PART A. ABSTRACT OF THE HABILITATION THESIS

ENTITLED

USEFULNESS OF VEGETAL EXTRACTS IN MANAGING POSTINFECTIVE STATUS WITHIN THE "ONE HEALTH" CONCEPT

Written by Senior Lecturer Dr. Carmen Dana ŞANDRU

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1. Relevance and impact of scientific research results

1.1 General data

• Doctor of Medical Sciences (PhD), field Veterinary Medicine, since 2007
1.2. Development of professional / teaching career

I am part of the teaching staff of the Faculty of Veterinary Medicine at the USAMV Cluj-Napoca since 2005. After graduation, I have worked as a veterinarian at IAS Alba, Teius Farm (1982-1985), hygienist veterinarian at SC Gorneşti, Mureş County (1985-1996), then the Doctor of Veterinary Medicine at the Faculty of Veterinary Medicine Cluj-Napoca (1996-2005), applied for the position of Lecturer in 2005 at the Department of Preventive Medicine, Veterinary Legislation and Deontology, Infectious Diseases, and in 2009, the contest for the position of Senior lecturer in the disciplines of Preventive Medicine, Veterinary Legislation and Deontology, Infectious Diseases, carrying out my academic teaching activity for 22 years.

1.3. Scientific results

Concerning the significance and the impact of scientific achievements during this period, it is necessary to mention, first of all, those obtained during the scientific work within the doctoral thesis. The subject of the PhD thesis "ASSESSMENT OF THE ADJUVANT AND IMMUNOSTIMULATING QUALITIES OF VEGETAL EXTRACTS IN FARMED AND COMPANION ANIMALS" has tackled aspects related to:

a) a distinction between the action of certain plant extracts obtained from different plants through the same extractive procedure on non-specific and specific immune responses;

b) Assessment of the solvent quality over the immunomodulating quality of the obtained extracts;
c) Differentiation of extracts’ activities based on their different potential influence on the two sectors of the cellular immune response (innate and adaptive immunity)

d) Differentiation of immune responses according to species (taxonomy) or peculiar morphological features in cattle, horses and goats, domestic carnivores (dogs) and wild (farm foxes), domestic (chickens) and wild birds (pheasants).

Based on our research, we came to the conclusion that *Calendula officinalis* extract inhibited phagocytosis in all herbivorous species during the first incubation period and subsequently induced phagocytic cell stimulation. The active principles of *E. angustifolia* stimulated this response in both periods, while the *Urtica dioica* extract was inhibitory in both reading periods. The dynamics of total leucocyte count increased after the treatment with unpolluted nettle. Nettle extracts stimulated phagocytosis, after a week of experiment, more intensely in the unpolluted area. This effect is short, until the end of the reading period, the effects being paradoxically reversed. The effects of nettle extracts, regardless of their origin, were more hyposensitising than hypersensitising chickens to antigen. By comparing the results obtained in pheasants with those in chickens, most of them were negative. The extracts acted as inhibitors in the used doses. Therefore, pheasants need a reduction in stress or a more adequate dose. A comparative assessment of blast transformation in the experiment showed that antigen priming was more effective in half of wild animals compared to domestic animals that were regularly treated.

Immunological reactivity, evaluated on the basis of spontaneous blast transformation in domestic species, was apparently lower, regardless of the phylogenetic class, than in wild mammals or birds, presumably due to environmental influences and/or vigor of artificially unselected animals.

The research activity carried out after the completion of the doctoral thesis was very broad, benefiting from the membership in the research teams
from the infectious disease discipline in which I first started as a researcher, then as a teacher. We have also collaborated on demand with the Hygiene and Welfare discipline team, monitoring the influences of the living environment on disease resistance and animal welfare, as well as the zoonotic risk they represent. This activity was carried out under the research contract I obtained through national competition (Evaluation of the immunomodulatory qualities of a whole and fractioned natural polivitamin concentrate on the immune system in phylogenetically different animal classes: 2002 - 2004 CNCSIS code 592), respectively the international grant obtained by competition (The zoonotic potential of polluting bacterial microflora of culture basins on Ontologically, the pond (Onchorhyncus mykiss) farms, Domus Hungarica Scientiarum et Artium, 2011), but also in the research contracts where I was active as a member (22 of which were relevant to the One Health concept), are the following: "Obtaining whole and fractional vegetal extracts with high bioavailability and potential adjuvant activity for animal species of economic interest". CEEX Adexplant nr. 99; 2006 - 2008 Module I CNMP BIOTECH; "Influence of the phylogenetic position on the immunomodulatory qualities of plant extracts useful as adjuvants in farmed herbivores" 2007 - 2008 CNCSIS - Code. 874; "Optimization of the production technology and implementation of the European food safety concept on the dairy branch in cattle farms in the counties of Alba, Bihor and Cluj" - Contract BM, Nr. Project: 1194/2004 / Nr. Grant Agreement: 5114 / 07.09.2004 / Nr. Act Arbitral: 6043 / 07.01.2005; 2004 - 2005, PN II - IDEI Exploratory Research Projects 2008; "Study of the Influence of the Shelter and Management Practices on the Welfare of Dairy Cows", Project Code: 1492 2009 - 2011, PNII - Complex Exploratory Research Program, CNCSIS; "Fundamental and applied studies of the ecoepidemiology, biology and molecular genetics of Lyme disease vectors" Project Code 84, 2010-2013, "Establishing a paradigm for assessing the pollution of heavy metals and pathogenic microflora in birds and fish and its application in biodiversity conservation in the Danube Delta Biosphere Reserve ", PNII PCCE 61/2012 (2016)). Some of the results obtained have been published in journals
indexed in international databases, presented at national and international conferences or used to complete doctoral theses (Duca Gheorghita Marius - "Immune status as a indicator of welfare of herbivorous species in different Growth Systems ", 2012 Bianu Grigore Traian - " Evaluation of the potential for immunological restructuring and the modulating impact of extrinsic factors in poultry ", 2014 Crisan Liliana Emilia -" Immunological implications of bacterial microflora variation in clinical and subclinical mamitis in cows ", 2012, Lazar (Diugan) Eva Andreea - "Relationship between welfare and resistance to infectious diseases in equines", Rindt (Doczi) Iulia Krisztina - "Evaluation of the therapeutic and immunostimulatory qualities of apiculture products", Kiss Timea - "The distribution and ecological particularities of Borrelia burgdorferi sensu lato genospecies spread in urban and silvic biocenoses in NorthWestern Transylvania" also supporting licensing works.

Thus, research can be synthesized in four main directions of study:

a) Evaluation of the efficacy of plant extracts as immunomodulators / stimulators in different animal species and estimation of antibacterial capacity (minimal inhibitory concentration, minimal bactericidal concentration);

b) Estimation of the potential for intervention of plant extracts in the treatment of immunopathological changes caused by different microbial agents (Pasteurella multocida, staphylococci and streptococci, E. coli, Mycobacterium tuberculosis, Mycoplasma agalactiae, viruses including infectious anemia);

c) Welfare monitoring in conjunction with infectious disease resistance by investigating factors of influence: growth technology, hygiene of the shelter, presence of gaseous noxes, drinking water quality, human-animal interrelation in immune system conditioning, microbial load the air in the shelters, etc.

d) Inventory of the potential intervention of vegetal extracts in conditioning the microbiota of the environment by exerting the
bacteriostatic/cidal effect, aiming at the isolation and identification of the microflora in different habitats and the way vegetal extracts act in vitro on these bacteria, compared to the classical drugs (antibiotics).

The results of the research were relevant in emphasizing the impact of the etiological-environmental agent interaction on the implementation of different therapies and prevention and control programs in order to avoid the spread of zoonotic agents and antibiotic resistance according to the concept of "One Health".

It has also been shown in various pathogenetic circumstances and on numerous feral or wild animal species the beneficial effect of using medicinal herb extracts that have proven to possess not only immunoactive qualities but also pronounced antibacterial potential, the medicine panacea.

Following the results of the studies on the segments of integrating the results into a whole "human-animal-environment, health", separating them for facilitating the discussions, we can conclude:

\[ a) \textit{Evaluation of the efficacy of plant extracts as immunomodulators / stimulators in different animal species and estimation of antibacterial capacity (minimal inhibitory concentration, minimal bactericidal concentration);} \]

In the case of monitoring the in vitro effects of plant extracts on immunity in mastitis cows it was observed that the values obtained for the transformation index (TI) were negative only in the case of \textit{Thymus vulgaris}; mixture of \textit{Thymus vulgaris} extract and honey; the mixture of \textit{Calendula officinalis} extract and the honey sample and also the \textit{Echinacea angustifolia} mixture and the honey sample, suggesting that the alcoholic extract of \textit{Thymus vulgaris} acted inhibiting in cell cultures every time they when evaluated, individually or in combination with a sample of honey. On the other hand, with regard to most variants that have been shown to be able to induce a stimulating effect on leucocyte growth, it is obvious that the level of biological effect mentioned can
not be considered very high. Differences established by the t-Student test can not be included in the statistically significant category (p > 0.05). The statistical evaluation of the results also showed the stimulatory effect determined by the honeydew honey sample, comparable to that of the extract of *Calendula officinalis* and *Hippophae rhamnoides*, well known in the literature for this effect. There are no statistically significant differences between blast index values (40.48%, 33.39% and 33.73% respectively), indicating the stimulating potential of the honey sample on the proliferation of avian leukocytes.

To investigate the effects of honey or propolis in a solid medium, the diffusion method was used on the plate. Two variants, with wells and tablets respectively, were applied, similar to Kirby-Bauer technique. Amoxicillin with clavulanic acid (AMC) and enrofloxacin (ENF), respectively, were the controls for antibacterial activity in this experiment. Bacteriostatic (CMI) and bactericidal (CMB) values obtained from staphylococci by the micrometode of dilutions are interconnected. These values suggest that honey, polychlorinated honey and propolis had bacteriostatic and bactericidal effects in similar concentrations. The antibacterial effect expressed by the increased size of the inhibition zones does not depend on the increased content in melezitose (e.g., at a melezitose content of 0.73% inhibition is 12.33 mm and at a melezitose content of 5.23% inhibition is 16.33 mm). Antimicrobial resistance as a result of irrational and excessive use of antibiotic therapy is a major concern for both human medicine and veterinary medicine.

**b)** *Estimation of the potential for intervention of plant extracts in the treatment of immunopathological changes caused by different microbial agents (Pasteurella multocida, staphylococci and streptococci, E. coli, Mycobacterium tuberculosis, Mycoplasma agalactiae, viruses including infectious anemia)*;

An estimation of the functionality of the various effectors involved in antiinfectious immunity during the clinical course of mycoplasma mastitis could
clarify some aspects of disease pathogenesis and recurrence in the constantly vaccinated flocks in regions with high epidemiological risk. The evolution of infection tends to be chronic in the affected animals and flocks. These insidious infections, associated with the carrier state in healthy animals, are difficult to diagnose and control. Positive effects of *Echinacea angustifolia* have been monitored to increase immune cell activity in diseased animals.

Spontaneous phagocytic activity during the first blood sampling (day 0) was higher than after in vivo antibiotic treatment due to the post-therapeutical decrease in leukocyte count or possibly due to the inhibitory effect of antibiotics. The results indicated a negative influence of the infection on the spontaneous phagocytic activity of the animals. *Echinacea purpurea* extract exerted a more pronounced inhibitory effect before therapy. Phagocytosis was significantly correlated with the total number of leukocytes previously, but not after treatment with antibiotics (r = 0.492, p <0.01).

The quantification of the effect of infection with the equine infectious anemia virus (EIAV) on cellular immunity was performed in vitro. The carbon particle inclusion test was used to monitor inborn spontaneous phagocytosis with 3 variants (control, alcohol and nettle extract). The in vitro blast transformation test was performed with 8 experimental variants (control, PHA-M, alcohol and alcohol extracts from *Calendula officinalis, Echinacea angustifolia, Echinacea purpurea, Urtica dioica* and *Aloe vera*).

A possible rapid ingestion of the carbon particles, followed by fragility of the cell membrane, could explain the stimulatory effects of nettle extract on phagocytosis in newly infected EIAV horses, while as a consequence of the lack of antigenic stimulation in vivo, indices was decreased in healthy animals. The duration of disease evolution highlights the incapacity of the response to PHA M, unlike the trend seen in young animals, probably due to the reduction in immune response to aging. In general, immunoreactivity and immune recovery effects of used plant extracts are more evident in newly infected young animals, suggesting
that viral infection has an increasingly negative effect on immunity to older horses.

c) *Welfare monitoring in conjunction with infectious disease resistance by investigating factors of influence: growth technology, hygiene of the shelter, presence of gaseous noxes, drinking water quality, human-animal interrelation in immune system conditioning, microbial load the air in the shelters, etc.*

Monitoring immune system’s changes in different environments could provide useful information to dairy cow breeders to ensure optimal growth and welfare conditions for their animals. The research looked at the overall influence of the maintenance system on humoral and cellular immunity in dairy cows aged between 5 and 10 years. The blood samples treated with alcoholic *Silybum marianum* extract were incubated with India ink at 37 °C for 0 (t0), 25 (t1) and 50 (t2) min.

Optical density measurements were converted to a log2 scale, and the phagocytic index was calculated as the negative of the regression of optical density regression (log2) (t0-t1, t1-t2). Tlg and CIC values were relatively high compared to physiological values (40.1 ± 16.6 and 7.50 ± 5.16, respectively), but remained lower than those seen in intensive growth. Phagocytosis was diminished in both control and treated versus normal values. The *Silybum marianum* extract exerted a negative effect on innate cell mediated activity. Intensive exploitation apparently had a more pronounced negative effect on innate cellular immunity than on innate humoral immunity in dairy cows.

Activation or stimulation of lymphocytes refers to a correlation of their *in vitro* activity with phenomena that occur regularly *in vivo* because the host interacts with an antigen. Thus, in vitro blast transformation capacity serves as a measure of reactive cell potency and also as a tool for monitoring the efficacy of various components (drugs, antigens, other active compounds) in modulating this response. The investigations were conducted to evaluate the efficacy of *Silybum*
marianum, Hippophae rhamnoides, Vaccinium myrtillus, Thymus vulgaris, and Aloe vera alcoholic extracts on cell mediated adaptive immunity. There were no significant differences between the experimental variants, except those treated with Vaccinium myrtillus and Thymus vulgaris (p < 0.05) versus the PHA and alcohol treated variants (36.68 ± 19.98% and 35.36 ± 10, 67% versus 58.26 ± 25.41% and 58.96 ± 15.25%, respectively). Although the control culture spontaneous index was a physiological one (56.78 ± 12.16%), none of the extracts had a stimulating effect. Furthermore, some of the extracts (Vaccinium myrtillus and Thymus vulgaris) exerted a strong inhibitory effect, showing that some of the plants found on pastures could negatively influence the adaptive cellular protection of high-quality cows.

**Inventory of the potential intervention of vegetal extracts in conditioning the microbiota of the environment by exerting the bacteriostatic/cidal effect, aiming at the isolation and identification of the microflora in different habitats and the way vegetal extracts act in vitro on these bacteria, compared to the classical drugs (antibiotics).**

In the framework of the conservation of the health of fish artificially and raised, more diseases have been included in the National Program for Surveillance, Prevention and Control of Animal Diseases, Transmissible Diseases, Animal Protection and Environmental Protection, especially Salmonids. Thus, surveillance and control of viral haemorrhagic septicemia, infectious haematopoietic necrosis, infectious pancreatic necrosis, bacterial kidney disease (Renibacterium salmoninarum), salmonid furunculosis (Aeromonas salmonicida) red mouth (Yersinia ruckeri) and Chondrococcus columnaris infection in trout were mentioned.

The health status of farmed fish depends largely on water quality, including oxygen levels, contamination with chemical or biological pollutants, as well as diet, growth techniques and management techniques. Investigations of bacterial pollution of waters and fish with regard to zoonotic species in
commercial trout farms are intended to signal the zoonotic risk posed by fish for consumption by facilitating measures to prevent disease in consumers and environmental pollution (“One Health”).

The rainbow trout seems to be more sensitive to an increased number of pathogenic bacteria (\textit{Pseudomonas aeruginosa, E. coli, Aeromonas salmonicida, Ochrobacterium anthropi}).

Following the microbial diversity in the culture basins, there has been an increase in the degree of pollution with zoonotic bacteria in culture waters for both rainbow and brown trout, such as \textit{Pseudomonas aeruginosa, E. coli, Acinetobacter baumannii / calcoaceticus, Ochrobacterium antrops, Pasteurella pneumotropica, Chrysoabacterium indoligenes, Photobacterium damselae}. Bacteria isolated from culture water and farmed fish were a potential risk to workers and other contact groups.

The recent introduction and spread of new pathogens, together with global climate change, have contributed to a considerable decrease in trout production. Increasing fish production means avoiding and controlling the limitation of harmful factors. The presence of potentially pathogenic bacteria in fish of different age groups and the level of bacterial contamination of culture waters in a commercial trout farm with mixed rainbow trout and brown trout population was monitored using ID 32 E, API 20NE and Staph API galleries. Both fish and culture water were highly polluted by bacteria, but the largest bacterial load was found in commercial trout (3-400g), suggesting the vulnerability of this age group. Bacteria with pathogenic potential for fish (\textit{Aeromonas hydrophila}) and humans (\textit{E. coli}) have indicated technological failure and potential faecal contamination of water, increasing the risk for consumers and consumers.

\section*{2. The capacity of the candidate to guide students or young researchers}
Throughout the entire teaching and research activity initiated in 1996, I had the opportunity to coordinate over 45 students within the debating society of Infectious Diseases, to conduct research and complete the DVM work both at the Faculty of Veterinary Medicine Cluj-Napoca, as well as at the Veterinary College, which worked within the faculty.

The various topics covered included subjects of anti-infectious immunity, veterinary medical prevention by non-specific and immunological means, evaluation of the immunological effects of medicinal herb extracts and their effects on the microbiome in different animal species.

These researches were presented at local, national and international student symposia. Similarly, I have been involved in supporting doctoral students in finalizing the research for the thesis and processing the data for publication in ISI rated journals.

Given the seniority in research, I have helped younger colleagues in acquiring laboratory techniques specific to the diagnostic field in infectious diseases, in order to accurately assess the complex changes these have induced in animals.

3. Teaching competences of the candidate

As a teacher, I taught the course of Veterinary Legislation and Deontology as well as the related practical part to the students of the 6th year of veterinary medicine. At the same time, I participated with my colleagues from the discipline to the clinical work of the V and VI years, and carried out trips to the production units and private farms for the clinical evaluation of animals and immunoprophylactic operations. When, on the recommendation of EAEVE (2004), the discipline of preventive medicine was introduced, I took over the course and developed the theoretical themes. Throughout this period, I have sought to enrich my theoretical and practical knowledge, to keep up with the
literature, to train the students in the day one skills and the development of logical thinking, analytical spirit and self-discipline.

I have edited and contributed with my colleagues from the discipline to the development of 7 textbooks and specialized books for the use of students. Through the emergence of "One Health" research orientation and the investigation into the widespread phenomenon of antibiotic resistance, we succeeded in publishing a chapter in an international InTech Open book titled "Heavy Metal Polutome and Microbial Resistome Reciprocal Interaction and its Impact on Human and Animal Matrices ". Every year I have directed at least one student to the scientific circle of infectious disease discipline in order to conduct the research and develop the DVM thesis. I contributed to the diversification of the didactic material by equipping the discipline with the necessary material from the research contracts to which I was a director or a team member. I have produced a collection of legislative material that we have provided to the students for improving their knowledge in veterinary legislation and guided them in their exercise through problem-based learning. These activities were aimed at improving the level of medical education of students, in particular the acquisition of Day 1 skills, according to the ARACIS instructions and the provisions of Directives 36/2005 and 55/2013, which regulate veterinary education at European level.

I contributed to the expansion of the discipline library through internationally developed relationships and book donations. As a result of these activities, I have obtained a very good rating at student ratings.

I also participated in a TEMPUS PHARE (Tempus JEP 14409/1999) educational educational program 1999 - 2001, in collaboration with the University of Liege, to evaluate the existing level of professional counseling of students and the perspectives of this field in veterinary medicine Romanian. Thanks to my participation in this project, I was appointed as First Deputy of the Professional Counseling Bureau newly established at USAMV Cluj in 2000.
I also repeatedly participated in the exchange of experience and courses for students in the ERASMUS and ERASMUS plus programs, with the Veterinary Medicine Faculties in Lisbon, Cordoba, Brno, Budapest and Warsaw.

4. The ability of the candidate to transfer knowledge and results towards the economic and social media, or to mediatise own scientific results

Between 1996 and 2017, I published 260 scientific papers (10 ISI, 6 ISI Proceedings and 82 BDI and 165 other publications - which appeared before indexing in the databases our work) to which I was the single author, first author or collaborator. Also, the research works elaborated with teachers from the Faculty of Veterinary Medicine Cluj-Napoca and other colleagues were presented orally or as a poster at symposia or scientific conferences in the country or abroad. Some of them were awarded by UEFISCDI in 2013 and 2016. Scientific reports on research contracts were communicated to the contractor MECŞ or UEFISCDI, and also to the economic and social environment, by publishing them on the project site, specialized sites or presented during lectures to practitioners in the work-shop or life-long learning courses. PNII PCCE Project 61/2012 – “Establishing a paradigm for assessing the pollution of heavy metals and pathogenic microflora in birds and fish and its application to biodiversity conservation in the Danube Delta Biosphere Reserve” has ended with a patent application, which is under work at OSIM.

At present I am a member of several professional and scientific associations in Romania and abroad, associations where congresses and symposiums are organized, where I presented the results of my research (CMAPSEEC, GA, MESMAP etc).
5. The capacity of the candidate to work on a team and the efficacy of his/her collaborations

Numerous national (21) and international (2) scientific research contracts, as well as the TEMPUS project (1), in which I participated, demonstrate the skills to work in a team. Collaboration in the field of infectious pathology with other groups (FMV Belgrade, FMV Warsaw) support the same principle.

The work resulting from collaborations of different levels to which I have contributed demonstrates my ability to participate in a complex research within a team. Most of the results obtained were published as scientific papers mainly in international databases, magazines, but also through presentations at various national and international symposiums and conferences.

I also contributed to decision-making teams of the students through my professional counseling, then participating in working groups.

6. The capacity of the candidate to lead research-development projects

The ability to conduct research projects is proven through the submission of project proposals in national competitions as a director and winning the grant obtained through competition from the Hungarian Academy of Sciences.

Throughout my teaching and research activities, I have also led research grants to service with the private sector. Through the grants I have contributed to equipping the discipline with modern equipment, materials and reagents necessary for the good development of didactic activity, but also for research. The involvement of students and PhD students in the relevant themes of the respective research projects facilitated the development and specialization in certain fields of diagnosis and development of the human resource.
Of the students contributing to these projects, some continued their scientific training at the doctoral level, remaining even in didactic positions within the discipline. Some of these students were accepted for studies but also permanent positions in prestigious research units in Europe (Leibnitz Institute for Zoo and Wildlife Research or Bernhard Nocht Institute for Tropical Medicine). The research results were disseminated by presenting and publishing scientific papers (national, international, ISI) at various symposia and congresses, thus contributing to the development of the knowledge of the respective field, as well as to increasing the international visibility of the research.

7. The professional experience of the candidate in other institutions than USAMV Cluj

For professional development and specialization, both before and after the presentation of my PhD thesis, I have benefited from several stages of training in prestigious universities in the country as well as abroad, as follows:

1998- Specialization Internship, Immunology Laboratory, INMV Pasteur, Bucharest
1999- Perfectionary Stage, Laboratory of Immunology, Electronic Microscopy Laboratory, INMV Pasteur Bucharest
2000- Specialization stage, Laboratory of Parasitology, INMV Pasteur Bucharest
2001 - Fleming consultancy model, a model that is specific in Belgium - University of Gent (Belgium)
2006 - Documentary Stage Agricultural University of Warsaw Faculty of Veterinary Medicine (Poland) Prof. Dr. Iaroslaw Kaba
2007 - Documentation Stage - Laboratory Diagnostic Tests - Cordoba University (Spain) Prof. Dr. Miguel Moreno Milan
2008 - Documentary Stage - Community Legislation. Sanitary veterinary regulations. Public Health and Environmental Protection, Technical University, Faculty of Veterinary Medicine, Lisbon (Portugal) Prof. Dr. Yolanda Vaz
Utilitatea unor extracte naturale în restaurarea postinfețioasă în cadrul conceptului “One Health”

2016 - Teaching course, SGGWU Warsaw, Poland
2017 - Teaching course, SGGWU Warsaw, Poland