NICULAE MIHAELA

AN ESTIMATE OF BIOAVAILABILITY OF CERTAIN IMMUNOMODULATING AND/OR ANTIMICROBIAL HERBAL EXTRACTS IN THERAPY OF CANINE OTITIS EXTERNA

SUMMARY OF Ph.D. THESIS

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SUMMARY

In small animals’ pathology, otitis externa (OE) represents an important chapter, taking into consideration its relative high prevalence, mainly in canine populations, also the course characteristics of the inflammatory process and the oscillating results of the current therapies.

Although, it is not a critical, life threatening condition, it can represent a challenge for the clinicians, as it is usually associated with a chronic or recurrent course and very modest respond to treatment. These aspects can be the direct consequences of minimizing or neglecting the multi-factorial etiology of otitis externa.

Current medical opinion on canine otitis externa specifies that the efficient management of this pathology is fundamentally dependent on the perception and control of the etiopathological phenomena. These phenomena are complex as they include three main categories of factors: predisposing, primary and perpetuating factors (Angus, 2004). Most of the veterinary dermatologists consider that the main factors favor the intervention of perpetuating factors, usually represented by opportunistic organisms (Pseudomonas aeruginosa) and/or normal bacterial flora of the ear canal (negative coagulase Staphylococcus spp., alpha-haemolytic Streptococcus spp., Proteus spp., Corynebacterium spp., non-pathogenic Bacillus spp.)

Unfortunately, the emergence of antibiotic resistance in case of bacterial species and also current practice of underestimating or ignoring the role of multiple etiopathogenetic factors result in augmentation of the number of otitis externa cases that are rebel to classic treatment, relapse and chronic course tendency. This situation is most frequently observed when testing bacterial susceptibility towards antibiotics is not used a medical tool.

Considering these aspects highlighted by the “Bibliographic study” and presented in the first part of the doctoral thesis (Chapter 1 and Chapter 2), the research of the thesis entitled: An Estimate of Bioavailability of Certain Immunomodulating and/or Antimicrobial Herbal Extracts in Therapy of Canine Otitis Externa, had as main objectives the study of microbial factors (bacteria and fungus) and the host (dogs with otitis externa) immune response towards this antigenic stimul, as well as an estimate of the antimicrobial and immunomodulating potential of certain herbal products in order to exploit further these natural products for the stated pathology therapy. The herbal preparations selected based on the results of the antimicrobial and immunomodulating effects screening were further evaluated both in vitro and in vivo for toxicity in order to avoid the development of undesirable phenomena, namely the last chapter of this thesis includes a preliminary study on the bioavailability of a polyherbal preparation proposed for the local therapy of otitis externa in dog.

These objectives were followed in the research conducted between 2005-2011 and the results are presented in the five chapters of the second part named “Personal researches” (Chapters 4 -9) which includes also Chapter 3 where the research motivation, objectives and activities are mentioned.

In the first personal research chapter (chapter IV), entitled ”Microbiological and Immunological Investigations in Canine Otitis Externa” we intended the study on canine external otitis associated microbial elements that act as perpetuating factors of inflammatory processes (the identification of different bacterial genera and species and
their analysis in terms of normal skin microecology and also the establishment of resistance spectrum of isolated bacterial strains towards different antibiotics), but also the evaluation of the level or of the functionality of non specific humoral immune factors: total immunoglobulins, circulant immune complexes, seric properdin, serum lyzozyme and serum bactericidal ability in case of dogs with otitis externa. The researches were performed between October 2005 and November 2006, considering the clinical cases of several Veterinary Clinics from Cluj-Napoca and the Infectious Disease Discipline cases (cases directed by the Emergency Hospital or other Clinics of the Faculty of Veterinary Medicine Cluj-Napoca). The study included 56 cases, 32 males and 24 females, belonging to several breeds and with ages between 2 months and 13 years. In order to ensure the homogeneity of cases registration, a protocol agreed with clinicians vets was followed, protocol that included: clinical examination, filling out an observation form and sampling from each affected ear. To isolate and characterize the microbial species, classic microbiological methods were used (microscopic evaluation, cultural examen, testing of susceptibility towards antimicrobials), while for the humoral reactivity assessment standardized the following immunological techniques were carried out: total immunoglobulins measurement, circulant immune complexes measurement, properdin serum concentration measurement, serum activity of lyzozyme evaluation, as well as serum bactericidal activity evaluation.

The results of the microbiological investigations confirmed in most cases an important microbial activity, as numerous strains belonging to genera *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp. and *Malassezia* spp. were isolated. Thus, the microbial flora isolated from dogs with otitis externa was represented by coagulase-positive staphylococi, mainly *Staphylococcus intermedius*, Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Proteus* spp. and as yeast *Malassezia pachydermatis*, proving their pathogenic role and ability. Analysis of microbial profile indicated the predominance of association between *Staphylococcus* spp. and *Malassezia pachydermatis*. Regarding the antimicrobial susceptibility, bacterial strains behaved inhomogeneous, some strains being sensitive, but not to all 14 tested antibiotics, probably as a consequence of excessive or non-rigorous use over time. Overall, isolated bacterial strains expressed a high level of antibiotic resistance more pregnant expressed in case of Gram-negative germs, and the resistance was observed mainly towards the antimicrobials included in otic preparations used in canine otitis externa therapy. Based on the antibiogram, several antimicrobial susceptibility patterns of isolated strains were determined and as active antimicrobials we suggested two quinolons: enrofloxacin and ciprofloxacin. The implication of multiresistant bacterial strains emphasizes the need for susceptibility testing in order to choose appropriate antimicrobial agents.

As for the immunological investigations, the obtained data underline the involvement of certain nonspecific humoral factors in otitis externa (namely properdin, lyzozyme and serum bactericidal activity against *Staphylococcus aureus*) (table 1). Significantly increased level of total immunoglobulins observed in dogs with otitis externa can be due to the multiple antigenic (multifactorial aetiology) and to the long lasting course (chronic course), but this type of immune reaction definitively possess a non specific humoral mediation, as well as a specific one (specific antibodies against bacteria and yeast). Statistically distinct significant difference between healthy and otitis externa groups’ properdin serum levels suggests the contribution of this immune factor to the
The aim of second personal research chapter (chapter V) entitled “In Vitro Evaluation of Antimicrobial Effects of Certain Herbal Extracts against Bacterial Strains Isolated from Dogs with Otitis Externa” was to investigate the in vitro antimicrobial properties of essential oils (EO) and ethanolic extracts (AE) derived from 14 herbal species (Calendula officinalis L., Matricaria chamomilla L., Hypericum perforatum L., Symphytum officinale L., Coriandrum sativum L., Hippophae rhamnoides L., Pelargonium graveolens L. Her., Lavandula angustifolia Mill., Mentha piperita L., Melissa officinalis L., Salvia officinalis L., Rosmarinus officinalis L, Ocimum basilicum L., Thymus vulgaris L. belonging to the following families: Asteraceae, Hypericaceae, Boraginaceae, Apiaceae, Elaeagnaceae, Geraniaceae and Lamiaceae) against clinical strains of Staphylococcus spp and Pseudomonas spp. (sensible and resistant strains) isolated from dogs with otitis externa. Herbal extracts were purchased from commercial sources or were obtained from Pharmacy Faculty Cluj-Napoca In order to characterize the antimicrobial efficacy of certain herbal extracts several methods were used; the antibacterial potential was evaluated by two variants of the diffusimetric method (disk and well assays, respectively), while for the elected herbal products after the screening, Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) were determined using a broth microdilution method (Carson et al, 1995). The MIC was defined as the lowest concentration (highest dilution) of herbal extractions that inhibited the visible growth (no turbidity), when compared to the control. The lowest concentration associated with no visible growth of bacteria on the agar plates was considered the MBC. Also, the bactericidal index (MBC/MIC) was calculated for each tested product (Pavithra et al, 2010) to determine whether the product is bactericidal (MBC/MIC<4) or bacteriostatic (4< MBC/MIC <32). Another study approach was represented by the estimation of synergistic relationships between herbal products and antimicrobials, considering essential oils and alcoholic extracts and gentamicin, polimixin B and neomicin, as these are the antimicrobial components of commercial otic drops. The interrelations between these product categories were studied by reference to the plate technique following the protocol described by Nweze and Eze Elizabeth (2009).

Diffusimetric method –both variants (disk and well) indicated based on the measurement of the bacterial growth inhibition diameter zone (mm) an important

### Table 1

<table>
<thead>
<tr>
<th>Investigated parameter</th>
<th>Group II</th>
<th>Group I</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total immunoglobulins</td>
<td>0.205±0.23 ODU 20.53±23.18 Vernes grades</td>
<td>0.043±0.01 ODU 4.37±1.47 Vernes grades</td>
<td>p&lt;0.05 (0.0248)</td>
</tr>
<tr>
<td>Immune complexes</td>
<td>4.51±6.8869</td>
<td>-0.545±3.751</td>
<td>p= 0.430</td>
</tr>
<tr>
<td>Properdin</td>
<td>0.194±0.052 mg/mL</td>
<td>0.303±0.113 mg/mL</td>
<td>p&lt;0.05 p= 0.0087</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.585±0.0421 µg/mL</td>
<td>0.522±0.150 µg/mL</td>
<td>p = 0.6504</td>
</tr>
</tbody>
</table>

| Normale values (U)     |  |  |  |

**augmentation of complement system functions with increased bacterial clearance.**
antimicrobial activity in case of certain herbal products, namely those obtained by several extraction procedures from the followin herbal species: *Coriandrum sativum* L., *Pelargonium graveolens* L.Hér., *Lavandula angustifolia* Mill., *Mentha piperita* L., *Melissa officinalis* L., *Salvia officinalis* L., *Rosmarinus officinalis* L., *Thymus vulgaris* L. The strongest antimicrobial potential was manifested by essential oils derived from *Pelargonium graveolens* L’Hér. (45±2 mm), *Mellisa officinalis* L. (48 and 50 mm, respectively) and *Salvia officinalis* L. (36 and 45 mm, respectively). Consistent with these values were found to be the MICs and MBCs values determined by a broth microdilution method (Carson *et al.*, 1995). The bacteriostatic (MICs) and bactericidal (MBCs) values obtained performing the microdilution protocol were well connected. Obtained results pointed out that the bacteriostatic and bactericidal effects are exhibited at similar concentrations of the active compounds.

The inhibitory effect was more intense against Gram-positive bacteria than against Gram-negative bacteria. Among the bacteria tested, *Pseudomonas* *spp.* was the most resistant towards both ethanolic extracts and essential oils (inhibition zone was observed only for essential oils and ranged between 10-28 mm, MIC and MBC = 2-4 % v/v), while *Staphylococcus* *spp.* strains were the most sensitive (d = 24-50 mm, MIC and MBC = 0,125-2 % v/v). The strongest antimicrobial activity was manifested by *Mellisa officinalis* essential oils (MIC and MBC = 0,125-2 % v/v). The level of bacterial growth inhibition induced by plant materials, as determined by the broth microdilution assay, proved to be dependent mostly on herbal source and type of extract, and then on bacterial strain. Regardless of the bacterial genera, some extracts, case of *Lavandula officinalis*, *Mentha piperita* and *Thymus vulgaris*, proved to be active only as essential oils, while *Salvia officinalis* and *Rosmarinus officinalis* exhibited antibacterial effects, both as essential oils and alcoholic extracts. Strain variation can be linked to intrinsic strain susceptibility towards the herbal preparations screened.

Also bactericidal index values suggested for most herbal extracts a bactericidal ability (MBC/MIC<4).

For some herbal products our results have not revealed the antimicrobial properties suggested by other authors in similar studies. This discrepancy in exerting the *in vitro* antibacterial potential may be caused by a variation that concerns the chemical composition as this one is determined at a greater extent by the distillation and extraction technique or by the different geographical chemotype.

*In vitro* interactions between antimicrobial agents and plant extracts were synergistic (essential oils) or additive (alcoholic extracts), with augmentation of the inhibition zone measured for the three tested antibiotics against resistant strains of *Staphylococcus intermedius*, except for the ethanolic extract of *Mellisa officinalis* L. and gentamicin combination that expressed an antagonistic effect. These observations may indicate the possibility of concurrent use of antimicrobial drugs and plant extracts in combination in treating infections caused by *S. intermedius* strains.

The aim of the third personal research chapter (chapter VI) entitled “*In vitro Evaluation of Immunomodulating Potential of Herbal Extracts*” was to investigate the effects of certain herbal extracts on canine leukocytes *in vitro* reactivity by performing the leukocyte blast transformation test. The investigation regarding the immunomodulatory effect of herbal extracts was carried out by the leukocyte blast transformation test on whole blood cultures, the micro method as described previously by
Spinu et al. This method allows the measurement of the in vitro reactivity of mononuclear cells to sensitizing (in vivo encountered) antigens. Cell growth was quantified by means of the glucose consumption technique.

This study was preceded by a selection of herbal extracts based on the results of their cytotoxic potential screening on canine T cells isolated using the Accuspin™–Histopaque®-1077 (SigmaAldrich) system. Cell viability after a 24 hours contact with tested herbal extracts (ethanolic extracts and essential oils of Ocimum basilicum L., Thymus vulgaris L., Salvia officinalis L., Lavandula angustifolia Mill., Rosmarinus officinalis L. AE, Mellisa officinalis L., Mentha piperita L., Calendula officinalis, Hippophae rhamnoides, and Pelargonium graveolens L’Hér. essential oil) was measured using Trypan Blue Exclusion Test.

As the cytotoxic potential was observed for certain extracts, mostly essential oils, the immunomodulating abilities were investigated only for the ethanolic extracts, considering sixteen experimental variants for each blood sample, namely (1) control (untreated blood culture), (2) lippopolysaccharides (LPS) (1 µl per well) treated culture, (3) phytohaemagglutinin-M (PHA) (1 µl per well) treated culture, (4) Concanavalin A (ConA) (1 µl per well) treated culture, (5) alcohol (2.5 µl per well) treated culture, (6–16) alcoholic extracts of Ocimum basilicum L., Thymus vulgaris L., Echinacea purpurea, Salvia officinalis L., Lavandula angustifolia Mill., Vaccinium myrtillus L., Calendula officinalis L., Hippophae rhamnoides L., Rosmarinus officinalis L., Mellisa officinalis L. and Mentha piperita L. (2.5 µl per well) treated cultures.

The stimulation index (TI) was calculated as follows: SI % = [(MG–SG)/MG]*100, where SI = blast transformation index, MG = glucose concentration in the initial culture medium and SG = glucose concentration of the sample after incubation. The data were statiscally processed and the different values obtained for the tested variants were analyzed using ANOVA post hoc followed by Dunnett test.

Stimulation indices determined by the above mentioned herbal extracts presented values comparable to those of the controls (untreated blood culture and alcohol treated culture) or those corresponding to standard mitogens (Con A, LPS, PHA); these values were between 48.28% and 63.80%, so that the statistical processing of results did not indicated significat diference (p>0.05) between the following experimental variants: control, LPS, PHA, ConA, 70% Alcohol, and extracts derivated from Ocimum basilicum L., Thymus vulgaris L., Echinacea purpurea, Hippophae rhamnoides L., Rosmarinus officinalis L., Mellisa officinalis L. and Mentha piperita L. Still, in case of four extracts, namely Salvia officinalis L., Lavandula angustifolia Mill., Vaccinium myrtillus L. and Calendula officinalis L. the stimulation indices had lower values compared to the other variants (SI = 22.31% -38.66%).

As the chart 1 indicates, the obtained value for the stimulation indices (SI) were significantly decreased compared to controls only in case of the Calendula officinalis L. treated culture (22.31%, p=0.0010), suggesting that this alcoholic extract acted inhibitory upon the cell cultures. On the other hand, the majority of the other experimental variants proved to be able to induce a stimulating effect on the canine leukocytes growth, with the highest values of SI recorded for Ocimum basilicum L. (63.80%) and Hippophae rhamnoides L. (61.39%). Still, it is obvious that the level of mentioned biological effect can not be considered very high or intense, as the differences between them, established by ANOVA post hoc followed by Dunnett test, cannot be included in the significant
As for the results concerning the other herbal extracts, they are relatively in contradiction with current literature data. These herbal products, especially *Calendula officinalis* L. and *Echinacea purpurea* are mentioned in literature for their intense immune modulating potential. This discrepancy in manifesting the biological potential may be related to the different chemical composition as there are a multitude of herbal extracts vegetal sources.

This *in vitro* evaluation of certain herbal alcoholic extracts, carried out using the blast transformation test, indicated an immunomodulating potential in terms of inhibitory activity determined by *Calendula officinalis* L. ethanolic extract on the canine leukocytes growth capacity. Further *in vitro* and also *in vivo* assays are required in order to explore this potential and consider this herbal product as immune modulating agent in dogs.

Chapter VII, entitled “*In vitro screening of toxicity of herbal extracts- study on cytotoxic potential of herbal extracts*”, was intended to point out the *in vitro* toxic potential of certain selected herbal extracts tested on cell cultures; this study included the herbal extracts selected based on the results of the antimicrobial and immunomodulating properties screening. Initially, the cytotoxicity evaluation was intended using primary culture of canine otic keratinocytes obtainment by explant method (biopsy material sampled from healthy dogs and dogs showing that external otitis - canine cutaneous keratinocytes and / or keratinocytes canine ear). Unfortunately, these keratinocytes failed to maintain the proper characteristics and after several passages we obtained mixt culture keratinocytes and fibroblasts. Without a homogeneous canine keratinocytes line, we used a stable cell line HFL-1 (*human lung fibroblast*) provided by the Laboratory of the Oncology Institute "Ion Chiricuță" Cluj-Napoca. The cytotoxic potential was estimated in terms of quality and quantity, using two experimental models that allow prediction of standard cellular response to potentially toxic product with greater confidence than using only one method: evaluation of biocompatibility on cell culture of human fibroblasts by

**Chart 1.** Immunomodulatory ability of tested herbal extracts
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, respectively evaluation of morphology and cell attachment level.

The MTT results, expressed as optic density (absorbance), were calculated as viability percentages by dividing the absorbance reading of cells under different herbal extracts concentrations by the absorbance reading of cells under normal growth (assumed 100% viability) The difference between the control and herbal extracts dilutions treated cells were analysed using ANOVA post hoc, followed by Dunnett test (against the control) or by Bonferroni test (against ten different dilutions).

The obtained data pointed out cytotoxic potential mostly in case of essential oils, consistent with the findings of other similar studies, and less intense for alcoholic extracts, particularly for lower concentrations.

MTT test results suggested in some cases the concentration dependence of tested herbal extracts-induced effect, some dilutions stimulated cell viability, while higher concentrations had moderate or highly expressed cytotoxicity, depending on herbal species and also on the investigated extract type (essential oil, hydroalcoholic extract or alcoholic extract) (chart 2). In case of alcoholic extracts derived from Ocimum basilicum L. and Lavandula angustifolia Mill., an in vitro stimulatory effect on HFL viability was observed.

Pooled analysis of tested herbal extracts indicated no adverse effects on the adhesion molecules, as the cells attachment degrees were unchanged. Cell viability was not affected except for cells treated with lavender essential oil dilution 1/1280, where 1 h after application, viability percentage was lower compared with untreated control cultures.

Considering the results of antimicrobial effects screening tests, it can be stated that certain essential oils can have major cytotoxic valences in the presence of remarkable antibacterial potential. However, we selected certain herbal products and recorded their pharmacologically active concentrations with antimicrobial activity and moderate or

![Chart 2. Effect of Lavandula angustifolia Mill. herbal products on HFL cells viability](chart2.png)
absent cytotoxic potential. In light of these aspects, in vitro evaluation of antimicrobial effectiveness and cell toxicity has provided various details for further in vivo toxicity studies, that represent the Chapter VIII main objective, entitled “In vivo evaluation of toxicity of certain herbal extracts”. In view of desired therapeutic purpose, in vivo toxicology studies were conducted in order to highlight the possible dermal toxicity manifested as local irritation effect and allergic sensitization potential, carrying out the following tests: acute dermal irritation assay and delayed allergic reaction test. The study of hypersensitivity phenomena is justified considering canine external otitis complex etiology where allergic mechanisms are listed, as stated in the research literature. Experimental models were performed between February 2011 - April 2011 at the Infectious Diseases Department including as biological material adult white female of the mice CD1 line. As a particularity of the study (no mention in the literature, except that such tests involve the use of products in ways that are relevant to their future use), for both tests the effect induced by ear topical application of a single dose and of repeated doses of tested plant products, but only for two concentrations (1% and 0.5%) was investigated.

The results obtained for the primary irritation index (PII) based on Draize protocol highlighted the toxic potential in case of Melissa officinalis L. essential oil for all tested concentrations and essential oil of Lavandula angustifolia Mill. 5% concentration, as we observed local irritation effects after application of a single dose, so for the second experimental study - delayed allergic reaction test, we considered appropriate to assess the skin sensitization potential only for alcoholic extract and essential oil of Lavandula angustifolia Mill., essential oil of Thymus vulgaris L., respectively alcoholic extract and essential oil of Ocimum basilicum L. and Salvia officinalis L. alcoholic extract and only for two concentrations (1% and 0.5%). These herbal products and their concentrations did not elicit a hypersensitivity response, except for Lavandula angustifolia Mill. essential oil. Regarding otic topical application, considered relevant for the future destination of herbal preparations similar aspects were concluded with one exception: Lavandula angustifolia Mill. essential oil. In two of the three mice allocated for this herbal product appearance of local ear edema was noted, accompanied by itching and agitation. Animals refused to eat for several hours, showed a visibly uncomfortable state, and after 24 hours in one animal, pinna displayed erythematous lesions that subsequently became infected.

In vivo toxicology studies indicated a dermal irritation potential acute in case of alcoholic extract of Melissa officinalis L. 5% concentration and of essential oil of the same herbal species, but with greater intensity and for all 3 concentrations tested. For other vegetable products included in the study it can be stated based on the values established for primary irritation index that they do not manifest acute dermal irritation effect for any of the tested concentrations. Selected for screening potential hypersensitivity induced no changes during the monitoring of animals, so that the can be considered safe in this respect, and some plant products can be considered safe for both dermal administration and for the topical otic.

Comparative analysis of pharmacological properties expressed in vitro and in vivo by alcoholic extracts and essential oils included in the present study resulted in providing information for selecting those herbal preparations with complex therapeutic potential to undergo further development of safe and effective drugs in therapy of canine external otitis.
Based on experimental data presented in previous chapters we appreciated that the use of herbal extracts may represent a promising option for the treatment of external otitis in dogs. This therapeutic hypothesis is supported by the highlighting of complex biological properties (antibacterial and/or immunomodulators), and also by the relatively low or no toxicity and few side effects (erythema). However, the response the medication is the result of complex interactions between highly active substance and target tissue, there are many factors that can exert significant influence, so that is not permitted the generalization of biological phenomena. In this context, Chapter IX is aimed as a Preliminary study on in vivo bioavailability of a polyherbal preparation proposed for topic therapy in canine otitis externa.

This study intended to perform a preliminary research using a small number of individuals, whose purpose was to evaluate clinical effects of application at the external ear canal of a solution based on herbal extracts, considering appropriate to include the following components: oily solution 0.125% (v/v) of thyme (Thymus vulgaris L.), hydroalcoholic solution 1% (v/v) of basil (Ocimum basilicum L.) and hydroalcoholic solution 1% (v/v) of sage (Salvia officinalis L.). Proposed therapeutic attitude assessment protocol survey was applied to 10 dogs presenting external otitis, which represented veterinarian casuistry in Cluj-Napoca. Canine subjects were considered eligible for inclusion in the study regardless of age, race or sex, under conditions which meet the following criteria: symptoms of otitis externa, intact tympanic membrane without visible lesions by otoscopic examination, positive cytology for the presence of bacteria, positive cultural examination (isolation of bacteria), cases where no local or general treatment. The animals were monitored throughout the treatment and clinical evaluation of the product efficacy based on the comparison of clinical scores determined by examining the affected area (external auditory canal) before topical administration of the product and after 48 hours and 7 days of treatment.

To establish the clinical scores for each animal we followed the evaluation protocol suggested by Hawkins et al. (2010), that takes into account the intensity and severity of six clinical indicators: the external ear canal occlusion, redness, appearance of otic exudate, the amount of ear discharge, lesions/erosions or ulcers, bad smell. The statistical significance of differences between the mean clinical scores determined for day 0 and day 2, and day 0 and day 7 respectively was determined using Student's t-test sample pairs (paired t test) for a confidence interval of 95% default.

The severity of symptoms observed was not uniform as clinical scores calculated according to the protocol proposed by Hawkins et al. (2010) were between 2 and 17 (almost maximum), with a mean clinical score of 9.7. Value of 5.518 calculated for standard deviation and variable pruritus intensity led to individual approach of each case, that each registered case was a different situation, and hence efficacy was assessed individually. Also, during polyherbal preparation treatment, changes in clinical parameters showed variations depending on the initial severity of otitis, recorded as a variability of the individual response, so it is appropriate to perform a post-therapy description of each case. At the end of testing, general assessments about the effectiveness of therapy depending on the evolution of symptoms (changes in clinical scores), but based on the results provided by the laboratory (cytological and bacteriological examination) and the safety of the preparation (analysis of adverse events: frequency occurrence, intensity, character) were made.
The five recorded clinical parameters, represented by: the external ear canal occlusion, redness, appearance otic exudate, the amount of ear discharge, lesions/erosions or ulcers, smell, allowed us to evaluate individually and globally the response to the proposed therapy. As chart 3 indicates, the first clinical evaluation after 48 hours of starting treatment all individuals showed a positive development, reducing clinical scores averaged 32.27% and on individual cases, case no. 1 to 66.66%, case No. 10 - 50% cases no. 2 and 6-40%, case no. 4-25%, while cases 3 and 7 to 20%. Case No. 8 did not express significant clinical improvement, but should be considered and complex pathology that involves erythema multiforme. The evolution of the cases by comparing the day 0 does not indicate a reduction statistically significant (p = 0.3781). At 7 days, significantly reduced lesion scores were recorded (mean score 4.22 ± 4.62 and p <0.05).

In all cases of the study an early antipruritic effect was found; this is important because most cases regardless of etiology are associated with itching, scratching, the animal is trying to remove the unpleasant feeling by rubbing on objects, situations that could lead to injuries of the ear external canal, lesions may become infected due to opportunistic pathogens from normal flora and altered immune response in terms of a (local or general) that will lead to increase inflammation. Cancellation or reducing the intensity of itching will avoid these complications. During treatment decreasing of symptoms intensity was observed, but not in all dogs simultaneously, which emphasizes the therapeutic properties of the polyherbal product, and specific reactivity of each individual. Reduce swelling suggests local inflammatory properties that may be the result of synergistic action of bioactive components of the preparation. Local therapy reduced the microflora isolated from the external ear canal, which indicates broad-spectrum for the antibacterial action (Gram positive and Gram negative) and antifungal effect. The absence of allergic phenomena indicate the safety of this topical preparation in accordance with data provided by the in vivo toxic potential evaluation, applied local therapy proved to be safe, well tolerated and with clinical and microbiological efficacy.
Thesis General Conclusions

By its complex therapeutic properties, manifested both in vitro in vivo, the tested preparation exhibited notable therapeutic potential in patients that received the treatment.

The therapeutic properties expressed during this experimental research recommend the association between the 3 herbal extracts for substantiation of technology in order to obtain products with therapeutic indications in canine external otitis.

The above mentioned aspects regarding the therapeutic potential could have applicability in the control and prevention of bacterial induced diseases in other animal species, namely development of technologies for new products that can be used in several species or in certain species.

The results and the observations on the bioavailability of herbal preparations in case of a local inflammatory process in terms of displaying antimicrobial and immunomodulating properties, in the absence of acute irritant effect or delayed allergic reaction, may represent a research direction for the control of systemic process with polymicrobial aetiology.