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HOST AND PLANT TAXONOMY AS INFLUENTIAL FACTORS IN CONTROLING *IN VITRO* SPECIFIC CELL-MEDIATED RESPONSE IN HERBIVORES

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Abstract

Objective: This study monitored several alcoholic vegetal extracts from the adjuvant and immune cell stimulating perspective in farmed herbivores, differing by their digestive physiology.
Material and Methods: Blood from randomly selected farmed ruminants (Romanian Spotted dairy cows, n=28 and Angora goats, n=19) and Romanian draft horses (n=27) was used to measure the *in vitro* effects on specific cell-mediated reactivity of alcoholic extracts of *Calendula officinalis* L., *Echinacea angustifolia* D.C. and *E. purpurea* (L.) Moench by the *in vitro* whole blood blast transformation test, in 96 well plates, after 60 to 72h of incubation. Cell growth was quantified by an orto-toluidine technique. Student's t- test was used to evaluate the statistical significance of the differences.
Results and Discussion: The *in vitro* growth indices were lowest in goats (*C. officinalis* – 58.52±10.02%, *E.angustifolia* – 50.06±11.67%, *E.purpurea* -50.79±10.98%) and higher in bovine (*C. officinalis* – 69.9±2.65%, *E.angustifolia* – 74.9±10.1%, $p<0.05$), and increased towards *E. angustifolia* versus *C. officinalis*.
In vitro responses to *C. officinalis*, *E. angustifolia and E. purpurea* were the most pronounced in horses and similar for all of these extracts.
Conclusion: All the extracts showed inhibiting effects in bovine and goats, but not in horses, supporting host-based differences. The biological activity of the tested extracts was plant species dependent.

Key Words: Ruminants, horses, plant extracts, blast transformation

1. Introduction

Antibiotic resistance represents in the actual world one of the biggest threats to human and animals health and food security, while it is a naturally occurring phaenomenon but the process is being enhanced by misuse of antibiotics in human and veterinary medicine (WHO, 2023). To prevent further aggravation of the antibiotic resistance, natural solutions are sought for, such as organic acids, feed enzymes, and pro- or pre-biotics to control microbes. Plant extracts represent a historical, inexhaustable, valuable and available tool to control health and welfare in pets and food animals as well (Windisch, 2008). The immune modulating properties demonstrated in case of several extracts derived from medicinal plants are

the subject of extended research as part of a global trend (Amirghofran et al., 2000; Barbour et al., 2004; Jiménez-Medina et al., 2006Amirghofran et al., 2009; Chand et al., 2011; Bhatt et al., 2014).

Essential oils from various plants positively influence the local gut immunity in non-ruminants (Zeng et al., 2015) while others such as noni (Nanak and Mengi, 2010), purple coneflower (Fors, 2015) or holy basil (Mukherjee et al., 2005) extracts increased the lymphocyte activation in people, horses and ruminants respectively. Some researches indicated that in different orders of carnivores the immunological activity of plants depended on species and also differed within the same plant family (*Compositae*) (Spinu et al., 2016). The results of such studies are valuable for the field of veterinary medicine given the negative impact of several factors related to inadequate husbandry and welfare on the immune system functionality in farmed animals (Disler et al., 2014). To our knowledge, there are no comparative studies on the combined influence of plant and animal taxonomic ranking on immune effects of plant extracts.

This study aimed to compare three alcoholic extracts from plants belonging to the same family (*Compositae*), but different genera (*Calendula, Echinacea*) and species (*E. purpurea* and *E. angustifolia*) for their ability to enhance the adaptive cell-mediated immunity in farmed herbivores, differing by their digestive physiology, as it is in ruminants (bovine, goats) and monogastric, non-ruminants (equine).

2. Material and Methods

2.1. Biological material

Randomly selected semi-intensively farmed ruminants (Romanian Spotted dairy cows, n=28 and Angora goats, n=19) and extensively farmed Romanian draft horses (n=27) were used to monitor the potential influence exerted by their differentiated immune morphology and physiology in eliciting responses to active principles from different vegetal extracts. Peripheral blood of the experimental animals obtained from the jugular vein, sampled on heparine (50 IU/ml), was used to investigate the potential of alcoholic vegetal extracts to improve leukocyte reactivity. The blood was transported to the laboratory under isotermal conditions (37°C) and processed in maximum 4 h after sampling.

2.2. Leukocyte blast transformation test

The *in vitro* leukocyte blast transformation test provides evidence on the potential of monocytes and lymophocytes to further react *in vivo* to sensitizing antigens. Commercial alcoholic extracts of *Calendula officinalis, Echinaceea purpureea and Echinaceea angustifolia* (Plantextract, Romania) for human use, produced according to the German Homeophatic Pharmacopeia, were used to *in vitro* treat the whole blood cultures.

One ml of each blood sample was diluted with four times the amount of RPMI 1640 (Sigma-Aldrich, USA) supplemented with 5% FCS (Sigma-Aldrich, USA) and antibiotics penicillin (1000 IU/ml, streptomycin 1000 µg/ml)(Sigma Aldrich, USA), at pH 7.4; when needed the pH was corrected with a sterile 2% sodium bicarbonate solution. Each blood+supplemented culture medium mixture was placed in 96-sterile-well plates (200 µl per well), in duplicate for each extract/species (Khokhlova et al., 2004). Six *in vitro* experimental variants were tested for each individual animal, namely (1) untreated control culture, (2) phytohaemagglutinin-M (PHA)(1µ per well), (3) 70° alcohol and (4–6) alcoholic vegetal extracts of *Calendula officinalis*, *Echinacea purpurea* and *Echinaceea angustifolia* (1.5 µl/well). The most effective *in vitro* concentrations of the extracts were established during preliminary studies by the same technique. Subsequent to an incubation at 37.5°C in a 5% CO_2 atmosphere for 60 h for equine cultures and

72 h for ruminant blood samples, glucose consumption was evaluated by an orto-toluidine colorimetric method with a subsequent spectophotometrical reading at 610 nm wavelength (SUMAL PE2, Karl Zeiss, Jena, Germany), using the reagent as a blank [17,18] . The stimulation/inhibition index (S/I) was calculated as: S/I %=[(IG–GR)/MG]x100, where S/I =blast transformation index, IG= the initial glucose concentration in the supplemented RPMI 1640 and GR=glucose residue in the sample after incubation (Spinu et al., 2016).

2.3. Statistical analyses

Average values and standard error were calculated by use of Excel program. Student's t test was applied to evaluate the statistical significance of the differences which program?

3. Results and Discussion

Traditional medicine worldwide uses numerous herbs and medicinal plants to prevent or treat most variable range of human and/or animal diseases. Observation of behavioral processes even indicated use of medicinal plants in nature by certain species of animals (Page et al., 1992).
The *Compositae* (*Asteracea*) family includes about 10% of all flowering plant species, consisting of over 1,900 genera and 32,000 species within the order *Asterales* with a vaste palet of forms and geographical distribution (Mandel et al., 2016, Marshall et al., 2023).

The uses of different *Asteraceae* are highly variable, with extremes as for cooking oils (*Helianthus annuus*, common sunflower) but also horticultural importance (*Calendula officinalis* marigold, *Echinacea* spp. (coneflowers). One important role of numerous members of this family are in herbal medicine use for human health (Jennifer et al., 2020). Similar to their high variety, there is a broad range of phytochemical compounds, including polyphenols, phenolic acids, flavonoids, acetylenes and triterpenes in the plants of *Asteracea*, responsible for their pharmacological effects (Hueza et al., 2019). Numerous plant compound are immunologically active such as flavonoids, polysaccharides, lactones, alkaloids, diterpenoids and glycosides (Jantan et al., 2015).

3.1. Dynamics of the leukocyte subpopulations

The trend of the *in vitro* leukocyte response to plant extracts was the most steady in equine (Fig. 1). The differences in responses to extracts from *Calendula* and *Echinacea* genera as well as to the different species of the same genus (*Echinacea*) indicated differences in composition and/or concentration of the active principles and also a different perception of the active plant principles by the immune system of each animal species.

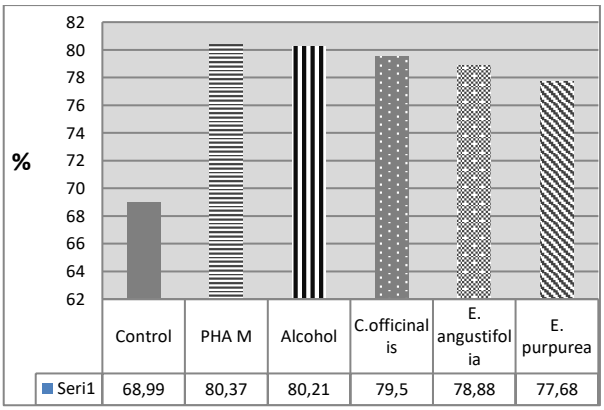

Fig. 1. Blast transformation indices in horse leukocytes treated with alcoholic vegetal extracts

In vitro responses to *C. officinalis*, *E. angustifolia and E. purpurea* were the most pronounced in horses (Fig. 1) and very close for all extracts: 79.50±6.39%, 78.88±3.74 and 77.68±5.93%, respectively.
The *in vitro* blast transformation indices were the lowest in goats (*C. officinalis* - 58.52±10.02%, *E. angustifolia* - 50.06± 11.67%, *E. purpurea* - 50.79±10.98%)(Fig 2).

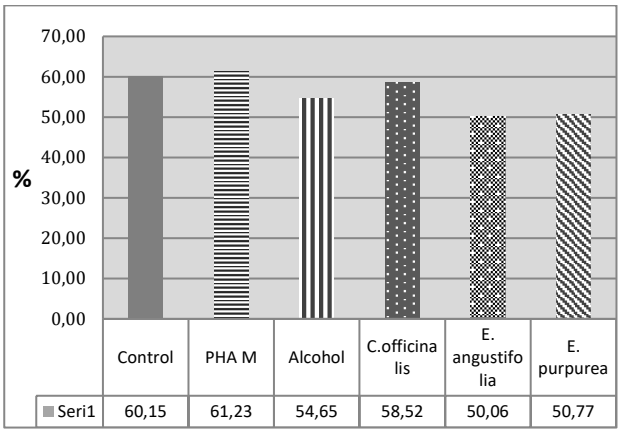

Fig. 2. Blast transformation indices in Angora goat leukocytes treated with alcoholic vegetal extracts (average) The decimal points in figure should be point not comma

In bovine (Fig. 3), the values were higher for all tested extracts (*C. officinalis* – 69.9±2.65%, *E.angustifolia* - 74.9±10.1%, *E. purpurea* -73.22±9.95% $p<0.05$ when compared to goats) and increased towards *E. angustifolia* versus *C. officinalis*.

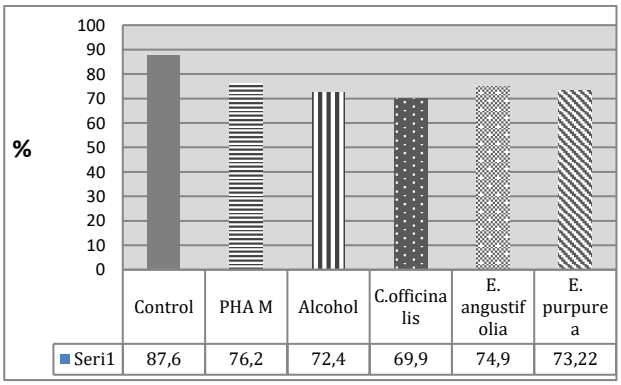

Fig. 3. Blast transformation indices in bovine leukocytes treated with alcoholic vegetal extracts (average)

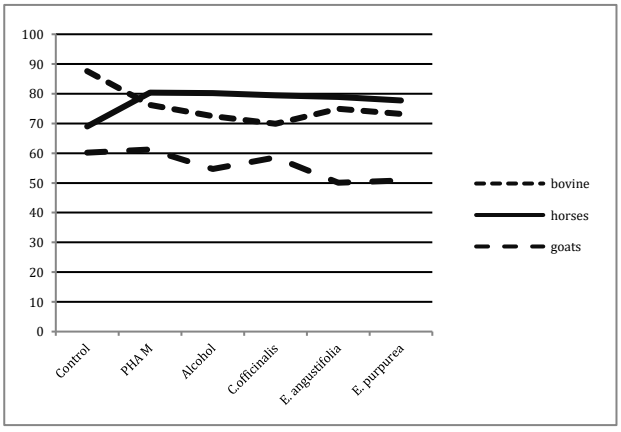

Fig. 4. Leukocyte blastogenic development trend in tested herbivorous species (average)

In this trial, there were few statistically significant differences between the blast transformation indices recorded for the three herbivorous species. When comparing goats with both bovine and horses, the cell-mediated response to both *Echinacea* species were statistically relevantly lower ($p<0.05$). Nevertheless, although some of the components of the tested plants are similar (polysaccharides, chlorogenic acid, caffeic acid) there are some differences in their chemical composition, even if belonging to the same genus (echinacoside is only present in *E. angustifolia* and absent in *E. purpurea*, while cynarin is typical for *E. angustifolia*) (Barnes et al., 2005). An aqueous extract of *C. officinalis* was mentioned to activate the lymphocyte and produce antitumor activity, mantioning the global composition (polysaccharides, proteins, fatty acids, carotenoids, flavonoids, triterpenoids and saponins) of the product (Jimenez-Medina et al., 2006). Although the *Echinacea* species were cited to influence both innate (Sun et al., 1999) and adaptive cell-meditede immunity (Zhai et al., 2007), *E. purpurea* was found less stimulating T_H cells than *E. angustifolia*.

The present study investigated the effects of the two *Echinacea* species in comparison with *Calendula officinalis*, without monitoring either the stimulated subpopulations of mononuclear cells or the separate activities of the plant extracts' individual compounds. Still, no statistically significant differences were observed between the plant species within the tested animals species, the SI induced by the two plant species being very similar for equine, bovine and goats. The variable in this respect was rather represented by the animals species than the plant taxonomy and its chemical composition.

The results obtained for the *C. officinalis* alcoholic extract, although statistically non significantly different from those induced by the *Echinacea* extracts within the frame of each animal species, were different between

horses, bovine and goats (Fig. 1, 2 and 3). Thus, the *Calendula* extract acted enhancing in horses and goats when compared to bovine.

4. Conclusion

When compared to the control, all extracts showed inhibiting effects in bovine and goats, but not in horses, at the tested doses, supporting host-based differences. The biological activity of the tested extracts was animal and plant species dependent, *Calendula officinalis* proving to better stimulate immune cell mitogenesis than *Echinacea* extracts, supporting differentiated effects of plants from different genera of the same family *Asteraceae*.

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Author Contribution

All authors declare equal contribution to the study design and experimental work, interpretation of the resultsand editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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