Praca oryginalna ***Original paper***

In vitro influence of plant extracts extending beyond taxonomy: A comparison of chicken and rabbit cell-mediated responses

AUREL VASIU[1], MARINA SPÎNU[1,2], EMOKE PALL[1,2], SILVANA POPESCU[1], CARMEN DANA ȘANDRU[1,2], JAROSŁAW KABA[3], GHEORGHIȚĂ DUCA[2]

[1]Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Str. Manastur no. 3-5, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology Str. XIII No. 147, Cristian, Sibiu, Romania
[3]Independent Department of Veterinary Epidemiology and Economics, Faculty of Veterinary Medicine, Institute of Veterinary Medicine, Warsaw University of Life Sciences, ul. Nowoursynowska 159, 02-776 Warsaw, Poland

Received 26.01.2024 *Accepted 05.02.2024*

Vasiu A., Spînu M., Pall E., Popescu S., Șandru C. D., Kaba J., Duca Gh.

In vitro influence of plant extracts extending beyond taxonomy: A comparison of chicken and rabbit cell-mediated responses

Summary

Detailed information on the *in vivo* activity of vegetal extracts on the immune system in birds and mammals, mainly the farmed ones, is helpful in improving their resilience and welfare. Therefore there is need to investigate the exact roles of active plant components in animals, mainly their influence on the immune system, which is crucial for antimicrobial resistance. The farming system, serving principally economic aims, overlooks the physiological support of several extremely stress-sensitive, short-lived species such as chickens or rabbits. Recovering from the damage caused by adverse microclimates or nutritional deficiencies in these species can be a prolonged process often standing beyond their economic lifespan. Additionally, the compromised condition makes them susceptible to various diseases, particularly microbial or parasitic infections which can easily take hold in the weakened state. Under these circumstances, it seems very important to select and study natural enhancers of the immune response. Setting up optimal therapeutic protocols and establishing the optimal age range and administration route boost their effects. The *in vitro* tests, performed mainly on isolated cell- or cell-line cultures, proved the beneficial influence of certain vegetal extracts, which diminished oxidative stress and tumor growth, as well as overcame the negative effects of active oxygen species, increasing the synthesis of cytokines. The study aimed a) to establish a potential dose-effect relationship for selected plant extracts, b) considering that belonging to either of groups *Aves* or *Mammalia* changes the response to these extracts. Blood samples taken from hens and rabbits were subjected to *in vitro* blast transformation tests (RPMI 1640 culture medium, 48 hours of incubation in a 5% CO_2 atmosphere) to estimate the effects of alcoholic extracts of *Calendula officinalis*, *Vaccinum myrtilus*, *Echinacea purpurea*, and *Hippophae rhamnoides* at 1.5% and 6.5% concentrations by a colorimetric assay (orto-toluidine test). The results in hens indicated maximal stimulation indices, in a reverse dose-dependent manner, for *Vaccinum myrtillus* (81.93 ± 18.74% for 1.5% concentration) and *Calendula officinalis* (74.70 ± 22.15% for 1.5% concentration). In rabbits, all the extracts had stimulating effects, *Calendula* (80.72 ± 6.82% for 6.5% concentration) and *Echinacea* (68.67 ± 17.04% for 6.5% concentration) being more effective than *Vaccinum* (65.06 ± 35.78% at 1.5% concentration). Obviously, the *in vitro* effects of the extracts depended on the phylogeny of the animal and the plant of origin. Thus, the birds reacted to certain extracts in an inversely dose-dependent manner, while in rabbits, the effects were directly dose-dependent. The results highlight the importance of preliminary tests in selecting an appropriate vegetal extract for immune stimulation/modulation of a given species of animals.

Keywords: *in vitro*, rabbits, chickens, immunity, blast transformation, alcoholic plant extracts

The usefulness of precise information about the ways in which plant extracts act on the immune system of animals, especially those raised in an intensive production system (2, 30), is indisputable. The technological advancement primarily aimed at economic progress, often overlook the physiological needs of certain species extremely prone to stress, such as chickens (36) or rabbits (33, 34, 35). Remedying the effects of disrup-

tions such as changes in microclimate (22, 28) or feeding/watering (15) can be prolonged, sometimes, depending on the species, exceeding the duration of their economic life. This neglect creates an environment conducive to various ailments particularly microbial or parasitic which can easily thrive. There is substantial evidence concerning the effects and relevance of oxidative stress, common to most infectious and parasitic or non-transmissible diseases, in swine (respiratory and digestive infections), bovines (mastitis) and equines (endotoxiemia, laminitis, obstructive airway disease, intense physical exercise) (24). The response of the individual/group depends on the level of the stressor (23).

Medicinal plants have been used for millennia for therapeutic purposes in humans and animals, especially in remote rural communities (5, 8, 26), because of their local availability and low cost, as stated in the 2019 WHO report (36). The traditional use of plants as medicines has continued for generations, but only recently their positive effects made them the subject of rigorous scientific studies (10). The selection and characterization of some natural immune enhancers could prove highly beneficial in this context. The high-performance techniques developed in recent years (PCR, genomic sequencing, HPLC, monoclonal antibodies, etc.) offer a profound insight into the mode of transport of plant compounds in receptor organisms (7, 20, 27, 31). The ways of interaction between these compounds and the patients seem to be extremely varied, depending on the metabolic balance, the degree of activation of the immune system, and the physiological state of the whole body. These are some of the reasons why it is sometimes difficult to predict the result of interaction between the compounds of plant extracts and the patient undergoing therapy (29). The effects of therapy can be maximized by developing optimal therapeutic protocols for animals and by determining the optimal age and the most suitable route of administration.

A special and extremely important category of compounds used in classical preventive medicine are adjuvants, including compounds with immune-modulating activity, not limited to those used alongside microbial vaccines. They are capable of restoring the optimal immune response in hypo- or hyper-reactive individuals. Such situations are relatively common in veterinary medicine, where many microbial diseases impair the functioning of the immune system in one way or the other.

In vitro tests, especially those carried out on cancer cell cultures or cell lines treated with isolated components of plants, have demonstrated the beneficial effects of certain extracts in reducing oxidative stress, tumor growth, and damage due to active oxygen species, increasing cytokine synthesis (9), or even suppressing viruses (18).

The literature data (14) on the testing of the adjuvant quality of plant extracts or the comparative evaluation of their effects in different species are extremely limited. Therefore, studies on the machisms of action and factors that influence the adjuvant capacity of plant extracts are valuable.

The present study aimed a) to establish a potential dose-effect relationship for selected alcoholic plant extracts and b) to clarify if belonging to either of groups *Aves* or *Mammalia* changes the response to these extracts.

Material and methods

Biological material. The experiment involved two equal groups of animals: adult chickens (n = 25) and rabbits (n = 25). The animals were raised on a small farm in a semi-intensive system, fed and watered according to the raising technology, with access to the courtyard. Blood collected on heparine (50 IU/ml in final dilution) from these animals in aliquots of 2 ml/animal served as biological material for the *in vitro* trial.

Plant extracts. The alcoholic plant extracts from marigold (*Calendula officinalis*), blueberry (*Vaccinum myrtilus*), purple coneflower (*Echinacea purpurea*), and sea buckthorn (*Hippophae rhamnoides*) were commercial ones. According to the manufacturer, all extracts were obtained based on the German Pharmacopoeia. Marigold, blueberry, and sea buckthorn were chosen due to their availability in the study area, whereas purple coneflower was used because it is a well-known immune stimulant.

Methods. The aliquots of blood were mixed at a ratio of 1 : 4 with RPMI 1640 culture medium supplemented with 5% fetal calf serum (SFV) and antibiotics (1000 IU penicillin and 1000 µg streptomycin/ml) at a pH of 7.2-7.4.

Subsequently, the blood + culture medium mixture was distributed into 96-well plates in amounts of 200 µl/well. To monitor cell growth, phytohemagglutinin (PHA) M (Bacto Difco, USA) was used as a classic mitogen in a quantity of 1 µl/well. The other variants were each treated with 70° alcohol (solvent extract) (Alc) and alcoholic extracts of *C. officinalis* (Cal), *E. purpurea* (Ech), *Vaccinum myrtilus* (Blueberry), or *Hippophae rhamnoides* (Cat). To compare the dose dependence of cell reactivity, doses of 1.5 µl and 6.5 µl were selected, based on previous experiments (unpublished data). All experimental variants were performed in duplicate, including an untreated control. The plates were then incubated for 48 hours in a 5% CO_2 atmosphere. At the end of the incubation period, cell growth was assessed by measuring glucose residue by the ortho-toluidine spectrophotometric method.

For that purpose, a 12.5 µl sample of culture supernatant medium was added to 0.5 ml of o-toluidine reagent in a silicone glass tube. The mixture was kept at 100°C for 8 minutes in a water bath. Then it was suddenly cooled, and 200 µl was transferred to 96-well plates for spectrophotometry at 610 nm and d = 0.5 cm (Sumal PE 2, Karl Zeiss, Jena).

The transformation index (TI) was calculated as follows: TI% = [(MG – SG)/MG] × 100, where TI = blast transformation index, MG = glucose concentration in the initial culture medium and SG = glucose concentration in the sample after incubation.

For each series of samples, a standard glucose sample (100 mg/dl) and a sample from the initial culture medium

were processed in parallel by the same method. The consumption of glucose corresponding to the stimulation index was calculated, expressed as a percentage, and compared to the initial culture medium glucose content and the glucose standard.

Statistical analysis of the data was performed by calculating the arithmetic mean and standard error with the Microsoft Excel program.

Results and discussion

Recent literature data emphasize the beneficial effects of plant-derived compounds or substances when used in conjunction with traditional treatments particularly in situations requiring immune system intervention (6). Within a scientific framework, such information encourages further exploration of the immunomodulatory components of plants, which may offer greater therapeutic potential and fewer side effects compared to those of synthetic products (12). Initially focused on investigating the anticancer and antimicrobial effects, based on the preliminary results obtained, research was later expanded to characterize the influence of whole or purified plant extracts on different branches of the immune system. However, the interaction between plant extract and immune effectors/mechanisms is far from being fully clarified (3, 13).

The results obtained, expressed as transformation indices and cell growth percentages versus control for each species, are presented in figures 1 to 4.

In chickens, the results of blast transformation showed a lower arithmetic mean value in the case of phytohemagglutinin compared to the control variant. This stands for a lower response to the classic mitogen, and therefore the transformation indices recorded for the alcoholic plant extracts look encouraging, since alcohol also has an inhibiting effect. Over time, purple coneflower has been used in both humans and animals to improve health and welfare because of its immune stimulating effects (25). Although the oral administration of hydro-alcoholic extract from purple coneflower induced a dose-dependent improvement in the immune response to disease (4) in this experiment, its extract, surprisingly, inhibited cell growth when compared to the untreated control. This kind of response might indicate differences in reaction of particular species to the active principles of the plant.

Overall, only three of the plants, i.e. marigold, sea buckthorn, and blueberry, stimulated the immune system. For those, the 1.5% concentration seemed to be more active, suggesting a potential "homeopathic" effect, rather than a straightforward dose-response relationship (Fig. 2). Contrary to the literature data (16) according to which marigold administration had no effect on humoral immunity in broilers, the results of the present experiment prompts further research to better understand the dose-response effect in this species. Berries, on the other hand, were claimed to improve broilers' immunity mainly by their antioxidant effect and by increasing T-cell activation (17). Our *in vitro* results supported this research, the lower dose of the blueberry extract being the most efficient in enhancing the cell growth by 21.4% when compared to the untreated control, a percentage which was the highest recorded ($P < 0.001$).

The mean transformation indices in rabbits were lower than those observed in chickens (Fig. 3). Nevertheless, the response to the phytohaemagglutinin was stronger than it was in the control variant, unlike in

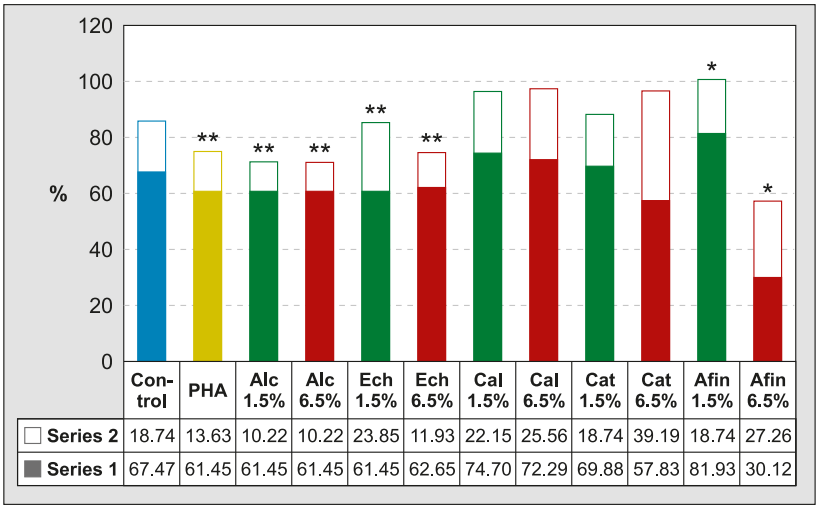

Fig. 1. Transformation indices in chickens
Explanations: series 1 = arithmetic mean; series 2 – st. error (*$p < 0.001$, **$p < 0.05$); the blueberry extract at a concentration of 1.5% had the strongest stimulating effect of all plants

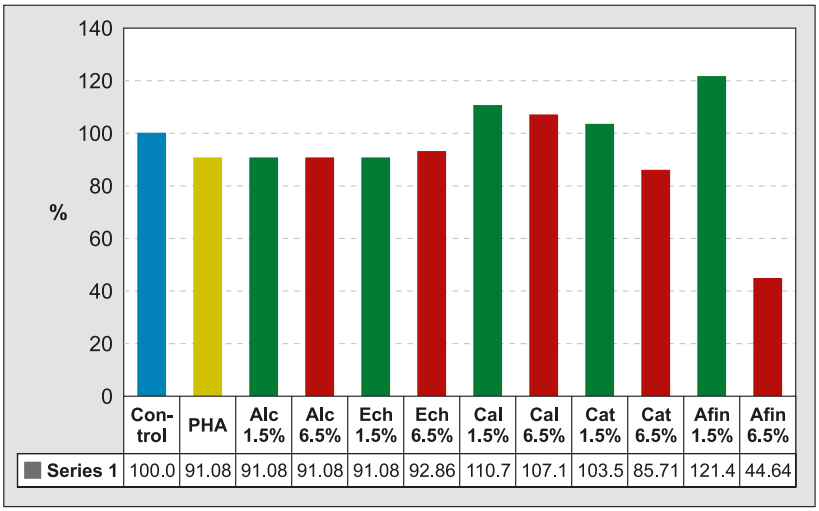

Fig. 2. Percentage growth for chicken leukocyte cultures treated with the in vitro plant extract compared with the untreated control
Explanations: all percentages above 100 indicate stimulation; the 1.5% concentration seems to be more effective in chickens for several plants (marigold, sea buckthorn and blueberry)

chickens, which underscored the even better response to the alcoholic plant extracts in this species.

It is noteworthy that, in rabbits, all extracts exhibited stimulating effects, with the maximum values observed for the higher doses of marigold and purple coneflower extracts, surpassing those of blueberry (Fig. 2). Although numerous Internet vendors claim marigold to be a valuable supplement for improving health and productivity of animals, no scientific studies supporting these claims have been found. Therefore, the massive (81%) increase in the *in vitro* cell growth observed for the alcoholic marigold extract encourages further research on the *in vivo* effects of the plant.

In the rabbits, unlike in the chickens, the higher concentration of each plant extract induced greater growth of the culture, with the exception of blueberry, whose 6.5% alcoholic extract reversed growth ($p < 0.05$ – $p < 0.001$) (Fig. 3). However the growth percentage was much higher overall compared to chickens, ranging from 2.6% (*Echinacea* 1.5%) to 81% (marigold 6.5%, $p < 0.001$) (Fig. 4). Although *Echinacea pallida* has been claimed to have no influence on the immune system of does (11), the present experiment clearly shows that rabbits respond to *in vitro* stimulation with alcoholic extracts of plants from the genus *Echinacea*. However, this response is directly dose-dependent, and its range is still lower than for the other plant extracts.

The response of the cells of the immune system to antigenic stimuli can be assessed by the blast transformation test (21), which makes it possible to calculate the blast transformation index, a parameter that indicates the stimulation or inhibition of cell growth. The results of the *in vitro* supplementation of the cultures with the extracts, whose adjuvant value was studied, confirmed the initial hypothesis about plant and animal species-dependent and dose-dependent responses.

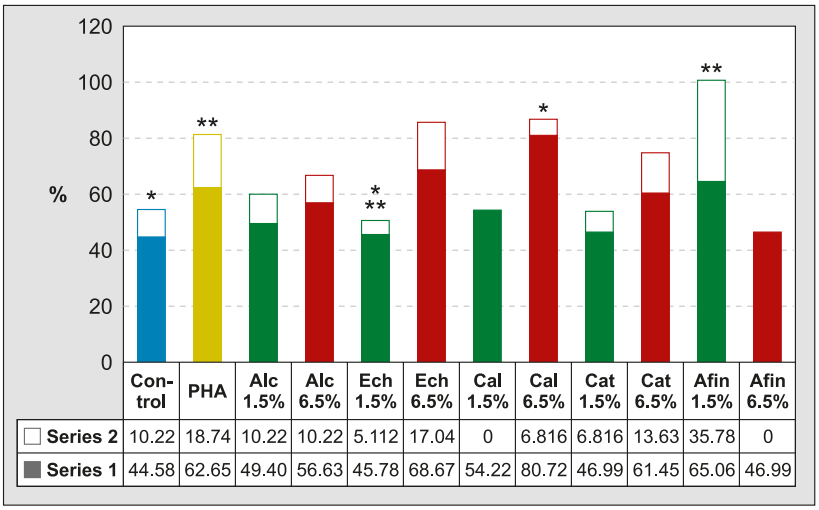

Fig. 3. Transformation indices in rabbits
Explanations: series 1 = arithmetic mean; series 2 – st. error (*$p < 0.001$, **$p < 0.05$); the marigold extract at a concentration of 6.5% had the strongest stimulating effect of all plants

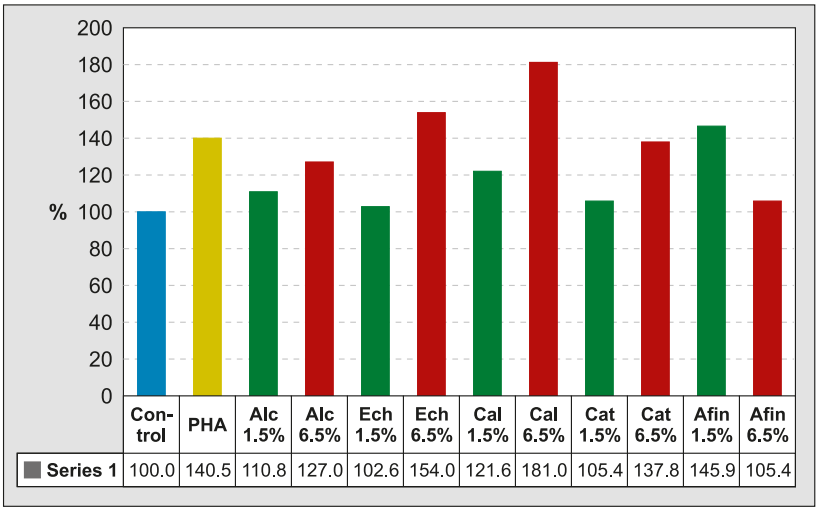

Fig. 4. Percentage growth for rabbit leukocyte cultures treated with the *in vitro* plant extract compared with the untreated control
Explanations: all percentages above 100 indicate stimulation; both concentrations, especially 6.5%, seem to be effective, except for the blueberry extract

The immune system of chicks has provided an ideal working model for investigations into mammalian immunity. In particular, the control of lymphocyte migration and survival is the key to immune development. CD4+ alpha beta T lymphocytes have a central role in the avian immune system, their activation being a precursor to the responses of other cells, including gamma-delta T lymphocytes (1, 32). In the case of the whole blood culture method, the cellular cooperation necessary to induce the immune response is maintained, but it becomes more difficult to attribute one reaction or the other to a certain cell category. Moreover, the reactivity to different compounds can only be estimated globally, without establishing precisely which cell category is most influenced.

The transformation activity of lymphocytes is influenced by environmental or individual factors. The *in vitro* blast transformation test allows for the evaluation of efficiency and comparison of the effectiveness of certain compounds, highlighting their influence on these factors, whether they are environmental (exogenous additions such as extracts) or individual, such as the animal's position in the phylogenetic tree.

It can thus be concluded that belonging to a specific taxonomic class, *Aves* or *Mammalia*, influenced the reactivity to the extracts. In birds, the lower concentration of the blueberry extract was the most active, both concentrations of marigold extract were less effective, and the sea buckthorn extract had little effect, whereas all other extracts were immune suppressive. In rabbits, on the other hand, all extracts and concentrations were stimulating compared to the control, with maximum effect for the marigold extract.

The results support the need to pursue research to better understand the effectiveness of plant extracts as immune stimulators/modulators before implementing any therapeutic scheme.

Conclusion:

• Taxonomic affiliation (classes *Aves* and *Mammalia*) obviously influences the reactivity of circulating white blood cells *in vitro* to active principles extracted from different plants, which emphasizes the need for testing before selecting active compounds as adjuvants or immune modulators.

• The comparative evaluation of the effects of selected plant extracts on circulating white blood cells in the leukocyte blast transformation test revealed that the most effective alcoholic extracts were those of *Calendula officinalis* in rabbits and *Vaccinum myrtillus* in birds, and that their effects were dose dependent.

References

1. *Arstila T. P.*: T cell subsets and the activation of gamma delta T cells. Current topics in microbiology and immunology1996, 212, 71-77.
2. *Artuso-Ponte V., Pastor A., Andratsch M.*: The effects of plant extracts on the immune system of livestock: the isoquinoline alkaloids model, [in:] Florou-Paneri P., Christaki E., Giannenas I. (eds.): Feed Additives. Academic Press. Cambridge, MA (USA) 2020, 295-310, doi: 10.1016/B978-0-12814700-9.00017-0.
3. *Attele A. S., Wu J. A., Yuan C. S.*: Ginseng pharmacology: multiple constituents and multiple actions. Biochem. Pharmacol. 1999, 58 (11), 1685-1693, doi: 10.1016/s0006-2952(99)00212-9.
4. *Ayrle H., Mevissen M., Bruckmaier R. M., Wellnitz O., Kaske M., Bieber A., Vögtlin A., Fricker R., Walkenhorst M.*: Effects of an oral hydro-ethanolic purple coneflower extract on performance, clinical health and immune parameters in calves. Research in veterinary science 2021, 138, 148-160, doi: 10.1016/j.rvsc.2021.05.022.
5. *Bartha S. G., Quave C. L., Balogh L., Papp N.*: Ethnoveterinary practices of Covasna County, Transylvania, Romania. Journal of ethnobiology and ethnomedicine 2015, 11, 35, doi: 10.1186/s13002-015-0020-8.
6. *Bauer R.*: Neue Erkenntnisse zur Wirkung und Wirksamkeit von Echinacea purpurea-Presssaftzubereitungen [New knowledge regarding the effect and effectiveness of Echinacea purpurea extracts]. Wien Med. Wochenschr. 2002, 152 (15-16), 407-411, doi: 10.1046/j.1563-258x.2002.02063.x.
7. *Berczi I., Bertók L., Chow D. A.*: Natural immunity and neuroimmune host defense. Ann. N Y Acad. Sci. 2000, 917, 248-257, doi: 10.1111/j.1749-6632.2000.tb05390.x.
8. *Bischoff T., Vogl C. R., Ivemeyer S., Klarer F., Meier B., Hamburger M., Walkenhorst M.*: Plant and natural product based homemade remedies manufactured and used by farmers of six central Swiss cantons to treat livestock. Livest Sci. 2016, 189, 110-125, doi: 10.1016/j.livsci.2016.05.003.
9. *Briskin D. P.*: Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiol. 2000, 124 (2), 507-514, doi: 10.1104/pp.124.2.507.
10. *Chakale M. V., Mwanza M., Aremu A. O.*: Ethnoveterinary knowledge and biological evaluation of plants used for mitigating cattle diseases: A critical insight into the trends and patterns in South Africa. Front Vet. Sci. 2021, 8, 710884, doi: 10.3389/fvets.2021.710884.
11. *Dabbou S., Rotolo L., Kovitvadhi A., Bergagna S., Dezzutto D., Barbero R., Rubiolo P., Schiavone A., Marco M., Helal A., Noureddine I., Zoccarato Gasco L.*: Rabbit dietary supplementation with pale purple coneflower. 1. Effects on the reproductive performance and immune parameters of does. Animal 2016, 10 (7), 1101-1109, 1-9, doi: 10.1017/S1751731115002979.
12. *Dattner A. M.*: From medical herbalism to phytotherapy in dermatology: back to the future. Dermatol. Ther. 2003, 16 (2), 106-113, doi: 10.1046/j.1529-8019.2003.01618.x.
13. *Dewick P. M.*: Medicinal Natural Products. John Wiley & Sons, West Sussex, UK 1997.
14. *Feng Q., Hu J. F., Chen H., Lou J. L., Gan Y., Hu Y., Shi Y. E.*: Studies on effect enhancement of the Schistosoma japonicum DNA vaccine pVIV02-IL12-Sj23 by vegetal polysaccharides. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 2005, 30, 23 (6), 401-403. Chinese. PMID: 16566205.
15. *Feng Y., Fan H., Liang X., Wang X., Gao G., Gun S.*: Environmental enrichment changes rabbits' behavior, serum hormone level and further affects cecal microbiota. Peer J. 2022, 10:e13068, doi: 10.7717/peerj.13068.
16. *Foroutankhah M., Toghyani M., Landy N.*: Evaluation of Calendula officinalis L. (marigold) flower as a natural growth promoter in comparison with an antibiotic growth promoter on growth performance, carcass traits and humoral immune responses of broilers. Anim. Nutr. 2019, 5 (3), 314-318, doi: 10.1016/j.aninu.2019.04.002.
17. *Hasted T. L., Sharif S., Boerlin P., Diarra M. S.*: Immunostimulatory potential of fruits and their extracts in poultry. Front. Immunol. 2021, 17, 12:641696, doi: 10.3389/fimmu.2021.641696.
18. *Kalvatchev Z., Walder R., Garzaro D.*: Anti-HIV activity of extracts from Calendula officinalis flowers. Biomed Pharmacother. 1997, 51 (4), 176-180, doi: 10.1016/s0753-3322(97)85587-4.
19. *Kaufman I.*: Structure and function of the major histocompatibility complex in chickens, [in:] Davison T. F., Morris T. R., Payne L. N. (eds.): Poultry Immunology. Abingdon United Kingdom Carfax. 1996, 67-82.
20. *Khokhlova I. S., Spinu M., Krasnov B. R., Degen A. A.*: Immune responses to fleas in two rodent species differing in natural prevalence of infestation and diversity of flea assemblages. Parasitol. Res. 2004, 94 (4), 304-311, doi: 10.1007/s00436-004-1215-4.
21. *Kikusato M., Toyomizu M.*: Mechanisms underlying the effects of heat stress on intestinal integrity, inflammation, and microbiota in chickens. J. Poult. Sci. 2023, 9, 60 (2), 2023021, doi: 10.2141/jpsa.2023021.
22. *Koolhaas J. M., Bartolomucci A., Buwalda B., de Boer S. F., Flügge G., Korte S. M., Meerlo P., Murison R., Olivier B., Palanza P., Richter-Levin G., Sgoifo A., Steimer T., Stiedl O., van Dijk G., Wöhr M., Fuchs E.*: Stress revisited: a critical evaluation of the stress concept. Neurosci. Biobehav. Rev. 2011, 35 (5), 1291-301, doi: 10.1016/j.neubiorev.2011.02.003.
23. *Lykkesfeldt J., Svendsen O.*: Oxidants and antioxidants in disease: oxidative stress in farm animals. Vet. J. 2007, 173 (3), 502-511, doi: 10.1016/j.tvjl.2006.06.005.
24. *McNeil B. K., Renaud D. L., Steele M. A., Keunen A. J., DeVries T. J.*: Effects of Echinacea purpurea supplementation on markers of immunity, health, intake, and growth of dairy calves. J. Dairy Sci. 2023, 106 (7), 4949-4965, doi: 10.3168/jds.2022-22862.
25. *Mussarat S., Ali R., Ali S., Mothana R. A., Ullah R., Adnan M.*: Medicinal animals and plants as alternative and complementary medicine in southern regions of Khyber Pakhtunkhwa, Pakistan. Front Pharmacol. 2021, 12, 649046, doi: 10.3389/fphar.2021.649046.
26. *Nahrstedt A., Butterweck V.*: Biologically active and other chemical constituents of the herb of Hypericum perforatum L. Pharmacopsychiatry. 1997, 30, 2, 129-34, doi: 10.1055/s-2007-979533.
27. *Oladimeji A. M., Johnson T. G., Metwally K., Farghly M., Mahrose K. M.*: Environmental heat stress in rabbits: implications and ameliorations. Int. J. Biometeorol. 2022, 66 (1), 1-11, doi: 10.1007/s00484-021-02191-0.
28. *Percival S. S.*: Use of echinacea in medicine. Biochem. Pharmacol. 2000, 15, 60 (2), 155-8, doi: 10.1016/s0006-2952(99)00413-x.
29. *Pisoschi A. M., Iordache F., Stanca L., Cimpeanu C., Furnaris F., Geicu O. I., Bilteanu L., Serban A. I.*: Comprehensive and critical view on the anti-inflammatory and immunomodulatory role of natural phenolic antioxidants. Eur. J. Med. Chem. 2024, 5, 265, 116075, doi: 10.1016/j.ejmech.2023.116075.
30. *Schulz V., Hansel R., Tyler V.*: Rational phytotherapy: A Physician's Guide to Herbal Medicine. Springer-Verlag, Berlin 1998.
31. *Sklan D., Melamed D., Friedman A.*: The effect of varying dietary concentrations of vitamin A on immune response in the turkey. Br. Poult. Sci. 1995, 36 (3), 385-392, doi: 10.1080/00071669508417785.
32. *Spînu M., Dancea Z., Şepşi R., Brudaşcă Gh. F., Oprea M., Spînu O.*: Dinamica unor indicatori ai profilului imunologic la păsări supuse imunostimulării. Simp. Actualităţi în patologia animal, Cluj-Napoca 1992, XVIII, 157.
33. *Spinu M., Degen A. A.*: Effect of cold stress on performance and immune responses of Bedouin and White Leghorn hens. Br. Poult. Sci. 1993, 34 (1), 177-185, doi: 10.1080/00071669308417573.
34. *Spinu M., Degen A. A., Rosenstrauch A.*: Effect of warm drinking water on the performance and immunological responses of broiler breeder hens raised at low air temperatures. Br. Poult. Sci. 1993, 34 (2), 361-366, doi: 10.1080/00071669308417591.
35. *Sumanu V. O., Naidoo V., Oosthuizen M. C., Chamunorwa J. P.*: Adverse effects of heat stress during summer on broiler chickens production and antioxidant mitigating effects. Int. J. Biometeorol. 2022, 66 (12), 2379-2393, doi: 10.1007/s00484-022-02372-5.
36. World Health Organization global report on traditional and complementary medicine, 2019.

Corresponding author: Vasiu Aurel, DVM, PhD, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Str. Manastur no. 3-5, Cluj-Napoca, Romania; e-mail: aurel_vasiu@yahoo.com