or exacerbates anxiety in sheep. Physiol Behav. 2012 Mar 20;105(5):1117-23

Growing concerns about the risk of addiction to benzodiazepines have led to increasing interest in alternative therapies to treat anxiety and depression. Lavender oil (Lavendula augustifolia) is reportedly anxiolytic in a number of species but little is known about how it affects individuals that are more or less anxious when faced with a stressor. In this study, we used changes in locomotor activity and the plasma concentrations of cortisol to test whether lavender oil would reduce behavioral and endocrine correlates of anxiety in calm and nervous sheep exposed to an isolation stressor. During the non-breeding season, 'calm' or 'nervous' female sheep from the UWA temperament flock were exposed to a mask containing either 1 mL of 10% lavender oil (calm: n=8; nervous: n=8) or peanut oil (calm: n=8; nervous: n=8). After 30 min, each sheep was isolated for 5 min and then returned to the group. Blood was sampled prior to the mask, prior to isolation, 1 min and 30 min after isolation to profile changes in the plasma concentrations of cortisol. Agitation score, locomotor activity and vocalizations were recorded as correlates of anxiety associated with the isolation stressor. Irrespective of whether they were exposed to lavender oil, calm sheep had a lower agitation score ($P<0.001$), crossed the central lines of the isolation box less frequently ($P<0.001$), expressed fewer vocalizations ($P<0.001$) and had lower plasma concentrations of cortisol immediately after isolation ($P<0.001$) than nervous sheep. Exposure of calm sheep to lavender oil decreased the agitation score ($P<0.001$), frequency of vocalizations ($P<0.05$), decreased the number of crosses of the central lines of the isolation box ($P<0.05$), and the plasma concentrations of cortisol prior to isolation ($P<0.05$) (after mask application) compared to calm control sheep. Exposure of nervous sheep to lavender oil increased the frequency of vocalizations ($P<0.05$), the number of sheep attempting to escape ($P<0.05$) and the plasma concentrations of cortisol 30 min after isolation ($P<0.05$)

compared to nervous control sheep. We conclude that genetic differences in temperament determine whether lavender oil alleviates or exacerbates the behavioral and/or endocrine correlates of anxiety in sheep

Giannenas I, Skoufos J, Giannakopoulos C, Wiemann M, Gortzi O, Lalas S, Kyriazakis I. Effects of essential oils on milk production, milk composition, and rumen microbiota in Chios dairy ewes. J Dairy Sci. 2011 Nov;94(11):5569-77.

The effect of the addition of an essential oil (EO) preparation (containing a mixture of natural and nature-identical EO) on the performance of dairy ewes of the Chios breed was investigated. Eighty lactating ewes were allocated into 4 equal groups in a randomized block design, each with 4 replicates of 5 ewes housed in the same pen. The 4 groups were fed the same total mixed ration allowance, the roughage being a mixture of corn silage, lucerne hay, and wheat straw, and the concentrate based on cereals and oil cakes. Control ewes were fed their daily allowance of total mixed ration without any EO. The other 3 groups were supplemented with EO at levels of 50, 100, and 150 mg/kg of the concentrated feed, respectively. Individual milk yield was recorded daily and feed refusals were recorded on a pen basis weekly during the first 5 mo of lactation. Milk samples were analyzed for chemical composition, somatic cell count, and urea content. Rumen samples were analyzed for pH, NH(3)-N content, and protozoa, cellulolytic, hyper-ammonia-producing, and total viable bacteria counts. Results showed that inclusion of EO increased milk production per ewe, the effect being dose dependent [1.565, 1.681, 1.876, and 2.119 L/d (standard error of the difference ± 0.176) for the control, 50, 100, and 150 mg of EO/kg of concentrate diets, respectively], and thus improved feed utilization. Although the inclusion of EO did not affect milk composition, it lowered urea concentration and somatic cell count in milk samples at the highest supplementation level compared with the control. Total counts of viable and cellulolytic bacteria and protozoa were not influenced by EO supplementation; however, counts of hyper-ammonia-producing bacteria were decreased at the 2 highest supplementation levels compared with the control group. Rumen pH was not affected by EO supplementation, but rumen NH(3)-N was reduced at the highest EO supplementation level, and acetate rumen concentrations tended to decrease and propionate to increase in a dose-dependent manner. In conclusion, EO supplementation may improve feed utilization and performance of the high-yielding dairy Chios ewes; however, the underlying mechanisms leading to this improvement merit further investigation.

Callander JT, James PJ. Insecticidal and repellent effects of tea tree (Melaleuca alternifolia) oil against Lucilia cuprina. Vet Parasitol. 2012 Mar 23;184(2-4):271-8

Laboratory studies were conducted to assess the effect of tea tree oil (TTO) from Melaleuca alternifolia (terpinen-4-ol chemotype) against different stages of the Australian sheep blowfly Lucilia cuprina. When applied to wool, 3% TTO formulation repelled gravid female L. cuprina and prevented oviposition for six weeks. Formulations containing 1% TTO caused 100% mortality of L. cuprina eggs and 1st instar larvae and 2.5% TTO caused mortality of most second and third instar larvae in agar feeding assays. In experiments where third instar larvae were dipped in TTO formulations for 60s, concentrations of up to 50% TTO gave less than 50% kill. TTO at concentrations of 0.5%, 2% and 5% was strongly repellent to third instar larvae and caused them to evacuate treated areas. Inclusion of TTO in formulations with diazinon, ivermectin and boric acid reduced mortality in comparison with the larvicides used alone, at least partially because of avoidance behaviour stimulated by the TTO. Addition of TTO to wound treatments may aid in wound protection and myiasis resolution by preventing oviposition by L. cuprina adults, insecticidal action against L. cuprina eggs and larvae, stimulating larvae to leave the wound and through antimicrobial and anti-inflammatory properties that aid in wound healing.

Tabassam SM[1], Iqbal Z, Jabbar A, Sindhu ZU, Chattha AI. Efficacy of crude neem seed kernel extracts against natural infestation of Sarcoptes scabiei var. ovis. J Ethnopharmacol. 2008 Jan 17;115(2):284-7.

This study was aimed to evaluate the efficacy of crude aqueous-methanol and aqueous extracts of neem (Azadirachta indica) seed kernel against sarcoptic mange of sheep. Crude aqueous-methanol (AME) and aqueous extracts (AE) of neem seed kernel (NSK) were prepared and formulated as 10% and 20% ointments (w/w), using Vaseline as vehicle. Forty-two lambs of Pak Karakul breed, having natural infection of sarcoptic mange were divided into seven experimental groups. Skin scrapings and clinical examination were carried out at scheduled intervals after treatment. Ivermectin (positive control) completely cleared infesting mites from animals after 10 days and 20% AME after 16 days. While, clinical mange was completely cured after 16 and 20 days with ivermectin and 20% AME, respectively, under field conditions. Only the higher concentration (20% AME) of NSK extracts completely cured the clinical mange, suggesting a dose-dependent response. Our results consolidate the belief that use of folk remedies can provide an effective and economic way of combating sarcoptic mange in sheep.

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IX-E Veterinary Botanical Medicine and Poultry

Lan L, Zuo B, Ding H, etal Anticoccidial evaluation of a traditional Chinese medicine--Brucea javanica--in broilers. Poult Sci. 2016 Apr;95(4):811-8.

The traditional Chinese medicinal plant Brucea javanica has received much attention for its significant antiprotozoal effects in recent years; however, little is known about its potential anticoccidial functions. In the present study, a series of experiments was conducted to investigate the prophylactic and therapeutic effects of ethanol extract from B. javanica on coccidiosis induced by Eimeria tenella in broiler chickens. Chickens infected with E. tenella were treated with B. javanica extract and compared either with broilers treated with the anticoccidial halofuginone hydrobromide (Stenorol) or with control groups that consisted of infected-unmedicated and uninfected-unmedicated broilers. The experiments revealed that the B. javanica extract could significantly ($P<0.05$) reduce bloody diarrhea and lesion scores. Additional, OPG output in these plant extract treated groups was reduced in comparison with non-treated groups ($P<0.05$). However, there was no evidence to show that the extract could promote BWG. Histological data showed that the number of second-generation schizonts in the medicated groups was substantially less than that in the infected-unmedicated control. In summary, our work showed that B. javanica extract exerted considerable anticoccidial effects, supporting its use as a promising therapeutic in controlling avian coccidiosis

Diaz-Sanchez S, D'Souza D, Biswas D, Hanning I. Botanical alternatives to antibiotics for use in organic poultry production. Poult Sci. 2015 Jun;94(6):1419-30.

The development of antibiotic resistant pathogens has resulted from the use of sub-therapeutic concentrations of antibiotics delivered in poultry feed. Furthermore, there are a number of consumer concerns regarding the use of antibiotics in food animals including residue contamination of poultry products and antibiotic resistant bacterial pathogens. These issues have resulted in recommendations to reduce the use of antibiotics as growth promoters in livestock in the United States. Unlike conventional production, organic systems are not permitted to use antibiotics. Thus, both conventional and organic poultry production need alternative methods to improve growth and performance of poultry. Herbs, spices, and various other plant extracts are being evaluated as alternatives to antibiotics and some do have growth promoting effects, antimicrobial properties, and other health-related benefits. This review aims to provide an overview of herbs, spices, and plant extracts, currently defined as phytobiotics as potential feed additives.

Brown AW, Stegelmeier BL, Colegate SM Etal The comparative toxicity of a reduced, crude comfrey (Symphytum officinale) alkaloid extract and the pure, comfrey-derived pyrrolizidine alkaloids, lycopsamine and intermedine in chicks (Gallus gallus domesticus). J Appl Toxicol. 2015 Jul 14. doi: 10.1002/jat.3205. [Epub ahead of print]

Comfrey (Symphytum officinale), a commonly used herb, contains dehydropyrrolizidine alkaloids that, as a group of bioactive metabolites, are potentially hepatotoxic, pneumotoxic, genotoxic and carcinogenic. Consequently, regulatory agencies and international health organizations have recommended comfrey be used for external use only. However, in many locations comfrey continues to be ingested as a tisane or as a leafy vegetable. The objective of this work was to compare the toxicity of a crude, reduced comfrey alkaloid extract to purified lycopsamine and intermedine that are major constituents of S. officinale. Male, California White chicks were orally exposed to daily doses of 0.04, 0.13, 0.26, 0.52 and 1.04 mmol lycopsamine, intermedine or reduced comfrey extract per kg bodyweight (BW) for 10 days. After another 7 days chicks were euthanized. Based on clinical signs of poisoning, serum biochemistry, and histopathological analysis the reduced comfrey extract was more toxic than lycopsamine and intermedine. This work suggests a greater than additive effect of the individual alkaloids and/or a more potent toxicity of the acetylated derivatives in the reduced comfrey extract. It also suggests that safety recommendations based on purified compounds may underestimate the potential toxicity of comfrey.

Haselmeyer A[1], Zentek J, Chizzola R. Effects of thyme as a feed additive in broiler chickens on thymol in gut contents, blood plasma, liver and muscle. J Sci Food Agric. 2015 Feb;95(3):504-8.

Aromatic herbs as feed additives in animal production are encountering growing interest, but data on the fate of the aromatic compounds from the plant in the animal body are very scarce. In the present study, thyme (Thymus vulgaris) herb consisting of leaves and flowers without stems was used as an ingredient in the diet for broilers. The herb was fed for 35 days to five groups of broilers (0, 0.1, 0.2, 0.3, and 1% w/w in the diet). Animal performance and the concentrations of the main essential oil component from thyme, thymol, were measured in gut contents, plasma and liver and muscle tissues using solid phase microextraction and gas chromatography/mass spectrometry.There were no differences between the groups in feed intake, daily weight gain, feed conversion and slaughter weight. Thymol was detected in gut contents, plasma and liver and muscle tissues. Increased intestinal thymol concentrations were found in the group with 1% thyme compared with the other groups ($P < 0.05$). In liver and muscle tissues the thymol levels were close to the limit of quantification.The data do not indicate a positive effect of thyme on animal performance. With high dietary levels of thyme herb, thymol concentrations increased in gut contents and plasma but were very low in edible tissues such as liver and flesh. © 2014 Society of Chemical Industry.

Wunderlich F, Al-Quraishy S, Steinbrenner H etal. Towards identifying novel anti-Eimeria agents: trace elements, vitamins, and plant-based natural products. Parasitol Res. 2014 Oct;113(10):3547-56.

Eimeriosis, a widespread infectious disease of livestock, is caused by coccidian protozoans of the genus Eimeria. These obligate intracellular parasites strike the digestive tract of their hosts and give rise to enormous economic losses, particularly in poultry, ruminants including cattle, and rabbit farming. Vaccination, though a rational prophylactic measure, has not yet been as successful as initially thought. Numerous broad-spectrum anti-coccidial drugs are currently in use for treatment and prophylactic control of eimeriosis. However, increasing concerns about parasite resistance, consumer health, and environmental safety of the commercial drugs warrant efforts to search for novel agents with anti-Eimeria activity. This review summarizes current approaches to prevent and treat eimeriosis such as vaccination and commercial drugs, as well as recent attempts to use dietary antioxidants as novel anti-Eimeria agents. In particular, the trace elements selenium and zinc, the vitamins A and E, and natural products extracted from garlic, barberry, pomegranate, sweet wormwood, and other plants are discussed. Several of these novel anti-Eimeria agents exhibit a protective role against oxidative stress that occurs not only in the intestine of Eimeria-infected animals, but also in their non-parasitized tissues, in particular, in the first-pass organ liver. Currently, it appears to be promising to identify safe combinations of low-cost natural products with high anti-Eimeria efficacy for a potential use as feed supplementation in animal farming.

Drăgan L, Györke A, Ferreira JF, Pop IA, Dunca I, Drăgan M, Mircean V, Dan I, Cozma V. Effects of Artemisia annua and Foeniculum vulgare on chickens highly infected with Eimeria tenella (phylum Apicomplexa). Acta Vet Scand. 2014 Apr 15;56:22.

Intensive poultry production systems depend on chemoprophylaxis with anticoccidial drugs to combat infection. A floor-pen study was conducted to evaluate the anticoccidial effect of Artemisia annua and Foeniculum vulgare on Eimeria tenella infection. Five experimental groups were established: negative control (untreated, unchallenged); positive control (untreated, challenged); a group medicated with 125 ppm lasalocid and challenged; a group medicated with A. annua leaf powder at 1.5% in feed and challenged; and a group treated with the mixed oils of A. annua and Foeniculum vulgare in equal parts, 7.5% in water and challenged. The effects of A. annua and oil extract of A. annua + F. vulgare on E. tenella infection were assessed by clinical signs, mortality, fecal oocyst output, faeces, lesion score, weight gain, and feed conversion. Clinical signs were noticed only in three chickens from the lasalocid group, six from the A. annua group, and nine from the A. annua + F. vulgare group, but were present in 19 infected chickens from the positive control group. Bloody diarrhea was registered in only two chickens from A. annua group, but in 17 chickens from the positive control group. Mortality also occurred in the positive control group (7/20). Chickens treated with A. annua had a significant reduction in faecal oocysts (95.6%; $P = 0.027$) and in lesion score (56.3%; $P = 0.005$) when compared to the positive control. At the end of experiment, chickens treated with A. annua leaf powder had the highest

body weight gain (68.2 g/day), after the negative control group, and the best feed conversion (1.85) among all experimental groups. Our results suggest that A. annua leaf powder (Aa-p), at 1.5% of the daily diet post-infection, can be a valuable alternative for synthetic coccidiostats, such as lasalocid.

Zeng ZK, Li QY, Piao XS, Liu JD, Zhao PF, Xu X, Zhang S, Niu S. Forsythia suspensa extract attenuates corticosterone-induced growth inhibition, oxidative injury, and immune depression in broilers. Poult Sci. 2014 Jul;93(7):1774-81.

Forsythia suspensa extract (FSE) has been demonstrated to attenuate physiological stress induced by high temperature or high stocking density. This experiment was conducted with 144 male Arbor Acre broilers (1-d-old, weighing 42.7 ± 1.7 g) to determine the effects of FSE on performance, nutrient digestibility, antioxidant activities, serum metabolites, and immune parameters for birds treated with corticosterone (CS). The birds were randomly allotted to 1 of 4 treatments in a 2 × 2 factorial arrangement that included FSE supplementation (0 or 100 mg/kg) and CS administration (0 or 20 mg/kg of diet for 7 consecutive days starting on d 14). The feeding program consisted of a starter diet from d 1 to 21 and a finisher diet from d 22 to 42. Corticosterone administration decreased ($P < 0.01$) ADG and impaired ($P < 0.01$) feed conversion ratio in both phases and overall, which were alleviated ($P < 0.01$) by dietary FSE supplementation in the finisher phase and overall. At d 21, CS administration caused decreases ($P < 0.05$) in the apparent digestibility of energy, relative weight of bursa and thymus, total antioxidant capacity, superoxide dismutase (SOD) activity, and antibody titers to Newcastle disease virus (NDV); however, serum malondialdehyde and uric acid were increased. All of these changes were attenuated ($P < 0.05$) by dietary FSE supplementation. At d 42, FSE supplementation improved ($P < 0.05$) the apparent digestibility of DM and CP, relative weights of bursa, SOD activity, and antibody titers to NDV, which were impaired by CS administration. Interactions ($P < 0.05$) were noted between CS and FSE for ADG and feed conversion ratio in the finisher phase and overall, as well as total antioxidant capacity, SOD activity, uric acid, and antibody titers to NDV at d 21, as well as relative weights of thymus at d 42. In conclusion, dietary FSE supplementation enhanced nutrient digestibility and performance of broiler possibly by reducing oxidative stress and immune depression challenged by CS.

Sun Y, Niu L, Song M, Zhao X, Sun N, He J, Wu C, Jiang J, Bai Y, Guo J, Li H. Screening compounds of Chinese medicinal herbs anti-Marek's disease virus. Pharm Biol. 2014 Jul;52(7):841-7.

Marek's disease (MD) seriously threatens the world poultry industry and has resulted in great economic losses. Chinese medicinal herbs are a rich source for lead compounds and drug candidates for antiviral treatments.The object of the study was to investigate the anti-MDV activity and mechanism of 20 compounds extracted from Chinese medicinal herbs. Antiviral assay, time of addition experiments, and virucidal assay were performed on chicken embryo fibroblast cells. The 50% cytotoxic concentration and 50% effective concentration were determined and, accordingly, selectivity index and inhibition ratio were calculated. Antiviral assay showed dipotassium glycyrrhizinate (DG) and sodium tanshinone IIA sulfonate (STS) exhibited significantly inhibitory activity against MDV in a dose-dependent manner. EC50 of DG and STS were 893.5 ± 36.99 μg/mL and 54.82 ± 2.99 μg/mL, and selective index (SI) were >3.36 and >9.12, respectively. Time of addition experiment and virucidal assay demonstrated DG inhibited viral replication in the full replication cycle and inactivated MDV particles in non-time-dependent manner, but STS interfered with the early stage of MDV replication and inactivated MDV particles in a time-dependent manner. Moreover, both DG and STS promoted apoptosis of cells infected by MDV. DG and STS have great potential for developing new anti-MDV drugs for clinic application.

Varmuzova K, Matulova ME, Gerzova L, Cejkova D, Gardan-Salmon D, Panhéleux M, Robert F, Sisak F, Havlickova H, Rychlik I. Curcuma and Scutellaria plant extracts protect chickens against inflammation and Salmonella Enteritidis infection. Poult Sci. 2015 Sep;94(9):2049-58.

After a ban on the use of antibiotics as growth promoters in farm animals in the European Union in 2006, an interest in alternative products with antibacterial or anti-inflammatory properties has increased. In this study, we therefore tested the effects of extracts from Curcuma longa and Scutellaria baicalensis used as feed additives against cecal inflammation induced by heat stress or Salmonella Enteritidis (S. Enteritidis) infection in

chickens. Curcuma extract alone was not enough to decrease gut inflammation induced by heat stress. However, a mixture of Curcuma and Scutellaria extracts used as feed additives decreased gut inflammation induced by heat or S. Enteritidis, decreased S. Enteritidis counts in the cecum but was of no negative effect on BW or humoral immune response. Using next-generation sequencing of 16S rRNA we found out that supplementation of feed with the 2 plant extracts had no effect on microbiota diversity. However, if the plant extract supplementation was provided to the chickens infected with S. Enteritidis, Faecalibacterium, and Lactobacillus, both bacterial genera with known positive effects on gut health were positively selected. The supplementation of chicken feed with extracts from Curcuma and Scutelleria thus may be used in poultry production to effectively decrease gut inflammation and increase chicken performance.

Lan L, Zuo B, Ding H, Huang Y, Chen X, Du A. Anticoccidial evaluation of a traditional Chinese medicine--Brucea javanica--in broilers. Poult Sci. 2016 Apr;95(4):811-8.

The traditional Chinese medicinal plant Brucea javanica has received much attention for its significant antiprotozoal effects in recent years; however, little is known about its potential anticoccidial functions. In the present study, a series of experiments was conducted to investigate the prophylactic and therapeutic effects of ethanol extract from B. javanica on coccidiosis induced by Eimeria tenella in broiler chickens. Chickens infected with E. tenella were treated with B. javanica extract and compared either with broilers treated with the anticoccidial halofuginone hydrobromide (Stenorol) or with control groups that consisted of infected-unmedicated and uninfected-unmedicated broilers. The experiments revealed that the B. javanica extract could significantly ($P<0.05$) reduce bloody diarrhea and lesion scores. Additional, OPG output in these plant extract treated groups was reduced in comparison with non-treated groups ($P<0.05$). However, there was no evidence to show that the extract could promote BWG. Histological data showed that the number of second-generation schizonts in the medicated groups was substantially less than that in the infected-unmedicated control. In summary, our work showed that B. javanica extract exerted considerable anticoccidial effects, supporting its use as a promising therapeutic in controlling avian coccidiosis.

Gholami-Ahangaran M, Rangsaz N, Azizi S. Evaluation of turmeric (Curcuma longa) effect on biochemical and pathological parameters of liver and kidney in chicken aflatoxicosis. Pharm Biol. 2016 May;54(5):780-7.

Aflatoxins as potent mycotoxins can influence vital parameters in chickens. Turmeric was used in decreasing toxic effect of mycotoxins on vital organs, traditionally. The study compared the protective effect of turmeric and Mycoad(TR) in broilers exposed to aflatoxin. Chickens (270) were divided into six groups. The chickens were fed a basal diet, turmeric extract (5 mg/kg diet), Mycoad(TR) (25 mg/kg diet), productive aflatoxin (3 mg/kg diet), aflatoxin plus turmeric extract (3 versus 5 mg/kg diet), and aflatoxin plus Mycoad(TR) (3 versus 25 mg/kg diet) in basal diet. At 28 d old, we determined plasma concentration of total protein, albumin, triglyceride, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), calcium, potassium, phosphorous, uric acid, aspartate transferase (AST), and alanine aminotransferase (ALT). Furthermore, liver and kidney were sampled for pathological examination. Chickens fed turmeric with aflatoxin had significant lower ALT, AST, and uric acid than chickens fed aflatoxin (11.4 ± 0.79, 228 ± 9, and 6 ± 0.4 versus 17.2 ± 1.7, 283 ± 5, and 7.7 ± 0.1) whereas, total protein, calcium, and HDL values in chickens fed aflatoxin plus turmeric increased significantly (2.66 ± 0.16, 8.4 ± 0.2, and 920 ± 4.1 versus 1.7 ± 0.17, 7 ± 0.2, and 690 ± 4.8). Pathological examination revealed severe congestion, degeneration, and necrosis in liver and kidney in chickens that received aflatoxin. The study showed that turmeric may provide protection against the toxic effects of aflatoxin on liver and kidney.

Nechita IS[1], Poirel MT[2], Cozma V[3], Zenner L[2]. The repellent and persistent toxic effects of essential oils against the poultry red mite, Dermanyssus gallinae. Vet Parasitol. 2015 Oct 24. pii: S0304-4017

The economic impact of the poultry red mite, Dermanyssus gallinae, the lack of new acaricides, the occurrence of resistance and tighter legislation have all led to the need to find new ways to control this pest. One promising alternative method of control focuses on employing repellent and/or toxic effects of selected plant essential oils against D. gallinae. Ten essential oils (basil, thyme, coriander, eucalyptus, lavender, lemon, fir tree, oregano, mint, and juniper) were tested for the persistence of toxic and repellent effects. In filter-paper toxicity bioassays against D. gallinae, the best results were observed for lavender (more than 97% mortality

after 48 and 72 h) and thyme (84% at 72 h) at a dose of 0.12 mg/cm(2). In addition, two oils showed significant persistent toxic effects 15 and 30 days post application to filter papers. Thyme was the most effective (100% mortality at 72 h), followed by lavender (nearly 80% mortality after 72 h). Out of the ten oils tested for their repellent effect, thyme was the strongest, with nearly 80% of the tested area avoided by mites; oregano caused a 60% avoidance and lavender exhibited an effect close to 40%. All other oils exhibited a repellent effect of less than 30%. None of the experiments showed a repellent effect for HM (commercial alimentary oil) or negative controls. We found that the thyme and lavender essential oils exhibited promising results when tested in vitro for toxic and repellent effects against D. gallinae; thus, we suggest that future experiments focus on in vivo tests using these oils in farm units.

Kim JE, Lillehoj HS, Hong YH, Kim GB, Lee SH, Lillehoj EP, Bravo DM. Dietary Capsicum and Curcuma longa oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. Res Vet Sci. 2015 Oct;102:150-8.

Three commercial broiler breeds were fed from hatch with a diet supplemented with Capsicum and Curcuma longa oleoresins, and co-infected with Eimeria maxima and Clostridium perfringens to induce necrotic enteritis (NE). Pyrotag deep sequencing of bacterial 16S rRNA showed that gut microbiota compositions were quite distinct depending on the broiler breed type. In the absence of oleoresin diet, the number of operational taxonomic units (OTUs), was decreased in infected Cobb, and increased in Ross and Hubbard, compared with the uninfected. In the absence of oleoresin diet, all chicken breeds had a decreased Candidatus Arthromitus, while the proportion of Lactobacillus was increased in Cobb, but decreased in Hubbard and Ross. Oleoresin supplementation of infected chickens increased OTUs in Cobb and Ross, but decreased OTUs in Hubbard, compared with unsupplemented/infected controls. Oleoresin supplementation of infected Cobb and Hubbard was associated with an increased percentage of gut Lactobacillus and decreased Selenihalanaerobacter, while Ross had a decreased fraction of Lactobacillus and increased Selenihalanaerobacter, Clostridium, Calothrix, and Geitlerinema. These results suggest that dietary Capsicum/Curcuma oleoresins reduced the negative consequences of NE on body weight and intestinal lesion, in part, through alteration of the gut microbiome in 3 commercial broiler breeds.

Alipour F, Hassanabadi A, Golian A, Nassiri-Moghaddam H. Effect of plant extracts derived from thyme on male broiler performance. Poult Sci. 2015 Nov;94(11):2630-4.

The effect of dietary thyme-oil extract (TOE) supplementation on immune functions of broilers were assessed by feeding graded levels (50, 100, 200, or 400 ppm) of TOE to male broiler chicks during a 42-d feeding trial compared with negative- or positive-control diets. Dietary control treatments included a negative-control diet with no feed-additive supplementation and 2 positive-control groups supplemented with either virginiamycin or zinc bacitracin. In total, 300 1-day-old Ross × Ross male broilers were randomly assigned to 6 dietary treatments that consisted of 5 replicates of 10 birds each. On d 21 and 42, 2 birds from each replicate were killed by cervical cutting to measure the relative weights of spleen and bursa of Fabricius. At 25 d of age, chicks were injected with 0.5 mL of 10% SRBC suspension. Broilers fed with 200 ppm of TOE had heavier weights of bursa of Fabricius than those fed other dietary treatments at d 42 of age. Furthermore, dietary inclusion of 100 ppm of TOE resulted in higher ($P < 0.05$) total immunoglobulin response in primary antibody titer against sheep erythrocytes compared with other dietary treatments. On the other hand, diet modifications had no significant effect on blood leukocyte subpopulations and heterophil-to-lymphocyte ratio. These results suggest that dietary supplementation with TOE, especially at the level of 100 ppm, can improve immunological responses of broiler chicks.

Varmuzova K, Matulova ME, Gerzova L, Cejkova D, Gardan-Salmon D, Panhéleux M, Robert F, Sisak F, Havlickova H, Rychlik I. Curcuma and Scutellaria plant extracts protect chickens against inflammation and Salmonella Enteritidis infection. Poult Sci. 2015 Sep;94(9):2049-58.

After a ban on the use of antibiotics as growth promoters in farm animals in the European Union in 2006, an interest in alternative products with antibacterial or anti-inflammatory properties has increased. In this study,

we therefore tested the effects of extracts from Curcuma longa and Scutellaria baicalensis used as feed additives against cecal inflammation induced by heat stress or Salmonella Enteritidis (S. Enteritidis) infection in chickens. Curcuma extract alone was not enough to decrease gut inflammation induced by heat stress. However, a mixture of Curcuma and Scutellaria extracts used as feed additives decreased gut inflammation induced by heat or S. Enteritidis, decreased S. Enteritidis counts in the cecum but was of no negative effect on BW or humoral immune response. Using next-generation sequencing of 16S rRNA we found out that supplementation of feed with the 2 plant extracts had no effect on microbiota diversity. However, if the plant extract supplementation was provided to the chickens infected with S. Enteritidis, Faecalibacterium, and Lactobacillus, both bacterial genera with known positive effects on gut health were positively selected. The supplementation of chicken feed with extracts from Curcuma and Scutelleria thus may be used in poultry production to effectively decrease gut inflammation and increase chicken performance.

Varmaghany S[1], Karimi Torshizi MA[2], Rahimi S[3], etal. The effects of increasing levels of dietary garlic bulb on growth performance, systolic blood pressure, hematology, and ascites syndrome in broiler chickens. Poult Sci. 2015 Aug;94(8):1812-20.

The effects of dietary garlic bulb were studied separately on hematological parameters, ascites incidence, and growth performance of an ascites susceptible broiler hybrid under both standard temperature conditions (STC:) and cold temperature conditions (CTC:). A total of 336 one-day-old male broiler chickens were allocated to 4 experimental groups with 4 replicates of 21 birds each under STC. In addition, the same grouping with another 336 birds was used for CTC. Under CTC, the birds were exposed to cold temperatures for induction of ascites. Experimental groups were defined by the inclusion of 0 (control), 5, 10 or 15 g/kg garlic bulbs in the diets under both STC and CTC. Growth performance, systolic blood pressure (as a measure of systemic arterial blood pressure), physiological and biochemical parameters, as well as ascites indices (right ventricle [RV:], total ventricle [TV:] weights, and RV/TV:) were evaluated. Systolic blood pressure was determined using an indirect method with a sphygmomanometer, a pediatric cuff, and a Doppler device. The final body weight decreased quadratically ($P = 0.003$), with increasing garlic bulb levels in the diets under STC. The feed conversion ratio showed no significant differences among all groups under both STC and CTC. No significant differences were observed in total mortality and ascites-related mortality in all groups under STC, although total mortality (L: $P = 0.01$; Q: $P = 0.001$) and ascites-related mortality (L: $P = 0.007$; Q: $P = 0.001$) were significantly different among the diets under CTC. Under STC, the systolic blood pressure, packed cell volume, hemoglobin, RV, TV, and RV/TV did not vary significantly among the diets. However, red blood cell count and erythrocyte osmotic fragility decreased linearly ($P < 0.005$) with increasing garlic bulb levels in the diets under STC. Under CTC, the systolic blood pressure, packed cell volume, red blood cell count, and erythrocyte osmotic fragility decreased ($P < 0.05$) with increasing garlic levels. It is concluded that the inclusion of 5 g/kg garlic bulb in susceptible broiler chicken diets has a systemic anti-hypertensive effect and could decrease ascites incidence without impairing broiler chicken performance.

Diaz-Sanchez S, D'Souza D, Biswas D, Hanning I. Botanical alternatives to antibiotics for use in organic poultry production. Poult Sci. 2015 Jun;94(6):1419-30.

The development of antibiotic resistant pathogens has resulted from the use of sub-therapeutic concentrations of antibiotics delivered in poultry feed. Furthermore, there are a number of consumer concerns regarding the use of antibiotics in food animals including residue contamination of poultry products and antibiotic resistant bacterial pathogens. These issues have resulted in recommendations to reduce the use of antibiotics as growth promoters in livestock in the United States. Unlike conventional production, organic systems are not permitted to use antibiotics. Thus, both conventional and organic poultry production need alternative methods to improve growth and performance of poultry. Herbs, spices, and various other plant extracts are being evaluated as alternatives to antibiotics and some do have growth promoting effects, antimicrobial properties, and other health-related benefits. This review aims to provide an overview of herbs, spices, and plant extracts, currently defined as phytobiotics as potential feed additives.

Müştak HK, Torun E, Özen D, Yücel G, Akan M, Diker KS. Effect of Lonicera japonica extract on Mycoplasma gallisepticum in naturally infected broiler flocks. Br Poult Sci. 2015;56(3):299-303

In this study, the effect of chlorogenic acid extract from Lonicera japonica Thunb. on Mycoplasma gallisepticum infections and the performance of broiler flocks was investigated. 2. A total of 360 Ross-308 broiler chicks taken from M. gallisepticum seropositive flocks were divided equally into three groups designated as control (nothing administered), antibiotic (Tylosin tartrate given for the first 3 d and d 20-22) and test group (chlorogenic acid extract given twice a day on d 16 and 22). 3. Broiler performance analysis, serological tests (slide agglutination), molecular identification (polymerase chain reaction) and histopathological examination were performed to detect M. gallisepticum. 4. The results show that chlorogenic acid not only increases live body weight but is also an alternative treatment option in M. gallisepticum-infected broiler flocks.

Song X, Zhang Y, Yin Z, Zhao X, Liang X, He C, Yin L, Lv C, Zhao L, Ye G, Shi F, Shu G, Jia R. Antiviral effect of sulfated Chuanmingshen violaceum polysaccharide in chickens infected with virulent Newcastle disease virus. Virology. 2015 Feb;476:316-22

Newcastle disease virus (NDV) belonging to the Paramyxovirinae subfamily is one of the most devastating pathogens in poultry. Although vaccines are widely applied to control the infection, outbreaks of Newcastle disease (ND) repeatedly happen. Currently, there are no alternative control measures available for ND. In the present study, we found that sulfated Chuanmingshen violaceum polysaccharide (sCVPS) were potent inhibitors of NDV in specific pathogen free chickens infected with a virulent strain. With sCVPS treatment, the survival rate increased by almost 20% and virus titers in test organs, including brain, lung, spleen and thymus, were significantly decreased. The sCVPS also exhibited the ability to prevent viral transmission by reducing the amount of virus shed in saliva and feces. Higher concentrations of interferon α and γ in serum were detected in chickens treated with sCVPS, indicating that one of the antiviral mechanisms may be attributed to the property of immunoenhancement. Histopathological examination showed that sCVPS could alleviate the tissue lesions caused by NDV infection. These results suggest that sCVPS are expected to be a new alternative control measure for NDV infection and further studies could be carried out to evaluate the antiviral activity of sCVPS against other paramyxoviruses.

Yang WC, Tien YJ, Chung CY, Chen YC, Chiou WH, Hsu SY, Liu HY, Liang CL, Chang CL. Effect of Bidens pilosa on infection and drug resistance of Eimeria in chickens. Res Vet Sci. 2015 Feb;98:74-81.

Extensive use of current anti-coccidial drugs together with drug resistance and residue has raised concerns about public health and poultry development. Here, we studied the anti-coccidial properties of Bidens pilosa. A phytochemical approach was developed for analysis of B. pilosa utilized as a feed additive. The protective effects of B. pilosa supplemented chicken diet were evaluated chickens infected with Eimeria tenella. B. pilosa, at doses of 0.5%, 1% and 5% of the chicken diet, significantly protected against E.tenella as measured by reduction in mortality, weight loss, fecal oocyst excretion and gut pathology in chickens. Finally, drug resistance of E. tenella to B. pilosa was assessed in chickens using the anti-coccidial index. This index showed that B. pilosa induced little, if any, drug resistance to Eimeria in chickens. Collectively, this work suggests that B. pilosa may serve as a novel, natural remedy for coccidiosis with low drug resistance in chickens.

Rusinek-Prystupa E, Tatara MR. Effect of a plant preparation Citrosept on selected immunity indices in blood of slaughter turkey hens. Ann Agric Environ Med. 2014;21(3):581-4

The objective of this study was to determine the effect of per os administration of 3 various dosages of a Citrosept preparation (a grapefruit extract)to growing turkey hens on changes in their selected haematological and immunological blood indices. An attempt was also undertaken to select the most efficient dose of the preparation with respect to the mentioned indices in turkey hens. The experiment was conducted on 180 turkey hens allocated at random to 4 groups, 45 birds in each group. Samples of their full blood were analyzed for haematological indices, such as red blood cell count (RBS), haemoglobin content (Hb), haematocrit value (Ht), and white blood cell count (WBC). Samples of blood plasma were assayed to determine the activity of lysozyme (chamber-diffusive method) and heterophils capability to reduce nitro blue tetrazolium (stimulated and spontaneous NBT test). Phagocytic activity of leucocytes against Staphylococcus aureus 209P strain was assessed and expressed as the percentage of phagocytic cells (% PC) and phagocytic index (PI). The administration of the grapefruit extract to turkey hens with drinking water caused a significant increase in

haemoglobin content in blood, as well as an increase in non-specific humoral immunity marker (activity of lysozyme) and non-specific cellular immunity marker (percentage of phagocytic cells; $P \leq 0.05$). The results obtained enabled the positive evaluation of the advisability of applying the Citrosept preparation in the feeding of turkey hens at the age of 6-9 weeks. Among the doses examined, the most efficient with respect to the stimulation of the non-specific humoral and cellular immunity was the dose of 0.021 ml/kg of body weight.

Zhong X, Shi Y, Chen J, Xu J, Wang L, Beier RC, Hou X, Liu F. Polyphenol extracts from Punica granatum and Terminalia chebula are anti-inflammatory and increase the survival rate of chickens challenged with Escherichia coli. Biol Pharm Bull. 2014;37(10):1575-82

Avian pathogenic Escherichia coli (APEC) causes inflammation in multiple organs of chickens called avian colibacillosis, and results in serious economic loss to the chicken industry. Polyphenolic compounds possess a wide range of physiological activities that may contribute to their beneficial effects against inflammation-related diseases. In this study, the curative effect and mechanism of action of the polyphenolic extracts from Punica granatum L. and Terminalia chebula Retz. in chickens challenged with APEC were studied. Specific-pathogen-free white Leghorn chickens (males, 21-d old) were challenged with APEC and then given oral administration of extracts of P. granatum and T. chebula. The extracts decreased the morbidity and inflammation induced by APEC. Data from quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay showed that the extracts of P. granatum and T. chebula polyphenols (GCP) reversed the over-expression genes of the Toll-like receptor (TLR) 2, 4, and 5, down-regulated the activation of nuclear factor-kappa B signal transduction pathways, and inhibited the production of pro-inflammatory cytokines. Naturally occurring GCP may be a potential alternative medicine for the prevention or treatment of avian colibacillosis.

Parker CD, Prins C, Saliba C, Gutierrez G, Serrar M. Effect of TEX-OE(®) treatment on the development of heat shock proteins in commercial broiler chicks and the impact on performance indicators in the grow-out period. Br Poult Sci. 2014;55(5):592-7.

Heat shock proteins (HSPs) are highly conserved proteins, shown to protect organisms against physical and physiological stress. TEX-OE(®) is a patented total extract of the fruit of Opuntia ficus indica, which has been demonstrated to accelerate the development of HSPs in several animal species. One-day-old commercial broiler chicks were treated with TEX-OE(®); HSP was measured by enzyme-linked immunosorbent assay (ELISA), and a large commercial field trial investigated key performance indicators (KPIs) in treated versus untreated controls chicks. TEX-OE(®) significantly increased HSP concentrations in treated chicks versus controls. Final cumulative mortality, liveweight and percentage factory-rejects were better than in controls. The accelerated HSP response may enable chicks to cope with early stressors, which is reflected in improved KPIs.

Zhai L, Wang Y, Yu J, Hu S. Enhanced immune responses of chickens to oral vaccination against infectious bursal disease by ginseng stem-leaf saponins. Poult Sci. 2014 Oct;93(10):2473-81.

Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), is an immunosuppressive infectious disease of global economic importance in poultry. This study was designed to evaluate the effect of oral administration of ginseng stem-leaf saponins (GSLS) on humoral and gut mucosal immunity in chickens vaccinated with live IBDV vaccine, and furthermore, to test its protective efficacy against virulent IBDV challenge following vaccination. In experiment 1, chickens were orally administered with GSLS at 5 mg/kg of BW for 7 d, and then immunized with live IBDV vaccine via the oral route. Serum was sampled on 0, 1, 2, 3, 4, and 5 wk postvaccination for detecting antibody titers by ELISA, and intestinal tissues were collected on 0, 1, 3, and 5 wk postvaccination for measurement of IgA-positive cells and intestinal intraepithelial lymphocytes by immunohistochemical and hematoxylin-eosin staining, respectively. Result showed that antibody titers, IgA-positive cells and intestinal intraepithelial lymphocytes were significantly higher in chickens drinking GSLS than the control, suggesting an enhanced effect of GSLS on humoral and gut mucosal immune responses. In experiment 2, chickens were delivered with GSLS and then vaccinated in the same way as in experiment 1. The

birds were challenged with virulent IBDV at wk 3 postvaccination. Then the birds were weighed, bled, and necropsied at d 3 postchallenge and the bursae were sampled for gross and histopathological examination. Results demonstrated that GSLS provided a better protection against virulent IBDV challenge following vaccination than the control. In conclusion, oral administration of GSLS enhances both humoral and gut mucosal immune responses to IBDV and offers a better protection against virulent IBDV challenge. Considering its immunomodulatory properties to IBDV vaccine, GSLS might be a promising oral adjuvant for vaccination against infectious diseases in poultry.

Pourhossein Z, Qotbi AA, Seidavi A, Laudadio V, Centoducati G, Tufarelli V. Effect of different levels of dietary sweet orange (Citrus sinensis) peel extract on humoral immune system responses in broiler chickens. Anim Sci J. 2015 Jan;86(1):105-10.

This experiment was conducted to evaluate the effects of different levels of sweet orange (Citrus sinensis) peel extract (SOPE) on humoral immune system responses in broiler chickens. Three hundred 1-day broilers (Ross-308) were randomly allocated to treatments varying in supplemental SOPE added in the drinking water. The experimental groups consisted of three treatments fed for 42 days as follows: a control treatment without feed extract, a treatment containing 1000 ppm of SOPE and a treatment containing 1250 ppm of SOPE. All treatments were isocaloric and isonitrogenous. Broilers were vaccinated with Newcastle disease virus (NDV), avian influenza (AI), infectious bursal disease (IBD) and infectious bronchitis virus (IBV) vaccines. Antibody titer response to sheep red blood cells (SRBC) was higher in the group fed 1250 ppm of SOPE ($P < 0.05$) as well as for immunoglobulin G (IgG) and IgM. Similarly, antibody titer responses to all vaccines were constantly elevated ($P < 0.05$) by SOPE enrichment in a dose-dependent manner. Relative weights of spleen and bursa of Fabricius were unaffected by treatments. Dietary SOPE supplementation may improve the immune response and diseases resistance, indicating that it can constitute a useful additive in broiler feeding. Thus, supplying SOPE in rations may help to improve relative immune response in broiler chickens.

Sun Y, Niu L, Song M, Zhao X, Sun N, He J, Wu C, Jiang J, Bai Y, Guo J, Li H. Screening compounds of Chinese medicinal herbs anti-Marek's disease virus. Pharm Biol. 2014 Jul;52(7):841-7.

Marek's disease (MD) seriously threatens the world poultry industry and has resulted in great economic losses. Chinese medicinal herbs are a rich source for lead compounds and drug candidates for antiviral treatments. This study was to investigate the anti-MDV activity and mechanism of 20 compounds extracted from Chinese medicinal herbs. Antiviral assay, time of addition experiments, and virucidal assay were performed on chicken embryo fibroblast cells. The 50% cytotoxic concentration and 50% effective concentration were determined and, accordingly, selectivity index and inhibition ratio were calculated.Antiviral assay showed dipotassium glycyrrhizinate (DG) and sodium tanshinone IIA sulfonate (STS) exhibited significantly inhibitory activity against MDV in a dose-dependent manner. EC50 of DG and STS were 893.5 ± 36.99 μg/mL and 54.82 ± 2.99 μg/mL, and selective index (SI) were >3.36 and >9.12, respectively. Time of addition experiment and virucidal assay demonstrated DG inhibited viral replication in the full replication cycle and inactivated MDV particles in non-time-dependent manner, but STS interfered with the early stage of MDV replication and inactivated MDV particles in a time-dependent manner. Moreover, both DG and STS promoted apoptosis of cells infected by MDV. DG and STS have great potential for developing new anti-MDV drugs for clinic application.

Tanweer AJ, Chand N, Saddique U, Bailey CA, Khan RU. Antiparasitic effect of wild rue (Peganum harmala L.) against experimentally induced coccidiosis in broiler chicks. Parasitol Res. 2014 Aug;113(8):2951-60.

Organic farming of poultry has increased in recent years as the prophylactic use of antibiotics has come into disfavor. This study was conducted to explore the antiparasitic effect of a methanolic extract of Peganum harmala in broilers challenged with coccidiosis. For this purpose, 200 1-week-old broiler chicks were divided into five treatments: negative control (basal diet, Ph-0/NC), positive control (basal diet with coccidiosis challenge, Ph-0/C), and three groups challenged with coccidiosis and supplemented with P. harmala at the rate of 200 mg L(-1) (Ph-200), 250 mg L(-1) (Ph-250), and 300 mg L(-1) (Ph-300) drinking water. Each group had three replicates of ten chicks each. Challenge with standard dose of the larvae of coccidiosis and supplementation of P. harmala were initiated on day 14 until 35 days of age. As expected, the results revealed

that weight gain, feed intake, and feed conversion ratio (FCR) were depressed significantly in Ph-0 group with significant mortality percentage. Weight gain, total body weight, and FCR increased linearly with increasing dose of P. harmala with the exception of feed intake. The growth and feed efficiency of Ph-0/NC was better in Ph-0/NC compared to that in Ph-0/C and comparable to that in P. harmala-treated birds. Similarly, mean ooccytes per gram (OPG) decreased linearly ($P < 0.05$) in supplemented groups compared to that in Ph-0/C. Histological evidences showed that cecal lesion and leucocyte infiltration decreased markedly in supplemented groups of P. harmala specifically the Ph-300 group compared to those in Ph-0/C. From the present experiment, we concluded the anticoccidial effect of P. harmala in broiler chicks.

Zeng ZK, Li QY, Piao XS, Liu JD, Zhao PF, Xu X, Zhang S, Niu S.Forsythia suspensa extract attenuates corticosterone-induced growth inhibition, oxidative injury, and immune depression in broilers. Poult Sci. 2014 Jul;93(7):1774-81.

Forsythia suspensa extract (FSE) has been demonstrated to attenuate physiological stress induced by high temperature or high stocking density. This experiment was conducted with 144 male Arbor Acre broilers (1-d-old, weighing 42.7 ± 1.7 g) to determine the effects of FSE on performance, nutrient digestibility, antioxidant activities, serum metabolites, and immune parameters for birds treated with corticosterone (CS). The birds were randomly allotted to 1 of 4 treatments in a 2 × 2 factorial arrangement that included FSE supplementation (0 or 100 mg/kg) and CS administration (0 or 20 mg/kg of diet for 7 consecutive days starting on d 14). The feeding program consisted of a starter diet from d 1 to 21 and a finisher diet from d 22 to 42. Corticosterone administration decreased ($P < 0.01$) ADG and impaired ($P < 0.01$) feed conversion ratio in both phases and overall, which were alleviated ($P < 0.01$) by dietary FSE supplementation in the finisher phase and overall. At d 21, CS administration caused decreases ($P < 0.05$) in the apparent digestibility of energy, relative weight of bursa and thymus, total antioxidant capacity, superoxide dismutase (SOD) activity, and antibody titers to Newcastle disease virus (NDV); however, serum malondialdehyde and uric acid were increased. All of these changes were attenuated ($P < 0.05$) by dietary FSE supplementation. At d 42, FSE supplementation improved ($P < 0.05$) the apparent digestibility of DM and CP, relative weights of bursa, SOD activity, and antibody titers to NDV, which were impaired by CS administration. Interactions ($P < 0.05$) were noted between CS and FSE for ADG and feed conversion ratio in the finisher phase and overall, as well as total antioxidant capacity, SOD activity, uric acid, and antibody titers to NDV at d 21, as well as relative weights of thymus at d 42. In conclusion, dietary FSE supplementation enhanced nutrient digestibility and performance of broiler possibly by reducing oxidative stress and immune depression challenged by CS.

Drăgan L, Györke A, Ferreira JF, Pop IA, Dunca I, Drăgan M, Mircean V, Dan I, Cozma V. Effects of Artemisia annua and Foeniculum vulgare on chickens highly infected with Eimeria tenella (phylum Apicomplexa). Acta Vet Scand. 2014 Apr 15;56:22.

Intensive poultry production systems depend on chemoprophylaxis with anticoccidial drugs to combat infection. A floor-pen study was conducted to evaluate the anticoccidial effect of Artemisia annua and Foeniculum vulgare on Eimeria tenella infection. Five experimental groups were established: negative control (untreated, unchallenged); positive control (untreated, challenged); a group medicated with 125 ppm lasalocid and challenged; a group medicated with A. annua leaf powder at 1.5% in feed and challenged; and a group treated with the mixed oils of A. annua and Foeniculum vulgare in equal parts, 7.5% in water and challenged. The effects of A. annua and oil extract of A. annua + F. vulgare on E. tenella infection were assessed by clinical signs, mortality, fecal oocyst output, faeces, lesion score, weight gain, and feed conversion. Clinical signs were noticed only in three chickens from the lasalocid group, six from the A. annua group, and nine from the A. annua + F. vulgare group, but were present in 19 infected chickens from the positive control group. Bloody diarrhea was registered in only two chickens from A. annua group, but in 17 chickens from the positive control group. Mortality also occurred in the positive control group (7/20). Chickens treated with A. annua had a significant reduction in faecal oocysts (95.6%; $P = 0.027$) and in lesion score (56.3%; $P = 0.005$) when compared to the positive control. At the end of experiment, chickens treated with A. annua leaf powder had the highest body weight gain (68.2 g/day), after the negative control group, and the best feed conversion (1.85) among all experimental groups. Our results suggest that A. annua leaf powder (Aa-p), at 1.5% of the daily diet post-infection, can be a valuable alternative for synthetic coccidiostats, such as lasalocid.

Habibi R, Sadeghi G, Karimi A. Effect of different concentrations of ginger root powder and its essential oil on growth performance, serum metabolites and antioxidant status in broiler chicks under heat stress. Br Poult Sci. 2014;55(2):228-37

This study was carried out to evaluate the impact of ginger (Zingiber officinale) feed supplementation on growth performance, antioxidant status, carcass characteristics and blood parameters in broiler chicks under conditions of heat stress (32 ± 2ºC for 8 h per d). 2. A total of 336 d-old male broiler chicks (Cobb-500) were randomly assigned to one of 6 dietary groups representing: basal diet with no supplement as control, basal diet containing 100 mg/kg vitamin E as positive control, basal diets containing either 7.5 or 15 g/kg of ginger root powder, and diets containing 75 or 150 mg/kg of ginger essential oil. 3. The results indicated that at 22 d of age, the group receiving 7.5 g/kg of ginger root powder experienced significantly increased body weight (BW) and body weight gain (BWG) compared to the control group. There were no significant difference among the diet groups regarding BW, BWG, feed intake (FI) or feed conversion ratio (FCR) at 42 and 49 d of age. 4. The inclusion of powder and essential oil of ginger in broiler diets did not affect carcass characteristics and blood parameters of the chickens. However, in the group receiving 150 mg/kg ginger essential oil, the total superoxide dismutase (TSOD) activity in liver increased compared to the control group. Malondialdehyde (MDA) concentrations in liver also decreased in the groups receiving ginger powder and essential oil compared to that in the control group. There were no significant difference between experimental groups regarding glutathione peroxidise (Gpx), TSOD and catalase (CAT) enzymes in red blood cells. All dietary groups increased total antioxidant capacity (TAC) and decreased MDA concentration in serum compared to the control group. 5. The results of this study suggest that ginger powder and essential oils may be a suitable replacement for synthetic antioxidants in broiler diets. Results also suggest that ginger powder might be better than extracted essential oil for improving antioxidant status in broilers.

Bozkurt M[1], Giannenas I, Küçükyilmaz K, Christaki E, Florou-Paneri P. An update on approaches to controlling coccidia in poultry using botanical extracts. Br Poult Sci. 2013;54(6):713-27.

This paper reviews the use of botanical extracts in the control of coccidial infection in poultry. 2. Some plants and their respective volatile oils and extracts have the potential to alleviate coccidiosis and reduce its severity. 3. Most plant bioactives improve some, but not all, aspects of coccidiosis with variable effectiveness against different species of Eimeria. 4. Difficulties in comparing research findings have arisen from the use of different experimental models, different active components and infectious dose of Eimeria. 5. Current knowledge of their potential anti-coccidial effects may provide guidance for the use of botanical extracts in the control of the coccidiosis.

Patial V, Asrani RK, Patil RD, Ledoux DR, Rottinghaus GE. Pathology of ochratoxin A-induced nephrotoxicity in Japanese quail and its protection by sea buckthorn (Hippophae rhamnoides L.).Avian Dis. 2013 Dec;57(4):767-79.

The present study was designed to study the protective effect of sea buckthorn (SBT) against renal damage induced by ochratoxin A (OTA) in Japanese quail. Day-old quail chicks were divided into six groups and fed a basal quail chick mash containing 2% SBT leaf powder (group SX), OTA at a dietary level of 3 ppm (group OX), 25 ppm L-beta-phenylalanine (Phe) plus 3 ppm OTA (group OP), 2% dietary level of SBT leaf powder plus 3 ppm OTA (group OS), SBT leaf extract at a level of 10%/L of drinking water plus 3 ppm OTA (group OSS), and a standard toxin-free feed (group CX, control) for 21 days. OTA at 3 ppm level in diet grossly revealed mild to moderate renal swelling in OX birds, and the severity was less in the case of OS, OSS, and OP birds. Microscopically, degenerative, necrotic, and inflammatory changes were observed in OX birds, but the changes were less severe in OS, OSS, and OP birds. Ultrastructural studies revealed remarkable and consistent changes in the proximal convoluted tubules (PCTs), with severe damage of mitochondria and endoplasmic reticulum in OX birds, whereas SBT-treated birds (groups OS, OSS) had mild changes in mitochondria. A moderate to marked increase in number of peroxisomes in the cytoplasm of PCTs was a consistent finding in the Phe- and SBT-treated groups kept on OTA in comparison to the group fed OTA alone. In conclusion, the inclusion of 2% SBT leaf powder in feed and SBT leaf extract in water provided partial protection against OTA-induced nephropathy in Japanese quail.

Almeida GF, Thamsborg SM, Madeira AM, Ferreira JF, Magalhães PM, Demattê Filho LC, Horsted K, Hermansen JE. The effects of combining Artemisia annua and Curcuma longa ethanolic extracts in broilers challenged with infective oocysts of Eimeria acervulina and E. maxima. Parasitology. 2014 Mar;141(3):347-55.

Due to an increasing demand for natural products to control coccidiosis in broilers, we investigated the effects of supplementing a combination of ethanolic extracts of Artemisia annua and Curcuma longa in drinking water. Three different dosages of this herbal mixture were compared with a negative control (uninfected), a positive control (infected and untreated), chemical coccidiostats (nicarbazin+narazin and, later, salinomycin), vaccination, and a product based on oregano. Differences in performance (weight gain, feed intake, and feed conversion rate), mortality, gross intestinal lesions and oocyst excretion were investigated. Broilers given chemical coccidiostats performed better than all other groups. Broilers given the two highest dosages of the herbal mixture had intermediate lesion scores caused by Eimeria acervulina, which was higher than in broilers given coccidiostats, but less than in broilers given vaccination, oregano and in negative controls. There was a trend for lower mortality (P = 0·08) in the later stage of the growing period (23-43 days) in broilers given the highest dosage of herbal mixture compared with broilers given chemical coccidiostats. In conclusion, the delivery strategy of the herbal extracts is easy to implement at farm level, but further studies on dose levels and modes of action are needed.

Kim DK, Lillehoj HS, Lee SH, Jang SI, Lillehoj EP, Bravo D. Dietary Curcuma longa enhances resistance against Eimeria maxima and Eimeria tenella infections in chickens.Poult Sci. 2013 Oct;92(10):2635-43.

The effects of dietary supplementation with an organic extract of Curcuma longa on systemic and local immune responses to experimental Eimeria maxima and Eimeria tenella infections were evaluated in commercial broiler chickens. Dietary supplementation with C. longa enhanced coccidiosis resistance as demonstrated by increased BW gains, reduced fecal oocyst shedding, and decreased gut lesions compared with infected birds fed a nonsupplemented control diet. The chickens fed C. longa-supplemented diet showed enhanced systemic humoral immunity, as assessed by greater levels of serum antibodies to an Eimeria microneme protein, MIC2, and enhanced cellular immunity, as measured by concanavalin A-induced spleen cell proliferation, compared with controls. At the intestinal level, genome-wide gene expression profiling by microarray hybridization identified 601 differentially expressed transcripts (287 upregulated, 314 downregulated) in gut lymphocytes of C. longa-fed chickens compared with nonsupplemented controls. Based on the known functions of the corresponding mammalian genes, the C. longa-induced intestinal transcriptome was mostly associated with genes mediating anti-inflammatory effects. Taken together, these results suggest that dietary C. longa could be used to attenuate Eimeria-induced, inflammation-mediated gut damage in commercial poultry production.

Bazh EK, El-Bahy NM.In vitro and in vivo screening of anthelmintic activity of ginger and curcumin on Ascaridia galli. Parasitol Res. 2013 Nov;112(11):3679-86.

Intestinal helmintic infection, continue to be a cause of major concern in several parts of the world, particularly in the developing nations. The use of plant extracts to control poultry helminths is increasing in different rearing systems. The anthelmintic activity of ginger and curcumin was studied on the nematode Ascaridia galli. In vitro and in vivo studies were allocated. Live parasites for in vitro studies were collected from the intestine of naturally infected chickens. Some living worms were incubated at 37 °C in media containing ginger at three concentration levels (25, 50, and 100 mg/ml), and others were incubated in media containing curcumin at the same concentration levels. Another living worm group was incubated in media containing albendazole at a dose of 7.5 mg/ml. The extracts' efficacy was exhibited in a concentration-time-dependent manner mainly at 100 mg/ml and after 48 h. The in vivo study takes place on experimentally infected chickens. Group of infected chickens was treated with ginger extract at dose of 100 mg, another group was treated with curcumin extract at dose of 100 mg, and a third group was treated with albendazole at dose of 7.5 mg. In vivo study of ginger and curcumin recorded lower mortality rates than the in vitro study. It is concluded that ginger and curcumin extracts have potential anthelmintic properties against A. galli. Ginger in all concentrations used exhibited a higher death rate observed than curcumin. Their wormicidal effect is concentration-time dependent.

Li XT, Wang B, Li JL, Yang R, Li SC, Zhang M, Huang W, Cao L. Effects of Dangguibuxue Tang, a Chinese herbal medicine, on growth performance and immune responses in broiler chicks. Biol Res. 2013;46(2):183-8.

The effects of Dangguibuxue Tang (DBT) on growth performance and immunity response in immunosuppressed broiler chicks were investigated in this study. 240 one-d-old broiler chicks (DaHeng S01) were randomly divided into 4 groups, 2.0% DBT-treatment (A), 0.5% DBT-treatment (B), cyclophosphamide-control (C), and control group (D). From 4 d to 7 d of age, chicks in group A, B and C were given cyclophosphamide (CY) at a dosage of 100mg/kg body weight (BW) daily by intraperitoneal injection to induce immunosuppression. Chicks in group D were given an equal volume of physiological saline daily by intraperitoneal injection and considered normal chicks. Groups A and B were supplemented with 2.0% or 0.5% of DBT in the drinking water from 8 d to 42 d of age. Groups C and D did not receive any additional medication. The results revealed that chicks from group B had lower feed:gain rate (FGR), lower total mortality, higher immunity organ indexes, higher levels of Newcastle disease (ND) antibody and infectious bursal disease (IBD) antibody, higher interleukin-2 and interleukin-6 levels, and greater lymphocyte proliferative responses to concanavalin A (ConA) during the experiment than those from group C. However, no significant difference in the immunity status in the two levels of DBT-treatment was observed. These results indicate that supplementation of 0.5% of DBT can improve both cellular immunity and humoral immunity in immunosuppressed broiler chicks.

Solcan C, Gogu M, Floristean V, Oprisan B, Solcan G. The hepatoprotective effect of sea buckthorn (Hippophae rhamnoides) berries on induced aflatoxin B1 poisoning in chickens 1. Poult Sci. 2013 Apr;92(4):966-74.

The leaves and berries of sea buckthorn (SB; Hippophae rhamnoides; family Elaeagnaceae) are medically claimed as having phytoantioxidant, antiinflammatory, and anticancerous properties in humans. This study evaluated the hepatoprotective activity of oil from SB berries against toxicity induced by aflatoxin B1 (AFB1) in broiler chickens. The toxicity of AFB1 led to lower total serum proteins and specifically reduced albumin ($P < 0.001$). Serum aspartate aminotransferase increased from 191.14 ± 11.56 to 218.80 ± 13.68 ($P < 0.001$). When chickens were simultaneously dosed with AFB1 and an extract of SB berries, subsequent histology of the liver showed a significant reduction of necrosis and fatty formation compared with chickens treated with AFB1 alone. Immunohistochemical results indicated that COX2, Bcl-2, and p53 were highly expressed in the liver of AFB1-treated chickens and their expression was significantly reduced by SB oil supplementation. The levels of AFB1 residues in chickens livers were significantly reduced by SB oil from 460.92 ± 6.2 ng/mL in the AFB1 group to 15.59 ± 6.1 ng/mL in the AFB1 and SB oil group. These findings suggest that SB oil has a potent hepatoprotective activity, reducing the concentration of aflatoxins in liver and diminishing their adverse effects.

Shang R, He C, Chen J, Pu X, Liu Y, Hua L, Wang L, Liang J. Hypericum perforatum extract therapy for chickens experimentally infected with infectious bursal disease virus and its influence on immunity. Can J Vet Res. 2012 Jul;76(3):180-5.

Hypericum perforatum extract (HPE) has been proved a drug effective to many viral diseases. The purpose of this paper was to investigate the therapeutic efficacy and immuno-enhancement of HPE for chickens which were already challenged with infectious bursal disease virus (IBDV BC-6/85). Chickens infected with IBDV were treated with HPE for 5 consecutive days, the observation of immune organ indexes and pathological changes index, determination of IFN-α and detection of IBDV with RT-PCR were employed to assess in vivo whether or not HPE had the certain therapeutic efficacy on infectious bursal disease (IBD), and if HPE was able to improve the immunologic function. The results showed that 1330 and 667.9 mg/kg body weight (BW) per day of HPE had significant therapeutic efficacy and improvement immunologic functions for chickens infected experimentally with IBDV.

Zhang DF[1], Sun BB, Yue YY, Zhou QJ, Du AF. Anticoccidial activity of traditional Chinese herbal Dichroa febrifuga Lour. extract against Eimeria tenella infection in chickens. Parasitol Res. 2012 Dec;111(6):2229-33.

The study was conducted on broiler birds to evaluate the anticoccidial efficacy of an extract of Chinese traditional herb Dichroa febrifuga Lour. One hundred broiler birds were assigned to five equal groups. All birds in groups 1-4 were orally infected with 1.5 × 10(4) Eimeira tenella sporulated oocysts and birds in groups 1, 2 and 3 were medicated with 20, 40 mg extract/kg feed and 2 mg diclazuril/kg feed, respectively. The bloody diarrhea, oocyst counts, intestinal lesion scores, and the body weight were recorded to evaluate the anticoccidial efficacy. The results showed that D. febrifuga extract was effective against Eimeria infection; especially 20 mg D. febrifuga extract/kg feed can significantly increase body weight gains and reduce bloody diarrhea, lesion score, and oocyst excretion in comparison to infected-unmedicated control group.

Akhtar M, Hai A, Awais MM, Iqbal Z, Muhammad F, ul Haq A, Anwar MI. Immunostimulatory and protective effects of Aloe vera against coccidiosis in industrial broiler chickens. Vet Parasitol. 2012 May 25;186(3-4):170-7.

This paper reports the immunostimulatory and protective effects of Aloe vera extracts (aqueous and ethanolic) against coccidiosis in industrial broiler chickens. The study was divided into two experiments. Experiment-I was conducted for the evaluation of immunostimulatory activity of A. vera and experiment-II demonstrated the protective efficacy of A. vera extracts against coccidiosis in chickens. Results of the experiment-I revealed significantly higher ($p<0.05$) lymphoproliferative responses in chickens administered with ethanolic extract of A. vera as compared to those administered with aqueous extract and control group. Microplate haemagglutination assay for humoral response on day 7th and 14th post primary and secondary injections of sheep red blood cells (SRBCs) revealed significantly higher ($p<0.05$) anti SRBC antibody (total Igs, IgG and IgM) titers in chickens of experimental groups as compared to the control group. None of the extracts, however, demonstrated significant effects on the development of lymphoid organs. Results of experiment-II revealed maximum protection (60%) in chickens administered with aqueous Aloe extract as compared to the ethanolic extract administered chickens (45%). Mean oocysts per gram of droppings in the control group was significantly higher ($p<0.05$) as compared to the chickens in both the experimental groups. Chickens administered with aqueous Aloe extract showed a minimal mean lesion score (2.3) followed by those administered with ethanolic Aloe extract (2.6) and control chickens (3.05) for caeca, and a similar pattern was observed for intestinal lesion scoring. Further, significantly higher weight gains and antibody titers ($p<0.05$) were observed in chickens administered with A. vera extracts as compared to those in the control group. It was concluded that A. vera may be a potential and valuable candidate to stimulate the immune responses and can be used successfully as an immunotherapeutic agent against coccidiosis in industrial broiler chickens.

IX-G Veterinary Botanical Medicine and Swine

Wang G, Kang S, Yin Z, etal Therapeutic effect of Chinese patent medicine "Wuhuanghu" on porcine infectious pleuropneumonia and its acute and subchronic toxicity as well as evaluation of safety pharmacology. Environ Toxicol Pharmacol. 2015 Sep;40(2):388-96.

Chinese patent medicines play an important role in veterinary clinical use. The aim of this study is to research the anti-infection effect of Chinese patent medicine "Wuhuanghu" for the treatment of porcine infectious pleuropneumonia and to evaluate the safety of "Wuhuanghu" in order to provide a comprehensive understanding of its toxicity. The anti-infection results showed that the treatment with "Wuhuanghu" could significantly inhibit pneumonia and decrement of the pneumonia in high, medium and low doses of "Wuhuanghu" groups were 70.97%, 61.29% and 58.06% respectively. The acute toxicity test showed that rats in the highest group (5000mg/kg) had no death and no abnormal response, suggesting the LD50 of "Wuhuanghu" was more than 5000mg/kg. The subchronic toxicity study showed that hematology indexes in all groups had no obvious differences; blood biochemical index, only albumin and total cholesterol in middle and low doses of "Wuhuanghu" groups were significantly decreased when compared with control group. The clinical pathology showed that the target organ of "Wuhuanghu" was liver. The safety pharmacology study indicated that "Wuhuanghu" had no side effects on rats. In conclusion, "Wuhuanghu" has therapeutic and protective effects to porcine infectious pleuropneumonia in a dose-dependent manner and "Wuhuanghu" is a safe veterinary medicine.

Maneewan C[1], Mekbungwan A, Charerntantanakul W etal Effects of dietary Centella asiatica (L.) Urban on growth performance, nutrient digestibility, blood composition in piglets vaccinated with Mycoplasma hyopneumoniae. Anim Sci J. 2014 May;85(5):569-74.

To investigate the effects of Centella asiatica (L.) on growth performance, nutrient digestibility and blood composition in piglets, 32 nursery pigs were fed 0.0, 0.5, 1.0 and 2.0% dietary C. asiatica (L.) from 15 to 90 kg BW. At 30 kg BW, nutrient digestibility was measured and at 35 kg BW piglets were vaccinated with Mycoplasma hyopneumoniae. Hematological parameters were checked at 40 and 80 kg BW. Compared with the control, growth performance was not affected. The ether extract, ash and calcium digestibility were lower at 0.5%, and dry matter, crude protein, crude fat, phosphorus and energy digestibility were lower at 1.0% ($P<0.05$). On hematological values, at 40 kg hematocrit, total white blood cells, neutrophils, eosinophils, basophils, monocytes and lymphocytes were higher at the 2.0% level ($P<0.05$). Most of these values except basophils and monocytes continued until at 80 kg, at which total white blood cells, neutrophils, eosinophils and lymphocytes were higher even at 1.0% ($P<0.05$); neutrophil-to-lymphocyte ratio tended to be higher at 2.0% ($P<0.03$). Cholesterol, triglycerides and antibody levels against M. hyopneumoniae did not differ except that at 40 kg the cholesterol of 0.5% was lower ($P<0.05$) and M. hyopneumoniae-specific antibodies tended to be higher with increasing levels of C. asiatica (L.) ($P<0.07$). The result that C. asiatica (L.) could not improve growth performance but increased values of serum hematocrit and white blood cells, and mycoplasma immunity to M. hyopneumoniae might suggest that C. asiatica (L.) has no function to elevate body weight but has the potential to enhance innate immunity.

Frankic T[1], Salobir K, Salobir J. The comparison of in vivo antigenotoxic and antioxidative capacity of two propylene glycol extracts of Calendula officinalis (marigold) and vitamin E in young growing pigs. J Anim Physiol Anim Nutr (Berl). 2009 Dec;93(6):688-94.

The objective of the study was to evaluate the protective effect of Calendula officinalis propylene glycol extracts against oxidative DNA damage and lipid peroxidation induced by high polyunsaturated fatty acid (PUFA) intake in young growing pigs. Forty young growing pigs were assigned to five treatment groups:

control; oil (linseed oil supplementation); C. officinalis 1 and 2 groups (linseed oil plus 3 ml/day of C. officinalis propylene glycol extracts); and vitamin E group (linseed oil plus 100 mg/kg of vitamin E). Lymphocyte DNA fragmentation and 24-h urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) excretion were measured to determine DNA damage. Lipid peroxidation was studied by analysing plasma and urine malondialdehyde (MDA), and urine isoprostane concentrations (iPF2α-VI), total antioxidant status of plasma and glutathione peroxidase (GPx) assays. C. officinalis 1 (extract from petals) effectively protected DNA from oxidative damage. It indicated a numerical trend towards the reduction of plasma MDA and urinary iPF2α-VI excretion. Its effect was comparable with that of vitamin E. C. officinalis 2 (extract from flower tops) showed less antioxidant potential than the extract from petals. We can conclude that the amount of C. officinalis extracts proposed for internal use by traditional medicine protects the organism against DNA damage induced by high PUFA intake.

van Krimpen MM[1], Binnendijk GP, Borgsteede FH, Gaasenbeek CP. Anthelmintic effects of phytogenic feed additives in Ascaris suum inoculated pigs. Vet Parasitol. 2010 Mar 25;168(3-4):269-77.

Two experiments were performed to determine the anthelmintic effect of some phytogenic feed additives on a mild infection of Ascaris suum in growing and finishing pigs. Usually, an infection of A. suum is controlled by using conventional synthetic drugs. Organic farmers, however, prefer a non-pharmaceutical approach to worm control. Therefore, phytotherapy could be an appropriate alternative. In the first experiment, a commercial available organic starter diet was supplemented with 3% of a herb mixture, adding 1% Thymus vulgaris, 1% Melissa officinalis and 1% Echinacea purpurea to the diet, or with 4% of a herb mixture, thereby adding the mentioned herbs plus 1% Camellia sinensis (black tea). A negative control group (no treatment) and a positive control group (treatment with conventional synthetic drug flubendazole) were included. In the second experiment, the anthelmintic properties against A. suum of three individual herbs, Carica papaya, Peumus boldus and Artemisia vulgaris, each in a dose of 1%, were tested. Pigs were infected with 1000 infective worm eggs each. Each experiment was performed with 32 individually housed growing pigs (8 replicates/treatment), which were monitored for 67 days. It was hypothesized that the herbs would block the cycles of the larvae, thereby preventing the development of adult worms. Therefore, phytogenic feed additives were not supplied during the whole experimental period, but only from the start until D39. Pigs were inoculated with infective worm eggs during five consecutive days (D17-D21). At D67 all pigs were dissected, whereafter livers were checked for the presence of white spots. Also numbers of worms in the small intestine were counted. In experiment 1, the numbers of worm-infected pigs were similar for both the herb supplemented (groups 3 and 4) and the unsupplemented (group 1) treatments (5-6 pigs of 8), while the treatment with flubendazole (group 2) resulted in 0 infected pigs. In experiment 2, herb addition (groups 2-4) did not significantly reduce the number of worm-infected pigs compared to the negative control (group 1). It can be concluded that the tested herb mixtures and individual herbs in the diets of growing and finishing pigs did not decrease the number of pigs which were infected with A. suum, although the herb mixture without black tea and also boldo leaf slightly ($P<0.10$) reduced the number of worms in the intestinal tract. The tested herb mixtures and individual herbs did not affect the performance of the pigs.

Kim HB[1], Lee CY[2], Kim SJ[3], etal Have Medicinal herb extracts ameliorate impaired growth performance and intestinal lesion of newborn piglets challenged with the virulent porcine epidemic diarrhea virus. J Anim Sci Technol. 2015 Oct 8;57:33.

The objective of this study was to evaluate effects of a combined use of extracts of medicinal herbs Taraxaumi mongolicum, Viola yedoensis Makino, Rhizoma coptidis, and Radix isatidis (MYCI) on porcine epidemic diarrhea (PED). Twenty-two 3-day-old piglets received an oral challenge with 3 × 10(3.5) TCID50 of the virulent PED virus (PEDV) in PBS or PBS only and daily oral administration of 60 mg of the MYCI mixture suspended in milk replacer or the vehicle for 7 days in a 2 × 2 factorial arrangement of treatments. Average daily gain (ADG) increased ($p < 0.05$) in response to the MYCI treatment in the PEDV-challenged piglets (-18 vs. 7 g for the vehicle- vs. MYCI-administered group), but not in unchallenged animals (27 vs. 28 g). Diarrhea score and fecal PEDV shedding, however, were not influenced by the MYCI treatment. The PEDV challenge caused severe intestinal villus atrophy and crypt hyperplasia, both of which were alleviated by administration of the MYCI mixture as indicated by an increase in the villus height and a decrease in the crypt depth due to the treatment. Overall, medicinal herb extracts used in this study ameliorated impaired growth performance and intestinal

lesion of newborn piglets challenged with the virulent PEDV. Therefore, our results suggest that the MYCI mixture could be used as a prophylactic or therapeutic agent against PED.

Stelter K[1], Frahm J, Paulsen J etal Effects of oregano on performance and immunmodulating factors in weaned piglets. Arch Anim Nutr. 2013 Dec;67(6):461-76.

Many health effects can be attributed to the Mediterranean herb oregano (Origanum vulgare L.) and several studies demonstrated the improving effect on performance, changes in blood count, antibacterial, antifungal and immunmodulating abilities. The majority of these investigations were carried out with processed essential oil, while whole plant material was only used in a few studies. Thus, the aim of the present experiment was to test the effect of increasing proportions of dried oregano in piglet feed on health and performance, with a special focus on immune modulation. A total of 80 male castrated weaned piglets (body weight [BW] 7.9 kg ± 1.0 kg) were used in a feeding experiment lasting 5 weeks. They were assigned to 4 experimental groups: a control diet, and three diets with an oregano supplementation at 2 g, 4 g and 8 g per kg feed, respectively, corresponding to 23.5 mg, 46.9 mg and 93.9 mg carvacrol/kg DM. After 3 weeks, half of each group was challenged with 5 µg lipopolysaccharides (LPS) per kg BW. Blood samples were collected 2 h after LPS stimulation and analysed for T-cell phenotypes, granulocyte activity, clinical-chemistry as well as white and red blood count. The results indicate no effects of oregano on performance. In contrast, oregano altered the lymphocyte proportion and the ratio of CD4(+) and CD8(+) T-cells as well as the triglyceride concentration in the serum of non-stimulated and in LPS-stimulated piglets. In conclusion, whole plant supplementation of oregano to piglet feed altered immune-related parameters, but did not modulate the acute inflammatory response induced by LPS stimulation.

Malo C[1], Gil L, Cano R, Martínez F, Galé I. Antioxidant effect of rosemary (Rosmarinus officinalis) on boar epididymal spermatozoa during cryopreservation. Theriogenology. 2011 Jun;75(9):1735-41

The objective of the present study was to evaluate the ability of rosemary to protect epididymal boar spermatozoa from freeze-thaw damage. Testis from eight boars were collected at the slaughterhouse in two trials. In the laboratory, sperm from epididymis were recovered by flushing and cryopreserved in lactose-egg yolk solution supplemented with various concentrations (low; medium; high) of rosemary. After thawing, total motility, viability, acrosome integrity, response to hypoosmotic swelling test (HOST) and malonaldehide (MDA) concentration were assessed. The results showed that there was an increase in motility at 1, 2 and 3 h in the presence of rosemary. The addition of this herb provided a significant beneficial effect on viability at 2 h of incubation, compared to the control group. Conversely, acrosome status was not affected by any extender. Higher concentration of rosemary produced significant improvement in percentages of positive HOST at 0 and 1 h, whereas no impact was observed at the end of incubation. Considering membrane lipid peroxidation, a greater decrease in MDA production was observed when rosemary content was raised. Rosemary-enriched freezing extender improved the post-thaw epididymis boar spermatozoa quality, showing a significant correlation between rosemary concentration and concentration of MDA. Further studies are needed to define the active component in rosemary that prevents peroxidation.

Chang CH[1], Chen YS, Chiou MT etal Have Application of Scutellariae radix, Gardeniae fructus, and Probiotics to Prevent Salmonella enterica Serovar Choleraesuis Infection in Swine. Evid Based Complement Alternat Med. 2013;2013:568528

Salmonella enterica serovar Choleraesuis, a host-adapted pathogen of swine, usually causes septicemia. Lactic acid bacteria (LAB) strains have been widely studied in recent years for their probiotic properties. In this study, a mouse infection model first screened for potential agents against infection, then a pig infection model evaluated effects of LAB strains and herbal plants against infection. Scutellariae radix (SR) and Gardeniae fructus (GF) showed abilities to reduce bacteria shedding and suppressing serum level of TNF- α induced by infection in swine. Bioactivities of SR and GF were enhanced by combining with LAB strains, which alone could speed up the bacteria elimination time in feces and boost immunity of infected pigs. Baicalein and genipin exhibited stronger cytotoxicity than baicalin and geniposide did, as well as prevent Salmonella from invading macrophages. Our study suggests LAB strains as exhibiting multiple functions: preventing infection, enhancing immunity to prepare host defenses against further infection, and adjusting intestinal microbes' enzymatic

activity in order to convert herbal compounds to active compounds. The SR/GF-LAB strain mixture holds potential infection-prevention agents supplied as feed additives.

Alfajaro MM[1], Kim HJ, Park JG etal Have Anti-rotaviral effects of Glycyrrhiza uralensis extract in piglets with rotavirus diarrhea. Virol J. 2012 Dec 18;9:310.

Since rotavirus is one of the leading pathogens that cause severe gastroenteritis and represents a serious threat to human and animal health, researchers have been searching for cheap, safe, and effective anti-rotaviral drugs. There is a widespread of interest in using natural products as antiviral agents, and among them, licorice derived from Glycyrrhiza spp. has exerted antiviral properties against several viruses. In this study, anti-rotaviral efficacy of Glycyrrhiza uralensis extract (GUE) as an effective and cheaper remedy without side-effects was evaluated in colostrums-deprived piglets after induction of rotavirus diarrhea. Colostrums-deprived piglets were inoculated with porcine rotavirus K85 (G5P[7]) strain. On the onset of diarrhea, piglets were treated with different concentration of GUE. To evaluate the antiviral efficacy of GUE, fecal consistency score, fecal virus shedding and histological changes of the small intestine, mRNA expression levels of inflammation-related cytokines (IL8, IL10, IFN-β, IFN-γ and TNF-α), signaling molecules (p38 and JNK), and transcription factor (NFκB) in the small intestine and spleen were determined. Among the dosages (100-400 mg/ml) administrated to animals, 400 mg/ml of GUE cured diarrhea, and markedly improved small intestinal lesion score and fecal virus shedding. mRNA expression levels of inflammation-related cytokines (IL8, IL10, IFN-β, IFN-γ and TNF-α), signaling molecules (p38 and JNK), and transcription factor (NFκB) in the small intestine and spleen were markedly increased in animals with RVA-induced diarrhea, but dose- dependently decreased in GUE treated animals after RVA-induced diarrhea. GUE cures rotaviral enteritis by coordinating antiviral and anti-inflammatory effects. Therapy of this herbal medicine can be a viable medication for curing rotaviral enteritis in animals and humans.

Yan C[1], Wang K, Chen L, He YM, Tang ZX. Effects of feeding an herbal preparation to sows on immunological performance of offspring. J Anim Sci. 2012 Nov;90(11):3778-82.

The objective of this study was to determine the effects of feeding Chinese herbal ultra-fine (CHU) powder to sows during the last week of gestation and during the lactation period on immunological performance of the offspring. In this experiment, 15 pregnant sows (mean BW = 235.6 ± 3.7 kg) were randomly assigned to 1 of 3 treatments including no additive (Control), 0.75% CHU powder (Group A), or 1.5% CHU powder (Group B) added to a maize- and soybean meal-based diet. Blood from 10 piglets per group was collected at d 7, d 14, or d 21 of age to measure serum metabolites, lymphocyte proliferating activity, and serum antibody and cytokine concentrations. Dietary supplementation of sows with CHU powder increased ($P < 0.05$) serum concentrations of total protein, albumin, and triglycerides of offspring, whereas the concentration of glucose was reduced ($P < 0.05$) compared with Controls. The CHU powder enhanced ($P < 0.05$) serum concentrations of IgG in Group B offspring on d 7 and IgM in Group A offspring on d 7 and d 14, increased IL-10 in Group A offspring on d 7, as well as IL-2 in offspring from Groups A and B on all days of determination. The CHU powder increased interferon gamma in Group A offspring on d 14 and in Group B offspring on d 14 and d 21, and increased tumor necrosis factor alpha in offspring of Group A on d 14 and in Group B on all days surveyed. Compared with Controls, a greater number ($P < 0.05$) of T lymphocyte subpopulations were detected in Group A and B offspring including CD4+ cells in Group A on d 7 and d 21, CD4+ cells in Group B on d 14 and d 21, and CD8+ cells in Group A on d 7 and d 14. Collectively, these findings indicate a beneficial effect of CHU powder treatment of sows in later gestation and during lactation on serum metabolism and cellular and humoral immune responses of their offspring.

Kang SN[1], Chu GM, Song YM etal The effects of replacement of antibiotics with by-products of oriental medicinal plants on growth performance and meat qualities in fattening pigs. Anim Sci J. 2012 Mar;83(3):245-51.

The effect of by-products of oriental medicinal plants (OMP; T1) containing 0.03% herb extracts (T2) or 0.1% aminolevulinic acid (T3) on the production performance of swine during the finishing period and on its meat quality were investigated. No significant differences were found in the weight gain, feed intake and feed conversion rate among the tested groups ($P > 0.05$). But the treated group showed higher ($P < 0.05$) moisture and ash and lower protein than the control group. The T3 group showed a lower meat cholesterol

content (38.42 mg/100 g) compared to the other groups ($P < 0.05$). The vitamin E content of the muscle in the treated groups was higher compared to the control group. No antibiotic content was detected in all treated and control samples. The values of the volatile basic nitrogen (VBN) and thiobarbituric acid reactive substance (TBARS) of the treated groups were significantly lower ($P < 0.01$) than the control group. The treated groups had significantly better ($P < 0.05$) sensory-test scores for color, flavor, off-flavor and total acceptability compared to the control group.

Guo KJ[1], Xu SF, Yin P etal Active components of common traditional Chinese medicine decoctions have antioxidant functions. J Anim Sci. 2011 Oct;89(10):3107-15

Many traditional Chinese medicine (TCM) decoctions are proven to have multiple functions in animal production. These decoctions are seldom recognized by the international scientific community because the mechanisms of action are not clearly elucidated. According to TCM theory, Cortex Phellodendri (COP), Rhizoma Atractylodes (RA), Agastache Rugosa (AR), and Gypsum Fibrosum (GF) can be used to formulate a medicinal compound that prevents or cures animal disease caused by heat stress. The aim of this research was to study the regulatory functions of the active components of TCM and to elucidate the effects of different TCM decoctions on antioxidant activity and lipid peroxide content, using in vitro and in vivo models of heat stress. For in vitro experiments, intestinal crypt-like epithelial cell line-6 (IEC-6) cells were employed to evaluate the effects of the active components of COP, RA, AR, and GF. For in vivo experiments, forty-eight 2-mo-old Chinese experimental mini-pigs (7.20 ± 0.02 kg) were randomly assigned to 4 groups: a normal-temperature group (NTG); a high-temperature group (HTG); HTG treated with COP, RA, AR, and GF (1:1:1:1, TCM1); and HTG treated with COP, RA, AR, and GF (1:1:1:0.5, TCM2). Results showed that the active components of the COP, RA, AR, and GF increased ($P < 0.05$) the proliferation and viability of heat-stressed IEC-6 cells and that the most effective treatment doses of COP alkaloid, RA Aetherolea, Herba Agastachis Aetherolea, and GF water extract were 200, 100, 100, and 200 µg/mL, respectively. All 4 active components increased ($P < 0.05$) superoxide dismutase, glutathione peroxidase activities, and glutathione content, and decreased ($P < 0.05$) malondialdehyde content with respect to the heat-stressed group to concentrations similar to those seen in NTG. In vivo experiments demonstrated that TCM1 and TCM2 improved ($P < 0.05$) the poor growth performance seen in HTG pigs. The superoxide dismutase, glutathione peroxidase activities, and malondialdehyde content in porcine jejunum treated with TCM1 and TCM2 were not different ($P > 0.05$) from those seen in the NTG and were better ($P < 0.05$) than results seen in the HTG. Overall, it appeared that TCM2 was more effective than TCM1 in ameliorating the effects of heat stress in pigs. In conclusion, this study revealed that the active components of common TCM decoctions have antioxidant functions.

Yeh HS[1], Weng BC, Lien TF Effects of Chinese traditional herbal medicine complex supplementation on the growth performance, immunity and serum traits of pigs. Anim Sci J. 2011 Dec;82(6):747-52.

The purpose of this study was to investigate the effects of a traditional Chinese herbal medicine complex supplementation on the growth performance, immunity and serological traits of pigs, and the feasibility of its use as a substitute for antibiotics. Thirty-six weaned pigs LYD with average initial body weight of 10 ± 0.55 kg were randomly divided into three treatments with three replicates. These constituted the control, the antibiotics group (chlortetracycline 100 µg/kg, oxytetracycline 100 µg/kg), and 0.3% Chinese herbal medicine complex group (CHM). Experiment results indicated that the CHM group exhibited significantly increased average feed intake and peripheral blood CD3(+)CD8(+) T cell percentage as compared with those of the antibiotics group ($P < 0.05$). High-density lipoprotein (HDL) level was greater while low-density lipoprotein + very low-density lipoprotein (LDL + VLDL) level was lower in the CHM group than the control group ($P < 0.05$). The in vitro results indicated that peripheral blood mononuclear cells (PBMC) stimulated by Con-A produced a greater interleukin (IL)-6 level in the CHM group and IL-6 level stimulated by lipopolysaccharide was greater than the antibiotics groups ($P < 0.05$). Above all, this study has indicated that the addition of Chinese traditional herbal complex to pigs' diets has beneficial results.

Huang CW[1], Lee TT, Shih YC, Yu B. Effects of dietary supplementation of Chinese medicinal herbs on polymorphonuclear neutrophil immune activity and small intestinal morphology in weanling pigs. J Anim Physiol Anim Nutr (Berl). 2012 Apr;96(2):285-94.

The purpose of this study was to evaluate the effects of dietary Chinese medicinal herbs (CMH) supplementation composed of Panax ginseng, Dioscoreaceae opposite, Atractylodes macrocephala, Glycyrrhiza uralensis, Ziziphus jujube and Platycodon grandiflorum, on the performance, intestinal tract morphology and immune activity in weanling pigs. Two hundred and forty weaned pigs were assigned randomly to four dietary groups including the negative control (basal diet), 0.1% CMH, 0.3% CMH and 0.114% antibiotic (Chlortetracycline calcium Complex, Sulfathiazole and Procaine Penicillin G) supplementation groups for a 28-day feeding trial. Results indicated that both CMH supplementation groups had a better gain and feed/gain than control group (CT) during the first 2 weeks of the experimental period. The 0.3% CMH had a significant decrease in the diarrhoea score in first 10 days of experimental period when compared with other groups. The CMH supplementation groups had a higher villous height, increased lactobacilli counts in digesta of ileum and decreased coliform counts in colon compared with CT. The immune activities of polymorphonuclear leucocytes (PMNs), including the respiratory burst and Salmonella-killing ability, were significantly enhanced in CMH supplementation groups at day 7 of experiment period. The CMH and antibiotic supplementations increased the nutrient digestibility such as dietary dry matter, crude protein and gross energy in weanling pigs. In conclusion, the dietary CMH supplementation improved intestinal morphology and immune activities of PMNs, thus giving rise to nutrient digestibility and reduce diarrhoea frequency in weanling pigs.

Liu JQ[1], Lee TF, Miedzyblocki M etal Effects of tanshinone IIA, a major component of Salvia miltiorrhiza, on platelet aggregation in healthy newborn piglets. J Ethnopharmacol. 2011 Sep 1;137(1):44-9.

Tanshinone IIA (STS), an active ingredient of the Chinese herb Danshen (Salvia miltiorrhiza) for angina and stroke in adults, has been reported to inhibit platelet function. However, its effect on platelet and underlying mechanism remain largely unknown, particularly in neonates. To investigate the effect of STS on the platelet aggregation and its interaction with various platelet activation pathways, platelet aggregatory function was studied in whole blood stimulated by collagen (2-10 µg/ml) ex vivo in newborn piglets receiving intravenous STS (0.1-10mg/kg, n=8) and in vitro in whole blood from newborn piglets (n=6) incubated with STS (0.1-100 µg/ml). The respective morphological changes of platelets were also examined by scanning electron microscopy. Plasma levels of nitrite/nitrate (NOx) and thromboxane B(2) (TxB(2)), matrix metalloproteinase (MMP)-2 and -9 activities were also examined. To further delineate the mechanistic pathway, the effect of STS on endothelial microparticles release from cultured human umbilical vein endothelial cells (HUVECs) was quantified by flow cytometry. STS impaired the ex vivo, but not in vitro, collagen-stimulated platelet aggregation. Infusion of STS elevated the plasma level of TxB(2) at 10mg/kg. However, STS had no effect on NOx level. Incubating cultured HUVECs with STS (1 and 10 µg/ml) caused a significant release of endothelial microparticles. Morphologically, STS elicited platelet activation in vivo, but not in vitro. STS impairs the ex vivo whole blood platelet aggregatory function by activating platelet in vivo in healthy newborn piglets. It implies that STS may elicit its effects by stimulating endothelial microparticles production and eicosanoid metabolism pathway.

Kong X[1], Wu G, Yin Y. Roles of phytochemicals in amino acid nutrition. Front Biosci (Schol Ed). 2011 Jan 1;3:372-84

Chinese herbal medicine (CHM) is often used as dietary supplements to maintain good health in animals and humans. Here, we review the current knowledge about effects of CHM (including ultra-fine Chinese herbal powder, Acanthopanax senticosus extracts, Astragalus polysaccharide, and glycyrrhetinic acid) as dietary additives on physiological and biochemical parameters in pigs, chickens and rodents. Additionally, we propose possible mechanisms for the beneficial effects of CHM on the animals. These mechanisms include (a) increased digestion and absorption of dietary amino acids; (b) altered catabolism of amino acids in the small intestine and other tissues; (c) enhanced synthesis of functional amino acids (e.g., arginine, glutamine and proline) and polyamines; and (d) improved metabolic control of nutrient utilization through cell signaling. Notably, some phytochemicals and glucocorticoids share similarities in structure and physiological actions. New research findings provide a scientific and clinical basis for the use of CHM to improve well-being in livestock species and poultry, while enhancing the efficiency of protein accretion. Results obtained from animal studies also have important implications for human nutrition and health.

Frydrychová S[1], Opletal L, Macáková K etal Effects of herbal preparation on libido and semen quality in boars. Reprod Domest Anim. 2011 Aug;46(4):573-8.

The objective of this study was to investigate the effects of a preparation from herbal extracts (PHE) on libido and semen quality in breeding artificial insemination boars. Ten fertile boars were divided into control and experimental groups according to significant difference of libido. There were no differences in semen quality between groups. Animals were fed a commercial feeding mixture for boars. The feeding mixture for the experimental group was enriched with PHE, which was prepared from Eurycoma longifolia, Tribulus terrestris and Leuzea carthamoides. Duration of the experiment was 10 weeks. Samples of ejaculate were collected weekly. Libido was evaluated according to a scale of 0-5 points. Semen volume, sperm motility, percentage of viable spermatozoa, sperm concentration, morphologically abnormal spermatozoa, daily sperm production and sperm survival were assessed. Amounts of mineral components and free amino acids were analysed in seminal plasma. Significant differences were found in these parameters: libido (4.05 ± 0.22 vs 3.48 ± 0.78; $p < 0.001$), semen volume (331.75 ± 61.91 vs 263.13 ± 87.17 g; $p < 0.001$), sperm concentration (386.25 ± 107.95 vs 487.25 ± 165.50 × 10(3) /mm(3); $p < 0.01$), morphologically abnormal spermatozoa (15.94 ± 11.08 vs 20.88 ± 9.19%; $p < 0.001$) and Mg concentration (28.36 ± 11.59 vs 20.27 ± 13.93 mm; $p < 0.05$). The experimental group's libido was increased by 20% in comparison with the beginning of the experiment. Results of this study showed positive effect of PHE on libido and some parameters of boar semen quality.

Chen Q[1], Liu Z, He JH Achyranthes bidentata polysaccharide enhances immune response in weaned piglets. Immunopharmacol Immunotoxicol. 2009 Jun;31(2):253-60.

The acquired immunity is underdeveloped at 3-4 weeks of age when piglets are usually weaned on commercial farms, and weaning is associated with compromised immunity. Dietary supplementation with immunomodulatory phytochemicals may enhance immune responses in the weaned piglets. This study is conducted to investigate the effects of dietary supplemental achyranthes bidentata polysaccharide (ABP) on proliferation activity of lymphocytes, and production of antibodies, complements and cytokines in weaned piglets. Results showed that lymphocyte proliferation activity in piglets fed diets supplementing with 1000 and 1500 mg/kg ABP increased ($P < 0.05$) on days 14 and 28 compared with the non-additive piglets, as well as serum contents of IgG, IgA, IgM, C(3), C(4), IL (interleukin)-2 and IFN (interferon)-gamma. The ABP had dose-dependent immunomodulatory activity and the dose of 1500 mg/kg presented the strongest stimulating activity in vivo. In addition, the ABP increased ($P < 0.05$) the proliferation activity and production of IL-2 and IFN-gamma of cultured lymphocytes in dose- or time-dependent manner. The proliferation activity of peripheral T cells and splenic lymphocytes in 400 microg/ml of ABP group arrived at their peak values, as well as the production of IL-2 and IFN-gamma at 72 and 12 h after the treatment, respectively. Collectively, these findings suggested that dietary supplementation with ABP to weaned piglets enhances cellular and humoral immune responses, and ABP addition to culture medium also increases the proliferation activity and cytokine production of lymphocytes cultured in vitro, which indicate that dietary supplementation with the herbal polysaccharide may offer an effective alternative to antibiotics for weaned piglets.

Kong XF[1], Yin YL, He QH etal Dietary supplementation with Chinese herbal powder enhances ileal digestibilities and serum concentrations of amino acids in young pigs. Amino Acids. 2009 Oct;37(4):573-82.

This study was designed to determine the effect of ultra-fine Chinese herbal powder as a dietary additive on serum concentrations and apparent ileal digestibilities (AID) of amino acids (AA) in young pigs. In Experiment 1, 60 Duroc x Landrace x Yorkshire piglets weaned at 21 days of age were randomly assigned to one of three treatments, representing supplementation with 0 or 2 g/kg of the powder, or 0.2 g/kg of colistin (an antibiotic) to corn- and soybean meal-based diets (n = 20 per group). Blood samples from five piglets per group were collected on days 7, 14, and 28 to determine serum AA concentrations. In Experiment 2, 12 barrows with an average initial body weight of 7.64 kg were randomly assigned to one of the three dietary treatments, followed by surgical placement of a simple T-cannula at the terminal ileum. All of the diets contained 0.1% titanium oxide as a digestibility marker. The samples of terminal ileal digesta were collected on day 7 for determining AID of AA. Results show that dietary supplementation with the herbal powder increased ($P < 0.05$) serum concentrations and AID of most AA by 10-50% and 10-16%, respectively. As an indicator of improved intestinal function, AID values of calcium were also enhanced in piglets supplemented with the herbal powder. Dietary supplementation of colistin increased serum concentrations and AID values of some AA by 8-44% and

10-15%, respectively, in comparison with the non-supplemented group. These novel findings demonstrate that the herbal powder can enhance the digestibility of dietary protein and the intestinal absorption of AA into the systemic circulation in post-weaning pigs, therefore providing a new mechanism for its growth- and immunity-promoting efficacy.

Xiao C[1], Rajput ZI, Liu D, Hu S. Have Enhancement of serological immune responses to foot-and-mouth disease vaccine by a supplement made of extract of cochinchina momordica seeds. Clin Vaccine Immunol. 2007 Dec;14(12):1634-9.

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animals. Vaccination against FMD is a routine practice in many countries where the disease is endemic. This study was designed first to investigate the extract of the seeds of Momordica cochinchinensis (Lour.) Spreng. (ECMS) for its adjuvant effect on vaccination of inactivated FMDV antigens in a guinea pig model and then to evaluate the supplement of ECMS in oil-emulsified FMD vaccines for its immunopotentiation in pigs. The results indicated that ECMS and oil emulsion act synergistically as adjuvants to promote the production of FMDV- and VP1-specific immunoglobulin G (IgG) and subclasses in guinea pigs. A supplement of ECMS in a commercial FMD vaccine significantly enhanced FMDV-specific indirect hemagglutination assay titers as well as VP1-specific IgG and subclasses in pigs. Therefore, ECMS could be an alternative approach to improving swine FMD vaccination when the vaccine is poor to induce an effective immune response.

Other papers

E. Peeters, B. Driessen and R. Geers Influence of supplemental magnesium, tryptophan, vitamin C, vitamin E, and herbs on stress responses and pork quality[1] Journal of Animal Science 2006 84: 7: 1827-1838

W. Windisch, K. Schedle, C. Plitzner and A. Kroismayr Use of phytogenic products as feed additives for swine and poultry[1] Journal of Animal Science 2008 86: 14_suppl: E140-E148

Y. Liu, M. Song, T. M. Che etal Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic *Escherichia coli*[1], Journal of Animal Science 2013 91: 11: 5294-5306

X. F. Mao, X. S. Piao, C. H. Lai, etal Effects of β-glucan obtained from the Chinese herb *Astragalus membranaceus* and lipopolysaccharide challenge on performance, immunological, adrenal, and somatotropic responses of weanling pigs[1] Journal of Animal Science 2005 83: 12: 2775-2782

K. Maenner, W. Vahjen and O. Simon Studies on the effects of essential-oil-based feed additives on performance, ileal nutrient digestibility, and selected bacterial groups in the gastrointestinal tract of piglets[1] Journal of Animal Science 2011 89: 7: 2106-2112

Y. Liu, T. M. Che, M. Song etal Dietary plant extracts improve immune responses and growth efficiency of pigs experimentally infected with porcine reproductive and respiratory syndrome virus[1] Journal of Animal Science 2013 91: 12: 5668-5679

Appendix X Documentation of Trends in the Public Acceptance of Botanical Therapies: Consumer Buying Patterns, Patterns of Use, and Industry Correlates.

ORGANIZING COMMITTEE REPORT:

D5: Documentation of Trends in the Public Acceptance of Botanical Therapies: Consumer Buying Patterns, Patterns of Use, and Industry Correlates

Prepared by: Robert J. Silver DVM, MS; Bill Bookout BS, MBA; Hubert J. Karreman, VMD

30 January 2016

Introduction:

The American College of Veterinary Botanical Medicine (ACVBM) is petitioning the American Board of Veterinary Specialties (AVBS) to be established as a specialty College under the criteria established by the American Veterinary Medical Association (AVMA) for the advancement of knowledge and education in the field of veterinary botanical medicine.

The purpose of this White Paper is to provide objective data to support the establishment of this College based on increased public acceptance and use of veterinary herbal medicine, as measured by consumer and industry trends over the past 17 years.

The information contained in this document was obtained from several sources:

1. National Animal Supplement Council[1] data enumerating the trends in administration of the top 25 herbal ingredients in products for dogs, cats and horses.
2. A peer-reviewed paper[2] submitted for publication containing a survey of members of the American Association of Bovine Practitioners (AABP) documenting an increase in the organic dairy industry and the consequent needs for this sector to have non-pharmaceutical options for production and veterinary care of problems like mastitis, metritis, etc.
3. Market research report[3] of the trending increase in consumer interest in herbal therapies for pets recently published by the oldest and most reputable association for herbal education and research, the American Botanical Council.
4. A National Pet Owners Survey from the American Pet Products Association (APPA) 2015-2016[4], documenting the trending increase in consumer interest in dietary supplements for their pets. By definition, the APPA includes herbal remedies under the general category of dietary supplements.

1. The National Animal Supplement Council

Established in 2001, the NASC is a non-profit trade organization dedicated to protecting and enhancing the health of companion animals and horses throughout the United States. As an all-industry association of stakeholders concerned with the issues surrounding the supply of health supplements for animals which are not intended for human consumption, NASC members include manufacturers of animal health supplements, raw material suppliers, distributors, veterinarians, retailers, and pet professionals.

The NASC works directly with the FDA-CVM and with state regulatory agencies to establish a regulatory environment that is fair, reasonable, responsible and nationally consistent.

By working with the FDA-CVM to establish a unique category of health supplements specifically for companion animals not in the human food chain known as "Unapproved Drugs", the NASC has allowed the health supplement industry to continue to grow with this federal sanction. Under this framework, the FDA-CVM exercises enforcement discretion with the caveat that companies act "responsibly". An important function of the NASC is to educate companies regarding FDA-CVM compliance.

The NASC established a Scientific Advisory Board, which, if the College of Veterinary Botanical Medicine had been established in 2001, would have contributed significantly to the work of this advisory board. This board reviewed over 900 ingredients found on the labels of animal supplements and assigned to each supplement one of four levels of risk, "ranging from generally recognized as safe" to "potentially toxic". Additionally, the NASC established an adverse event reporting website, and has recorded millions of administrations of health supplements and has documentation regarding the percentage of adverse events associated with those supplements in dogs, cats and horses.

It is this administration data which is presented below, that has been mined specifically to generate this numerical report and to chart the trends over the past 17 years of the number of administrations of the top 25 selling herbal ingredients for dogs, cats, and horses.

Mr. Bill Bookout, President and Founder of the NASC, in providing this confidential and proprietary information writes:

"We are providing the attached data and usage trends for the top 25 herbal ingredients used in supplements for Dogs, Horses and Cats.

The NASC Adverse Event Reporting Database NAERS™ tracks over 2000 individual ingredients contained in over 7000 products which are entered by NASC member companies. Our organization is the leading non-profit trade association for these types of products representing over 90% of sales in the US. Total bytes of data in our system exceeds 100 Billion and these data may be utilized by regulatory agencies in North America when questions arise about products and/or individual ingredients.

In an effort to assist the American College of Veterinary Botanical Medicine in its documentation for its petition for membership in the ABVS, we are pleased to provide the trends for products (similar to human dietary supplements) that contain the top 25 herbal ingredients. Please note the following:

- These data reflect the following botanical ingredients: Alfalfa, Barley grass, Boswellia, Cayenne, Celery seed, Chamomile, Cinnamon, Cranberry extract, Devil's Claw, Echinacea, Garlic, Ginger, Gingko Biloba, Grape Seed extract, Kelp, Marshmallow root, Milk thistle, Nettles, Oregon Grape root, Parsley, Slippery Elm, Spirulina, Turmeric, Valerian root, Yucca.
- Administrations are calculated and grouped from all NASC Member companies, tracked by the target species indicated on the product label.
- Data reflects units shipped to the first distribution point for products purchased and does not necessarily indicate all administrations were 100% consumed by the animals.
- Data reflects purchases in all market channels.
- All data is the property of The National Animal Supplement Council and may be used only for the purposes described in this document. Any other use requires written permission from the President of the NASC.
- This information is confidential and proprietary and should not be provided to anyone for any purpose other than to support the objectives of the ACVBM."

Discussion of Trends from NASC Data:

Canine Data: Total administrations of the top 25 herbal ingredients for dogs was 42,087,369 in 1999, the first year of tabulating this data. The number of administrations grew over the 17 years recorded to 244,797,878 administrations, estimated for the year 2015. This is 500% growth.

Equine Data: Total administrations of the top 25 herbal ingredients for horses was 8,385,566 for 1999, the first year of recording this data. This value increased in 17 years of data recording to 42,476,440 administrations estimated by the end of 2015. This is also a 500% growth in number of administrations, just as with the canine data.

Feline Data: Total administrations of the top 25 herbal ingredients for 1999 for cats was 5,638,172. This value it is estimated will increase to 81,495,270 by the end of 2015. Although cats are experiencing a down turn in number of visits to their veterinarians, the number of administrations of the top 25 herbal ingredients increased 14.5 times during this 17 year period being measured!

Combined Data: Total administrations of the top 25 herbal ingredients for Dogs, Cats and Horses combined for 1999 was 56,111,107. This value increased over 17 years to an estimated 368,769,588 by the end of 2015. This is a 650% increase in the administrations of these top 25 herbal ingredients, which implies a similar increase in use of these herbal supplements in all species measured.

ANALYSIS: The National Animal Supplement Council was established from the need that arose as a result of the rapid growth in the animal supplement industry, including the increased use of herbal remedies by veterinarians and consumers.

With the growth of interest in herbal medicine among veterinarians and consumers, most animal supplement companies are in need of scientifically-derived information regarding the safety, herb-drug interactions, and clinical applications regarding herbal therapies in veterinary species, such as the establishment of a College of Veterinary Botanical Medicine would facilitate.

Additionally, as the job of FDA-CVM oversight of product safety and compliance with FDA labeling guidelines becomes more difficult due to the significantly increased number of companies selling herbal products, the value of this non-profit trade organization has become indispensable.

TABLES and GRAPHS of NASC DATA can be found at the end of this report.

2. The Growth in Interest Of Bovine Practitioners Toward Complementary And Alternative Medicine Based on Surveys with AABP Veterinarians in 2006 and 2010

Goal: To investigate whether bovine veterinarians are interested in complementary and alternative medicine, and which diseases the use of alternative therapies would be of most interest to them.

Methods: Members of the American Association of Bovine Practitioners were invited by mail (2006) or email (2010) to participate. The survey was anonymous and included six closed-ended and two open-ended questions. It was published

Questions: Focused on the practitioner's perceptions of complementary and alternative therapies and products, as well as how many organic clients they had.

Results: 181 veterinarians in 2006 and 185 veterinarians completed the survey. In both years, approximately 80% of the veterinarians were interested evidence-based alternative therapies, and in particular for the treatment of mastitis. From 2006 to 2010 interest increased significantly ($p<0.01$) in alternative treatment approaches for calf diarrhea, metritis, infertility, pneumonia and digital dermatitis/foot rot. In general veterinarians with organic clients were more interested in these alternative non-drug therapies than those veterinarians without organic clients.

Conclusions: The majority of bovine veterinarians were interested in evidence-based alternative or complementary therapies for bovine disease and the interest in alternative therapies for common cattle diseases increased between 2006 and 2010.

ANALYSIS: The majority of evidence-based complementary and alternative therapies are based on botanical remedies. This survey of the growth of bovine veterinarians' interest in CAVM between 2006 and 2010 supports the need for the establishment of a College of Veterinary Botanical Medicine as a resource for these veterinarians to better establish the science that underlies these therapies. For industry, the graduation of Board-certified veterinarians with scientific and clinical expertise in botanical medicine and phytopharmacology will be invaluable as these companies develop and bring evidence-based botanical products to the marketplace to address the needs of these bovine practitioners.

3. Market Research Reports

A. The American Botanical Council:

Founded in 1988, The ABC is the leading non-profit association in the United States that provides educational material using science-based and traditional information to promote the responsible use of herbal medicine—serving the public, researchers, educators, healthcare professionals, industry and media.

Recently, this past November 2015, the results of a market research study, performed by one of the national leaders that follow the pet industry, SPINS, located in Chicago, Ill, was published in an article in the journal of the American Botanical Council. The data used by SPINS did not include sales information from Whole Foods Market or direct sales from businesses that sell solely on the internet. These data do include products sold in the natural specialty/gourmet and mass-market channels in the United States.

For a 52 week period, ending August 9, 2015 SPINS recorded aggregate sales of $43,044,385 for herbal supplements which provide a solid dosage format such as tablets or capsules or liquids, and for treats and snacks, which provide the botanical or blend of botanicals in a baked biscuit, cookie or other tasty format. This market grew by 25% between the years of 2013-2014, but for 2014-2015 a 25% decrease in this market was noted.

This marketplace is growing, in spite of year to year variation, overall sales continue to climb for botanical products, both for the consumer, and also for healthcare professionals such as veterinarians, trainers, and rehabilitation experts. This year marked the second annual industry trade show, The Petfood and Animal Nutrition Conference, which had a heavy representation of companies offering botanical raw materials and finished products.

Several large national supplement companies are now offering pet specific supplements to meet the increased consumer demand for these types of products.

Types of conditions for which consumers are buying botanical products was summarized in this article based on a ranking of individual botanical sales by the SPINS data. Supplements are being purchased to address immune system function, support of the digestive system and oral health. Omega three oils are being sourced from Flax, Chia or Hemp seed and their oils. Chamomile and parsley were cited as two very popular botanicals. Parsley is commonly used for improving gastrointestinal and urinary symptoms, including joint disorders such as gout and arthritis. Chamomile can be used topically as a salve for insect bites, allergies, bacterial or fungal infections, and orally, provides a gentle degree of calming and is a mild tonic for the digestion.

The article in Herbalgram closes with a discussion of consumer concerns regarding the quality and purity of these animal products, and how the National Animal Supplement Council has effectively addressed those concerns with its efforts to secure a reliable and consistent supply chain for manufacturers as well as direct oversight by third party inspections of manufacturing facilities and random product analysis of member products to assure they match label claims of botanical content and concentration.

B. The American Pet Products Association National Pet Owners Survey 2015-2016

The American Pet Products Association is the leading not-for-profit trade association serving the interests of the pet products industry since 1958. APPA's membership consists of over 1000 pet product manufacturers, importers, manufacturer's representatives and livestock suppliers worldwide.

The APPA was established to promote, develop and advance responsible pet ownership and the pet products industry. To this end, APPA supports industry-related market research, monitors and responds to industry legislation and regulation, and sponsors educational seminars.

Every two years the APPA distributes a survey questionnaire amongst pet owners to determine current consumer trends. The survey methodology was changed in 2012 to an on-line survey. Despite changes in methodology for this survey since 2012, the results indicate a faithful mapping of consumer trends based on the recurrent patterns in pet ownership based on geographic region, market size, family structure (children versus no children), household size, household composition, home ownership and marital status when compared to the results of each prior study performed by the APPA since 1990.

The APPA survey (a 2' thick, 5 pound publication) was mined for data regarding the trends in the use of herbal supplements by pet owners of dogs, cats and horses. The compilation and summary of those results follows.

NOTE:

The 2014 APPA Survey added the new category of dietary supplements as a result of the increase in the number of these products for sale, and sales in this sector since the inception of this APPA survey in 1990. Interestingly, in the survey they mis-use the word "Homeopathic" in describing this sector. The APPA erroneously defines homeopathic as: "…alternative remedies including holistic, herbal, floral or plant-based products." Homeopathy is a separate system of medicine using very dilute substances. This term is commonly misused by individuals with no understanding of complementary and alternative therapies. With the establishment of the College of Veterinary Botanical Medicine, members of this College will be engaged by industry to better describe these products.

General Information About Pet Ownership Summarized from APPA Survey 2015-2016

By Household.

Significant growth in household pet ownership has been noted since the survey began in 1990. Currently 79.7 million US households own a pet, compared to 52.6 million households in 1990. That is greater than a 50% gain in pet-owning households.

By Generation.

The Baby Boomers (BB) have been an integral part of the growth of pet ownership over the past 20 years.

Baby boomers represent 37% of the Survey sample. BB are the largest segment of horse owners (44%)

By Pet Species Owned.

77.8 million dogs are estimated to be owned in the US. That averages to 1.43 dogs per dog-owning household and are estimated to cost their owners an average of $551/year.

85.8 million cats are estimated to be owned in the US. That averages to 2.0 cats per cat-owning household and are estimated to cost their owners an average of $398/year.

7.5 million horses are estimated to be owned in the US. That averages to 3.0 horses per household, and are estimated to cost their owners $416, which does not include the cost of food, which annually averages $2121 per horse per year.

Trends in Consumer Use of Herbal Therapies for Pets

The percentage of dogs and cats given medications of any kind increased to 77% over the past 12 months. 10 years ago this was 52%, indicating a trend toward better acceptance of administration of medications, and better palatability strategies. More than 90% of horses have been administered medication or supplements this past year.

The percentage of pets receiving dietary supplements excluding vitamins was 12% for dogs (9.3 million dogs), 6% for cats (5.15 million cats), and 5% for horses (375,000 horses).

Consumers source their dietary supplements from the following outlets: *(Multiple response question, therefore total may exceed 100%)*

1. **Dogs**: from Veterinarian (28%), Internet (22%), Pet chain superstore (16%), Pet store independent (13%), Discount/Mass marketing (16%) Hardware store (6%), Other (6%)

2. **Cats**: from Veterinarian (17%); Pet chain super store (25%); Internet (17%); Grocery store (17%); Discount Mass marketing (25%)

3. **Horses**: from Internet (33%); Veterinarian (25%); Feed store (17%) Tack shop (8%) Other (17%)

Consumers source their information about dietary supplements from the following outlets:
(Multiple response question, therefore total may exceed 100%)

1. **Dogs**: from Veterinarian (65%), Internet (44%), Friends & Relatives (28%); past experience (32%); Pet store personnel (16%), Television (12%); Groomer (15%)

2. **Cats**: from Veterinarian (47%); Internet (42%); Friends & Relatives (33%); past experience (35%); Pet store personnel (13%); Television (10%); Other (8%)

3. **Horses**: from Veterinarian (73%); Internet (48%); past experience (60%); Breed club and societies (17%); Feed store personnel (25%), Farrier/Trainer (50%) Other

ANALYSIS OF SURVEY DATA

1. Veterinarians are the major source of supply to pet owners for herbal supplements
2. Veterinarians are the major source of information about using herbal supplements to pet owners
3. The market share that herbal supplements have, in comparison to more commonly used products like food, bedding, tack, collars is relatively small (average 11% for dogs, cats and horses).
4. The market share for herbal remedies and other alternative therapies has been growing sufficiently over the years since 1990 that this survey has been conducted, such that the APPA has now created a specific category in this survey to measure the trends in this growing segment.

CONCLUSIONS DRAWN FROM THE ABOVE FOUR SOURCES OF DATA
(NASC Report, Bovine Veterinarian Survey, American Botanical Council report of a market survey of herbal use in pets, APPA Pet Owner Survey 2015-2016)

Since 1990 there has been a steady growth in consumer demand for dietary supplements that contain herbal ingredients. Most consumers consult with their veterinarian regarding supplements, and most consumers purchase their dietary supplements from their veterinarian.

Thus, veterinarians are in a unique position of providing evidence-based information to their clients about products they believe, based on the best information available, will augment their existing clinical protocols.

It is known that herbs can interact adversely with pharmaceutical therapies, and that not all herbs are safe or effective. Currently we lack an adequate body of evidence-based information, or Board certified veterinarians, to guide the use of herbal therapies concurrent with conventional therapies. In some cases, herbal therapies can serve as complete substitutes, where appropriate, for conventional therapies. Without these safeguards and without a College of Veterinary Botanical Medicine to graduate Board-certified specialists in herbal therapies, the consumer is left without adequate protections that would provide safe and effective options for the use of the botanical therapies that they are requesting and are currently using anyway.

The consumer, the marketplace and the Veterinary profession are ready for the establishment of the College of Veterinary Botanical Medicine for all of the reasons stated above.

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NASC DATA: TABLES AND GRAPHS

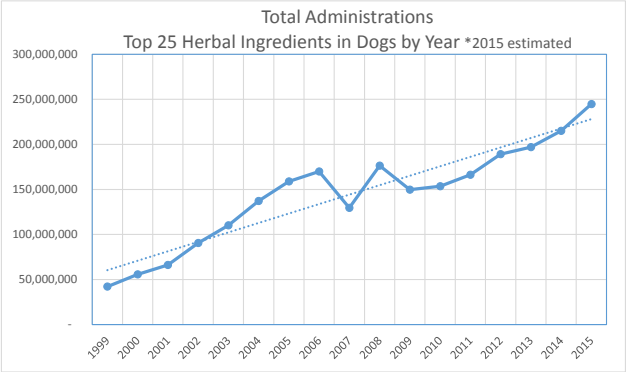
Trends of Top 25 Herbal Ingredients in Animal Health Supplements

All information is the property of The National Animal Supplement Council and may be used only for the purposes described in the cover letter accompanying this document.

Total Products Containing Top 25 Herbal Ingredients: 2,179

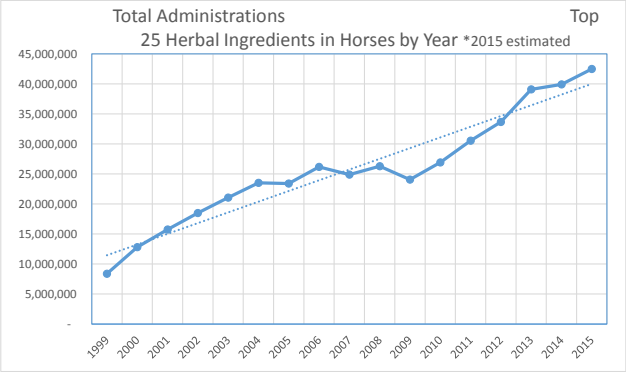
Canine Data

Year	Administrations
1999	42,087,369
2000	55,622,828
2001	66,185,689
2002	90,589,629
2003	110,066,071
2004	137,186,212
2005	158,831,832
2006	169,876,093
2007	129,522,132
2008	176,323,256
2009	149,719,076
2010	153,623,867
2011	166,291,096
2012	189,169,577
2013	196,998,779
2014	214,948,834
*2015	244,797,878



Equine Data

Year	Administrations
1999	8,385,566
2000	12,823,169
2001	15,742,371
2002	18,489,412
2003	21,072,094
2004	23,511,247
2005	23,397,120
2006	26,189,829
2007	24,896,047
2008	26,281,836
2009	24,054,093
2010	26,911,744
2011	30,538,424
2012	33,658,446
2013	39,088,702
2014	39,906,398
*2015	42,476,440



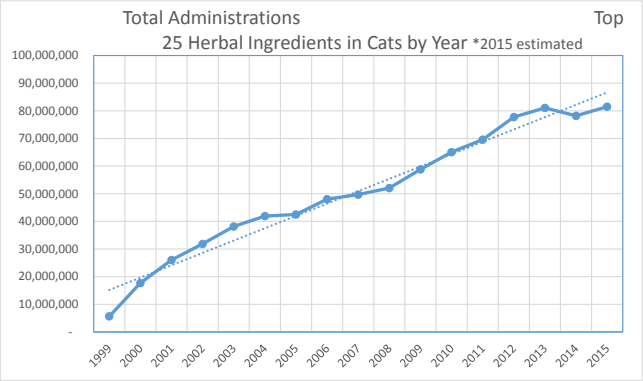
Trends of Top 25 Herbal Ingredients in Animal Health Supplements

All information is the property of The National Animal Supplement Council and may be used only for the purposes described in the cover letter accompanying this document.

Total Products Containing Top 25 Herbal Ingredients: 2,179

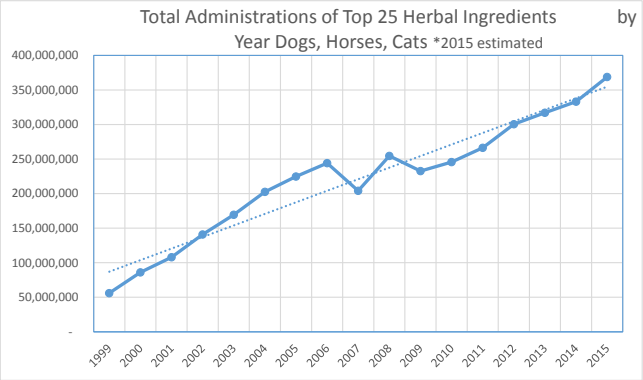
Feline Data

Year	Administrations
1999	5,638,172
2000	17,708,052
2001	26,085,510
2002	31,838,672
2003	38,187,536
2004	41,903,163
2005	42,487,220
2006	48,014,498
2007	49,712,281
2008	52,042,402
2009	58,798,209
2010	65,076,637
2011	69,587,467
2012	77,755,627
2013	81,084,175
2014	78,214,777
*2015	81,495,270



Combined Data

Year	Administrations
1999	56,111,107
2000	86,154,049
2001	108,013,570
2002	140,917,713
2003	169,325,701
2004	202,600,622
2005	224,716,172
2006	244,080,420
2007	204,130,460
2008	254,647,494
2009	232,571,378
2010	245,612,248
2011	266,416,987
2012	300,583,650
2013	317,171,656
2014	333,070,009
*2015	368,769,588



COPIES OF DOCUMENTS CITED IN THIS REPORT

NASC Letter from Bill Bookout:

P.O. Box 2568
Valley Center, CA 92082

760.751.3360
Fax: 760.751.5027

Adverse Event Reporting
866.833. NASC (6272)

www.nasc.cc
b.bookout@nasc.cc

The National Animal Supplement Council, Inc., Incorporated under Utah Law, is a non-profit industry coalition dedicated to improving the health and welfare of Companion Animals, including horses, dogs, cats and other animals not intended for human consumption, by working cooperatively with regulators to bring about reasonable, responsible, nationally consistent regulation of the labeling and manufacture of supplements intended for Companion Animals.

Document Control Number - 1.1.4

Date: November 3, 2015

To: Robert J Silver DVM, MS, CVA

From: Bill Bookout, President

RE: Data to Support Use of Botanical Ingredients in Dogs, Horses and Cats

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Dear Dr. Silver,

I am providing the attached data and usage trends for the top 25 Herbal Ingredients used in supplements for Dogs, Horses and Cats.

The NASC Adverse Event Reporting Data Base, NAERS™ tracks over 2,000 individual ingredients contained in over 7,000 products which are entered by NASC Member

Companies. Our organization the leading non-profit industry trade association for these types of products representing over 90% of sales in the US. Total bytes of data in our system exceeds 100 billion and these data may be utilized by regulatory agencies in North America when questions arise about products and / or individual ingredients.

In an effort to assist the Veterinary Botanical Medical Association (VBMA) in working with the American Veterinary Medical Association (AVMA) we are pleased to provide the trends for products (similar to human dietary supplements) that contain the top 25 herbal ingredients. Please note the following:

- These Data Reflect the following Botanical / Herbal Ingredients: Alfalfa, Barley Grass, Boswellia, Cayenne, Celery Seed, Chamomile, Cinnamon, Cranberry Extract, Devils Claw, Echinacea, Garlic, Ginger, Ginkgo Biloba, Grape Seed Extract, Kelp, Marshmallow Root, Milk Thistle, Nettle, Oregon Grape Root, Parsley, Slippery Elm, Spirulina, Turmeric, Valerian Root, Yucca\
- Administrations are calculated and grouped from all NASC Member Companies, tracked by the target species indicated on the product label
- Data reflects units shipped to the first distribution point for products purchased and does not necessarily indicate all administrations were 100% consumed by the animals
- Data reflects purchases in all market channels
- All data is the property of The National Animal Supplement Council and may be used only for the purposes described in this document. Any other use requires written permission from the President of NASC

This information is confidential and proprietary and should not be provided to anyone for any purpose other than to support the objectives of VBMA.

If you have any questions please do not hesitate to contact me directly and we hope you find this information helpful.

Thank you for supporting NASC and our members' products.

Bovine Practitioners Survey:

Interest of Bovine Practitioners in Complementary and Alternative Veterinary Medicine in 2006 and 2010.

U.S. Sorge[1*], A. Bastan[2], H. Karreman[3]

[1] College of Veterinary Medicine, Dept. Veterinary Population Medicine, University of Minnesota, Saint Paul, MN 55108 USA
[2] Ankara University Faculty of Veterinary Medicine Department of Obstetrics and Gyneacology. 06110, Dışkapı, ANKARA. Turkey
[3] Red Hill Road, Narvon, PA 17555

* Corresponding author: sorge@umn.edu

ABSTRACT

Objectives: The aim of this survey was to investigate whether bovine veterinarians are interested in complementary and alternative medicines and for which diseases alternative therapies would be of interest to them.

Methods: Members of the American Association of Bovine Practitioners were invited by mail (2006) or email (2010) to participate. The survey was anonymous and included six closed and two open-ended questions. The questions focused on the practitioner's perception of complementary and alternative veterinary medicines (CAVM), for which diseases they would be interested in alternative therapies and products, as well as how many organic clients they had.

Results: In the end, 181 veterinarians and 185 veterinarians completed the survey in 2006 and 2010, respectively. In both years, approximately 80% of veterinarians were interested in evidence-based alternative therapies and in particular for the treatment of mastitis. Interest in alternative treatment approaches for calf diarrhea, metritis, infertility, pneumonia, and digital dermatitis/foot rot significantly increased over the years. Veterinarians with organic clients were more interested in CAVM than those without.

In *conclusion*, the majority of veterinarians were interested in evidence-based alternative or complementary therapies for bovine diseases and the interest in alternative therapies for common cattle diseases increased between 2006 and 2010.

Keywords: alternative, bovine, complementary, therapy, veterinarian

INTRODUCTION

Antibiotics and other synthetically produced drugs are commonly used for the treatment of diseases in people and livestock (McEwen and Fedorka-Cray, 2002; Laxminarayan et al., 2013). However, the use of antibiotics on livestock operations has become under increasing public scrutiny due to concerns that it may add to the creation of multidrug resistant bacteria (Laxminarayan et al., 2013; PCAST, 2014). Whether this is indeed the main culprit for increases in multidrug resistant infections in humans remains to be seen (Laxminarayan et al., 2013). Nevertheless, changes have already been introduced in some sectors of the agricultural industry. The furthest reaching change would be the National Organic Program (NOP) of the United States (USDA, 2015) which prohibits the use of antibiotics or hormones on organically certified livestock operations. Animals treated with such therapies need to leave the farm and neither they nor their products must ever be sold as organic again. Instead, organic producers are encouraged to use preventive practices or non-synthetic treatments that do not violate guidances of the Food and Drug Administration (FDA), such as Pasteurized Milk Ordinance (FDA, 2007) or Animal Medicinal Drug Use Clarification Act (FDA, 1994). Unfortunately, data on dosage, efficacy or even withhold times of alternative therapies (e.g. herbal) are sparse and formally FDA approved alternative therapies are virtually not existent. Yet, FDA prohibits the use of unapproved animal drugs to prevent harm to the animal and minimize the risk of an adulteration of the food supply (FDA, 1994 & 2007). This provides a potential challenge for veterinarians. We hypothesize that veterinarians would be interested in alternative therapies as long as data exists regarding their efficacy and withhold times.

Therefore, the aim of this survey was to investigate whether bovine veterinarians are interested in complementary and alternative medicines and for which diseases alternative therapies would be of interest.

MATERIAL AND METHODS

In the late fall of 2005, veterinary practitioners who were listed in the directory of the American Association of Bovine Practitioners as "dairy" or "mostly dairy" practitioners and located within

the North East, Upper Midwest and Western United States were invited by mail to participate (n = 750). The questionnaire was sent out on a pre-stamped postcard. The survey was anonymous and included six closed and two open-ended questions. The questions focused on the practitioner's perception of complementary and alternative veterinary medicines (CAVM), for which diseases they would be interested in alternative therapies and products, as well as how many organic clients they had. The disease options given were mastitis, metritis, infertility, pneumonia, calf diarrhea, foot rot/digital dermatitis and the open ended choice of "other" diseases.

The same questionnaire was applied again as online survey (SurveyMonkey) in the spring 2010. Only the wording of the last question was changed from "Would you be interested in receiving information for a few specific products" to "Would you be interested in receiving more information about products allowed for use in organic herds?". This time, bovine veterinarians who subscribed to the list-serve of the American Association of Bovine Practitioners (AABP-L) were invited via email. Veterinarians could provide their email address at the end of the survey, if they were interested in receiving more information regarding specific alternative therapies. The data was analyzed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA, 2010-2012). The significance level was set at $\alpha=0.05$. The data was first summarized with frequency statistics and then comparisons between the 2 surveys were completed with Fisher's exact statistics. Likewise, comparisons between practitioners that had organic clients and those without were also done with a Fisher's exact test within years of the survey.

RESULTS

In the end, 181 (response rate: 24.1%) and 185 veterinarians completed the survey in 2006 and 2010, respectively. While the response rate was 24.1% in 2006, the response rate for 2010 could not be calculated as the exact number of subscribers from the United States was not available.

Approximately half of the participating veterinarians had organic dairy clients (Table 1). Of these veterinarians most (64%) had less than 3 organic clients, but one practitioner had over 100 organic clients (range 1-111 herds); the most commonly reported average herd size for organic dairy herds was less than 100 milking cows.

Many veterinarians were interested in using CAVM, but fewer showed interest in the use of CAVM or information about specific products in the second survey. However, in both years approximately 80% of veterinarians were interested in CAVM, if pharmacologic data were available for the alternative products or approaches. Veterinarians with organic clients were more interested in CAVM (interest yes: 68%, no: 5%) and specific alternative products (94% vs 78%, $P < 0.01$) compared to those with no organic clients (interest yes: 43%, no: 10%; $P < 0.01$). However, while in 2006 almost two-thirds of veterinarians thought that their conventional clients would be interested in CAVM, only 25% of veterinarians responded correspondingly in 2010. Yet in general few (16%) thought that none of their conventional clients would be interested in CAVM. This response was independent of them having organic clients or not ($P = 0.74$).

Overall, most veterinarians indicated interest in alternative treatment approaches for all listed diseases and the interest in CAVM for all listed diseases increased significantly between the years ($P < 0.01$). In both years the majority of practitioners were interested in CAVM approaches for mastitis, calf diarrhea and metritis (Table 2). The interest in CAVM for infertility, pneumonia and digital dermatitis or foot rot increased even by 23 to 24 percent points between surveys ($P < 0.01$). In addition to the listed diseases, veterinarians also expressed interest in alternative treatments for coccidiosis (n=2), adult enteric diseases (n=1), cystic ovaries (n=1) and

acupuncture for downer cows (n=1) in 2006. Again, veterinarians with organic clients were more interested in CAVM for the different diseases than veterinarians with conventional clients only ($P < 0.01$).

Further comments from veterinarians in 2006 and 2010 are listed in Table 3. Comments in 2006 mostly focused on interest in more scientific information (n=5) regarding efficacy, side effects or interactions as well as residue avoidance, that some of their clients considering to transition to organic (n=2) and the suggestion to use oregano for mastitis (n=1). The comments in 2010 were different. Two veterinarians strongly expressed their opinion opposed to organic farming ("'Organic' is somewhat of a lie […]." and "The organic movement is crap […]") and their concern about the regulations, residues avoidance as well as animal welfare. Another comment also focused on their confusion regarding allowable substances under NOP regulations, while the other two comments broached the issues of management for disease prevention and acupuncture against pain on organic farms.

DISCUSSION

Although other studies have speculated about the interest of veterinary practitioners in alternative treatment approaches and their need for accompanying pharmacological data (Arlt and Heuwiser, 2014; Mathias 2004 & 2007), this is the first survey to quantify bovine veterinarians' interest in CAVM over multiple years.

The response rate was reasonable and probably fairly consistent between surveys as probably approximately the same number of people was initially invited through AABP-L. Both surveys were anonymous and so no direct comparison between answers of respondents was possible. Although the organic dairy industry is a niche market and increased only from 1 to 3% of the dairy industry between 2005 and 2010, (ERS, 2012) about half of the participants had at least one organic client. Therefore, there may have been an overrepresentation of participants with organic clients. However, this bias may be negligible as most veterinarians had less than 3 organic clients and the distribution of organic herd sizes of the clients mirrored the distribution of dairy herd sizes in the United States: most herds were milking less than 100 cows and approximately 6% of the organic clients' herds had over 500 milking cows (NASS, 2007).

As expected, the majority of veterinarians were interested in using evidence-based CAVM in both years. However, several things are noteworthy. First, it is evident that the data supporting the efficacy of CAVM became more important to practitioners over the years: The number of positive respondents regarding the use of CAVM without mentioning specific efficacy data dropped significantly, most comments 2006 already focus on the need for more data on alternative practices and in 2010 most comments reflected the challenges veterinarians may face when trying to balance both NOP as well as FDA guidelines. Interestingly, 4% of respondents were absolutely against CAVM – even if data to support its use would be provided. The reason for this is unknown. One might speculate that those respondents were possibly still concerned about violating FDA regulations such as PMO (FDA, 2007) and AMDUCA (FDA, 1994), which prohibit the use of unapproved veterinary drugs on livestock operations. Similarly while veterinarians provided positive comments regarding herds transitioning to organic dairying in 2006, several comments in 2010 were rather opposed to organic farming, which, as aforementioned, may be the result of the veterinarians' frustration trying to interpret and align several regulations and balance the wishes of clients with their professional ethics (Rollins, 2006; Ludbrook, 2007). Second, veterinarians with organic clients were more interested in alternative treatment approaches compared to those without. Veterinarians with organic clients probably want to remain a resource for animal health concerns to their clients while accommodating the different needs and management approaches of those organic clients before having to use NOP prohibited antibiotics or hormones.

Last, the interest in CAVM for the diseases listed in the survey increased over the years. It is not surprising that the need for alternative therapies for mastitis was indicated by over 80% of respondents. After all, mastitis therapy accounts for most used antibiotics on dairy farms in the United States (USDA, 2010). Furthermore, not all bacterial causes are susceptible to available antibiotics (Schukken et al., 2013) and various approaches to reduce antibiotic usage for mastitis have been discussed for years (Royster and Wagner, 2014). Similarly, the efficacy of antibiotics against calf diarrhea (Smith, 2014) and respiratory diseases of cattle (Fulton et al., 2009) is not guaranteed and alternative approaches are needed. In particular pneumonia is a cause of losses on dairy farms. It is the most common disease (5.4%) and cause of death for weaned heifers – almost half of all weaned heifer deaths are due to respiratory disease (46.5%) (USDA, 2009). Similarly, although, on average, only 3.3% of mature cows experience pneumonia, it is the 4th most common cause of mature cow losses on dairy farms (USDA, 2009) and veterinarians may want alternative therapy approaches to safe those cows.
In conclusion, most veterinarians, but in particular veterinarians with organic clients, are interested in CAVM. The interest in alternative therapeutic approaches for common cattle diseases increased between the survey years, while the need for evidence-based approaches remained high in both years of the survey.

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Table 1. Responses of veterinarians regarding their client base as well as their interest in complementary and alternative veterinary medicine (CAVM). Results are presented as frequencies of overall, 2006 and 2010 answers. The reported P-value is based on Fisher's exact statistics and compares frequencies of 2006 and 2010 responses.

Question	Answer	Overall		2006		2010		P-Value
		N	%	n	%	n	%	
Do you work with organic or transitioning-to-organic dairy herds?	No	174	48	81	45	93	50	0.30
	Yes	192	52	100	55	92	50	
Average number of milking cows of organic clients	1-50	91	49	49	52	42	46	0.29
	51-100	51	28	25	27	26	29	
	101-500	33	18	16	17	17	18	
	501-1000	4	2	0	0	4	4	
	>1000	6	3	4	4	2	2	
Would you or your clients be interested in the use of CAVM?	No	26	7	9	5	17	9	0.03
	Yes	206	56	114	63	92	50	
	Maybe	134	36	58	32	76	41	
Would you be interested in CAVM products, if they are presented from a rational, pharmacologic basis?	No	14	4	7	4	7	4	>0.99
	Yes	291	80	144	80	147	79	
	Maybe	61	16	30	16	31	17	
Do you feel that any of your conventional clients/herds would be interested in CAVM?	No	61	16	35	19	26	14	<0.01
	Yes	161	44	115	64	46	25	
	Maybe	143	39	31	17	112	61	
Would you be interested in receiving information for a few specific products (2006)/products allowed for use in organic herds (2010)?	No	51	13	8	4	43	23	<0.01
	Yes	314	86	173	96	141	77	

Table 2: Interest in complementary and alternative veterinary medication by bovine veterinarians for specific cattle diseases reported as overall, 2006 and 2010 responses as well as by veterinarians with or without organic clients. The reported P-value is based on Fisher's exact statistics and compares frequencies of responses of 2006 and 2010 as well as those from veterinarians with and without organic clients.

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	Overall		Year					Organic Clients				
			2006		2010			No		Yes		
Disease	N	%	n	%	n	%	P-Value	n	%	n	%	P-Value
Mastitis												
No	73	19	35	19	38	21	0.79	48	28	25	13	<0.01
Yes	293	80	146	81	147	79		126	72	167	87	
Calf Diarrhea												
No	117	32	75	42	42	23	<0.01	74	43	55	29	<0.01
Yes	248	67	105	58	143	77		100	57	137	71	
Metritis												
No	129	35	80	44	49	26	<0.01	78	45	65	34	0.03
Yes	237	64	101	56	136	74		96	55	127	66	
Infertility												
No	143	39	93	51	50	27	<0.01	83	48	65	34	<0.01
Yes	223	60	88	49	135	73		91	52	127	66	
Pneumonia												
No	148	40	95	52	53	28	<0.01	53	30	64	34	0.58
Yes	218	59	86	48	132	72		121	70	127	66	
Digital Dermatitis/Foot Rot												
No	159	43	99	55	60	32	<0.01	83	48	76	40	0.14
Yes	207	56	82	45	125	68		91	52	116	60	

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Table 3: Comments from veterinarians at the end of the survey either written in the margins of the postcard (2006 survey) or in the comment section of the "Thank you for participation" page in Survey Monkey.

Additional Comments
Survey 2006
A 1000 cow dairy is considering!
2 are considering (120cows each)
Main interest would be residues (concern slaughter)
No testimonials as scientific evidence, please
Interested in side effects and Interactions (with conventional meds) of CAVM products
Need more info to rationally reply
More scientific proves
Possibliy oregano for mastitis
Survey 2010
"Organic" is somewhat of a lie. Exemptions exist, allowing deworming in "emergency" situations, but not for routine health maintenance. Unproven, and often ineffective, products are promoted as superior. The consumer gets screwed, the farms go broke, and the cows suffer.
The organic movement is crap and it is set up to use government money to declare one citizen's products as superior to another's. This is not free market and is wrong.
I have been working with just a few organic herds. As I've do more reading, I sometimes become more confused. For instance, I read that if a cow has a follicle, and you want her to show a heat, you can treat her with Folliculin. A quick internet search of Folliculin reveals that it is a synthetic estrogen. I feel like that shouldn't even be legal for conventional herds. These are the kinds of questions I would appreciate clarification about. Thanks.
The point is of production medicine is prevention. The diseases you list are all management induced. We shouldn't have to treat. That is where our interest lies.
This stimulates a question regarding how much is acupuncture used for pain management in these animals?

Flax for Fido and Seaweed for Kitty

HerbalEgram November 2015

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Flax for Fido and Seaweed for Spot: The Growing Market for Herbal Pet Care in the United States

Health-conscious consumers are not only using botanicals in record numbers for their own well-being but, increasingly, they also are turning to herbal supplements for their pets. "What's good for the goose is good for the gander," and many herbs that show up on the supplement aisle, such as chlorophyll/chlorella (*Chlorella vulgaris*, Chlorellaceae) and flax (*Linum usitatissimum*, Linaceae), are being marketed for companion animals, mainly cats, dogs, and horses.

According to the labeling used by Chicago-based marketing firm SPINS, the market for "pet supplements" comprises two broad categories: supplements, which deliver the botanicals in what is usually a solid dosage form, and treats and snacks, which include the botanical or blend of botanicals as a part of a baked biscuit, cookie, or other tasty reward. The market so far shows uneven growth: for a 52-week period that ended August 9, 2015, SPINS recorded total aggregate sales of $43,044,385 across both categories, a 25% decrease from the same time period in 2014. However, the market in 2014 grew by 12% over 2013. These data include products sold in the natural, specialty/gourmet, and mass-market channels in the United States. They do not take into account sales from Whole Foods Market, which does not report its sales to marketing firms, or direct sales from businesses that sell solely on the Internet.

Natural Medicine Practice for Pets

While complementary and integrative therapies for animals, including herbal supplements, are gaining popularity and visibility in the United States, they are not new practices. Traditional Chinese veterinary medicine (TCVM), along with traditional Chinese Medicine for humans, has been practiced for thousands of years, using both herbal remedies and practices such as acupuncture, nutritional therapy, and therapeutic massage.

"Far and away, I used Chinese herbal formulations most often because that is where most of my training lies," wrote Clay Bernard, DVM, head veterinarian at Even Flow Veterinary Herbal and Acupuncture in Austin, Texas (email, October 28, 2015). "I see great results from them…. Many pet owners come to me looking for another option for treatment when conventional options have either been exhausted [or] unsuccessful."

The United States has several professional societies and training programs for the practice of integrative veterinary medicine, including the American Holistic Veterinary Medical Association, Veterinary Botanical Medicine Association, and the American College of Veterinary Botanical Medicine. "I think there are still a few skeptics among clinicians and pet guardians," Dr. Bernard admitted, "but the number seems to be shrinking. I think most people have seen results first-hand, or know someone whose animal has benefitted from acupuncture, herbs, food therapy, chiropractic, or other means of natural healing."

The market is responding. In October 2015, the second annual Petfood and Animal Nutrition Conference was held in Chicago, Illinois. Exhibitors and speakers from all aspects of the animal nutrition industry, including natural supplements, were represented. In 2015, natural supplement brand NOW® Foods released its own line of pet supplements. In 2009, former *HerbalGram* Managing Editor Courtney Cavaliere examined the pet supplement market from a regulatory perspective in issue 82.[1]

According to Bill Bookout, president and chair of the board of directors of the National Animal Supplement Council (NASC), an industry trade organization, little change has been made regarding the regulatory status of animal supplements (oral communication, November 3, 2015). The Dietary Supplement Health and Education Act of 1994 (DSHEA) applies only to products intended for human consumption, and no parallel law creating a "dietary supplement" category for animal products exists. Therefore, products for animals are classified as either a "food" or a "drug." Legally, most animal supplements are food additives, said Bookout. Since they are not dietary supplements, they cannot be labeled with or advertise a structure/function claim.

Long-term solutions pertaining to the regulation of supplements for animals have been debated since the passing of DSHEA, and still are being sought at the state and federal levels. However, "the industry has been able to operate very successfully under a framework of enforcement discretion, provided companies act responsibly," said Bookout.

The Herbal Pet Supplement Market in the United States

For the past three years, chlorophyll/chlorella supplements have taken the first spot in both the supplement and treats/snacks categories (Table 1, Table 2). These ingredients primarily are used to support immune

system function in humans, and have similar benefits for animals, including support of the digestive tract and oral health. Other ingredients that are popular across both categories include flax seed and/or oil, parsley (*Petroselinum crispum*, Apiaceae), and chamomile (*Matricaria recutita*, Asteraceae).

Table 1. The 20 Top-Selling Herbal Supplements for Animals in the United States, August 2014 – August 2015 (per SPINS)*

	Botanical†	Latin Binomial	Total Sales	% Change from 2014**
1	Chlorophyll/Chlorella	NA/*Chlorella vulgaris*	$4,548,754	-64.80%
2	Parsley	*Petroselinum crispum*	$3,246,821	5.89%
3	Peppermint and Other Mints	*Mentha* spp.	$1,281,459	7.94%
4	Chamomile	*Matricaria recutita*	$725,928	-15.29%
5	Garlic	*Allium sativum*	$495,399	-2.01%
6	Alfalfa	*Medicago sativa*	$285,237	2.79%
7	Flax seed and/or oil	*Linum usitatissimum*	$208,423	-24.17%
8	Lavender	*Lavandula angustifolia*	$134,844	-55.58%
9	Grass (Wheat or Barley)	*Triticum aestivum* or *Hodeum vulgare*	$75,906	-25.63%
10	Valerian	*Valeriana officinalis*	$51,419	22.78%
11	Mullein	*Verbascum thapsus*	$33,309	-0.37%
12	Cranberry	*Vaccinium macrocarpon*	$29,273	5.09%
13	Cayenne	*Capsicum annuum*	$21,572	-88.14%
14	Yucca	*Yucca* spp.	$12,828	-54.82%
15	Red Clover	*Trifolim pretense*	$4,733	1897.05%
16	Barberry	*Berberis vulgaris*	$3,735	-35.51%
17	St. John's Wort	*Hypericum perforatum*	$3,414	61.88%
18	Goldenseal	*Hydrastis canadensis*	$3,179	2272.39%
19	Skullcap	*Scutellaria lateriflora*	$2,887	N/A
20	Boswellin or Boswellia	*Boswellia glabra*	$2,885	-13.13%
	All Other Herbs Total		$11,172	
	Total Sales		**$11,183,177**	**-43.67%**

*Source: SPINSscan Natural, SPINSscan Specialty Gourmet, and SPINSscan Conventional Multi-Outlet powered by IRI, 52 weeks ending August 9, 2015.
†Herb coded as primary ingredient.
**52 weeks ending August 10, 2014.

Flax seed and its oil contain alpha-linolenic acid, linoleic acid, and omega-3 fatty acids, which aid in thedevelopment and maintenance of the brain, liver, and heart. Animal models have shown that these are vital to the healthy development of young animals and may also improve their skin, coat, and nails.[2,3] Flax can be administered either in supplement form or the ground seeds and oil can be added directly to the animal's food.[4] The consumption of parsley can improve gastrointestinal and urinary symptoms, as well as joint disorders, such as gout and arthritis. Chamomile can be used in the diet as a tea or tincture, or applied as a salve or ointment for a multitude of benefits. Given orally, chamomile acts as a mild sedative and gentle digestive tonic. Applied topically, chamomile preparations can relieve mild inflammation due to insect bites, allergies, or bacterial or fungal infections.[5]

SPINS data identified new or more robust sales for several ingredients in the supplement and treat market in 2015. The latest botanicals considered beneficial for pets include slippery elm bark (*Ulmus rubra*, Ulmaceae) and skullcap (*Scutellaria lateriflora*, Lamiaceae) supplements, and cranberry (*Vaccinium macrocarpon*, Ericaceae) has been added to treats. Slippery elm is commonly used in pets for its mucilaginous and anti-inflammatory properties, and can be administered in cases of gastrointestinal distress, such as diarrhea and constipation.[4] Skullcap, considered a nervine tonic, can benefit animals as an analgesic and anti-spasmodic for

jittery conditions. Cranberry is a source of proanthocyanidins, antioxidants that give cranberry its dark red color. This makes it a useful addition to pet treats, which generally are given daily and can help maintain urinary tract health.

Table 2. The 15 Top-Selling Herbal Treats and Snacks for Animals in the United States, August 2014 – August 2015 (per SPINS)*

	Botanical†	Latin Binomial	Total Sales	% Change from 2014**
1	Chlorophyll/Chlorella	NA/*Chlorella vulgaris*	$20,623,873	1.02%
2	Flax seed and/or oil	*Linum usitatissimum*	$6,415,345	-44.64%
3	Parsley	*Petroselinum crispum*	$3,167,581	-12.89%
4	Alfalfa	*Medicago sativa*	$1,451,904	-0.07%
5	Chamomile	*Matricaria recutita*	$93,124	-70.01%
6	Chia seed or oil	*Salvia hispanica*	$24,320	309.77%
7	Turmeric	*Curcuma longa*	$22,937	-2.23%
8	Clove	*Syzygium aromaticum*	$20,092	136.96%
9	Cherry fruit	*Prunus avium*	$17,147	26.67%
10	Ginkgo	*Ginkgo biloba*	$8,676	39.71%
11	Kelp	*Laminaria digitate*	$8,169	34.76%
12	Cranberry	*Vaccinium macrocarpon*	$2,729	N/A
13	Lemon balm	*Melissa officinalis*	$1,558	360.95%
14	Grass (Wheat or Barley)	*Triticum aestivum* or *Hodeum vulgare*	$1,522	-39.22%
15	Thyme	*Thymus vulgaris*	$873	-4.69%
	All Other Herbs Total		$1,358	
	Total Sales		**$31,861,208**	**-15.11%**

*Source: SPINSscan Natural, SPINSscan Specialty Gourmet, and SPINSscan Conventional Multi-Outlet powered by IRI, 52 weeks ending August 9, 2015.
†Herb coded as primary ingredient.
**52 weeks ending August 10, 2014.

As with the human supplement market, consumers have concerns regarding the quality and purity of the products given to their animals. Bookout acknowledges the need for vigilant oversight and accountability to ensure consumer confidence. He called product quality "not a destination, but a journey," and said that the NASC "supports a philosophy of continuous improvement."

In an effort to ensure a trustworthy supply chain for manufacturers, the NASC has recently instituted its "Preferred Supplier Program," in which interested parties must provide the NASC with an ingredient profile sheet for each botanical, vitamin, or other product they produce. The standards are stringent, but manufacturers who qualify for the Preferred Supplier Program will be available for viewing to NASC members, along with their ingredient profiles, testing data, and regulatory inspection audits. Bookout said that the NASC consults with leaders in the supplement industry to determine what tests are necessary to maintain high quality and purity standards.

Off-Market Considerations: Cannabis

With a growing number of states legalizing medical and/or recreational marijuana (*Cannabis sativa*, Cannabaceae) use, it follows, of course, that products containing marijuana are becoming an increasingly popular — and dubiously legal — option for pet owners. Even in states with legal medical marijuana use, however, veterinarians cannot by law prescribe or recommend cannabis for animals (though in Nevada, that may soon change: a law, SB372, which was introduced to the legislature in March 2015, has a provision called "pot for pets"[6]). Another option for the cannabis-minded pet owner is hemp and hemp products. Industrial hemp farming has been legal in Canada since 1998, and to date, 22 American states have legalized hemp production, as well.[7,8] Unlike marijuana, hemp contains a negligible amount of the psychoactive compound tetrahydrocannabinol (THC) and cannot be used to produce psychoactive effects. Hemp can be processed as a source of fibers for rope, cloth, and paper, and its seed is a nutritious food product that contains protein, vitamin E, and the essential fatty acids omega-3 and omega-6.

Because of marijuana's strict Schedule I classification in the United States, research into its efficacy for humans is minimal, and efficacy for animals even more so. However, anecdotal evidence from owners who used marijuana or marijuana products to ease their animal's end-of-life care, joint pain, or degenerative condition (including cancer) indicates the possibilities for future research.[9] "I get asked about it at least weekly, so the demand is there," Dr. Bernard noted. "I'm all for 'pot for pets' and anything natural that can [facilitate] healing and eliminate/minimize pain or discomfort. I think there is still much to learn, however, about its use in the animal world…. To not explore that further would be a shame."

But pet owner beware: cannabis pet products have no regulatory oversight from the US Food and Drug Administration (FDA), and marijuana does not always present a safe, non-toxic treatment option. Though no lethal human overdoses have been recorded, marijuana ingestion can be injurious or fatal to animals. States with legalized medical marijuana have seen increasing reports of marijuana toxicosis in pets. According to one study, it was responsible for the death of two dogs.[10]

Conclusion

The practice of herbal medicine for animals has a millennia-long history, especially in the Chinese tradition. In the United States, the market for herbal pet products is so far uneven, but pointing towards a trend of growth overall. Pet owners increasingly are seeking out alternative therapies for their companion animals, embracing holistic practitioners and natural medicines — including medicinal marijuana. The growing mainstream interest and introduction of new products indicate that the US market for animal-oriented herbal remedies, though unstable at the moment, may be at the start of an impressive upswing.

—Hannah Bauman

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American Botanical Council, 6200 Manor Rd, Austin, TX 78723
Phone: 512-926-4900 | Fax: 512-926-2345 | Email: abc@herbalgram.org

About APPA

The American Pet Products Association (APPA) is the leading U.S. not-for-profit trade association serving the interests of the pet products industry. Founded in 1958 with 35 member firms, APPA's membership currently includes over 1,000 pet product manufacturers, importers, manufacturers' representatives and livestock suppliers representing both large corporations and growing enterprises worldwide.

APPA was established to promote, develop and advance responsible pet ownership and the pet products industry. To this end, APPA supports industry-related market research, monitors and responds to industry legislation and regulation, and sponsors educational seminars, networking and PR opportunities, giving members the tools they need to make important business decisions. APPA also works closely with other major organizations dedicated to similar goals to accomplish these and other important objectives.

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255 Glenville Road, Greenwich, CT 06831
Phone: 203-532-0000/Fax: 203-532-0551
www.americanpetproducts.org

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