

Original article

Target cell type dependent immune activity of plant extracts in bovine raised under different technologies

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Abstract

Innate and adaptive immune responses, key to appropriate defense against diseases differ in their development dynamics and are both influenced by raising technologies in bovine. This study aimed to identify the differences in activity of plant extracts on neutrophil phagocytic function and blast transformation of lymphocytes/monocytes from bovine raised in extensive and semi-intensive systems.

Blood samples from dairy cows raised extensively and semi-intensively were subjected to *in vitro* testing for their phagocytic activity (carbon particle inclusion test) and blast transformation capacity (glucose consumption test). Alcoholic extracts of medicinal plants were used as potential stimulating agents. Stimulation indices (%) were calculated as compared to a glucose control. Minitab 16.0 was used for statistical interpretation.

The phagocytosis was highly enhanced by the *Silybum marianum* L. (Gaernet.) extract in semi-intensively raised (0.023 ± 0.04) when compared to the extensively raised cows (0.160 ± 0.069). The *S. marianum* extract enhanced lymphocyte/monocyte growth more in extensively (55.22 ± 11.10 %) than in the semi-intensively raised animals (50.56 ± 9.82 %), with significant differences when compared to the other extracts ($p < 0.0002$). All the used plant extracts acted inhibiting in the blast transformation test, but to a lesser extent in semi-intensively raised animals. Correlation coefficients between the phagocytosis and blast transformation in the presence of *S. marianum* extract were non-significant.

The immune effects of various plant extracts were dependent on cell type (small phagocytes or lymphocytes/monocytes) and also on the raising system, allowing the choice of immune modulating compounds in dairy cows.

Key words: Medicinal plants, neutrophils, lymphocytes, raising system, dairy cows

1. Introduction

Active compounds of medicinal plants have multiple physiological effects with a specific role in environmental adaptation and resistance (Cariddi *et al.*, 2017). Milk thistle (*Silybum marianum* L. Gaernt.) is an annual to biannual plant of the Asteraceae family, which is a native plant in Southern Europe, Southern Russia, Asia Minor and Northern Africa, and it is naturalized in North America, South America and South Australia. *S. marianum* is composed of complex mixtures of phytochemicals (Abenavoli *et al.*, 2010). The active constituents of milk thistle seeds are three isomeric flavonolignans, namely; silibinin (silybin), silychristin, and silidianin, collectively known as silymarin, which is extracted from the dried milk thistle seeds. Silibinin is the most biologically active. The seeds also contain other flavonolignans, betaine, apigenin,

silybonol, proteins, fixed oil, and free fatty acids, which may contribute to the health-giving effects of milk thistle seeds (DerMarderosian and Beutler, 2014; Evans, 2002). The main component of *S. marianum* fruit extract (silymarin) is silybin, a flavonolignan (Shaker *et al.*, 2010), with anti-inflammatory, antiangiogenic, antimetastatic and immunomodulatory activity in various structures and pathways of the cell. Silymarin is also with a strong antioxidant and free radical scavenger by different mechanisms (Esmaeil *et al.*, 2017). Milk thistle extract can also alleviate allergic airway inflammation, atopic dermatitis and allergic rhinitis (Bakhshae *et al.*, 2011; Bijak, 2017, Mady *et al.*, 2016). Due to the differences in external factors present in their habitat, bovine raised in different environments and exploited under different technologies, show different non-specific and specific immune responses. The macrophages and neutrophils key factors of innate immunity are essential for the control of common bacterial infections, but there are microorganisms that cannot be eliminated. The lymphocytes from adaptive immune system offer a more complex defence with improved protection against ensuing reinfection (Janeway *et al.*, 2011). Impaired immune response in individuals subjected to various stress factors can cause: low protective responses following vaccination; sometimes disease; concurrent

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infections/diseases due to lower protection and economic losses. Innate and adaptive immune responses, key to appropriate defense against diseases differ in their development dynamics and are both influenced by raising technologies in bovine. This study aimed to identify the differences in activity of *S. marianum* extracts on phagocytosis and blast transformation of cells from bovine raised in extensive and semi-intensive systems.

2. Materials and Methods

2.1 Animals

The study design was carried out in accordance with the regulations and guidelines set forth by the Bioethics Commission of the Faculty of Veterinary Medicine, Cluj-Napoca. Blood samples from dairy cows raised extensively (n=12) and semi-intensively (n=12) were subjected to *in vitro* testing for their phagocytic activity (carbon particle inclusion test) and blast transformation capacity (glucose consumption test). Jugular blood (5 ml /50 IU heparin/ml) was collected from each cow after the morning milking and before the morning feeding. Blood samples were processed within 1 h of collection. Stimulation indices (%) were calculated as compared to a glucose control.

2.2 Plant extracts

Commercial alcoholic extracts for human use of *S. marianum*, *Vaccinium myrtillus* and *Thymus vulgaris* (Plant extract SRL Romania) produced according to the German Homeopathic Pharmacopeia, were used to treat the whole blood cultures. The identification of the plant species was carried out jointly by Professor M. Tamas, Department of Pharmaceutical Botany, University of Medicine and Pharmacy, Cluj-Napoca, Romania and Plant extract, SRL Romania. The dosage of plant extracts used in this experiment as well as exposure times to the extract were identified in repeated preliminary studies aiming at the obtainment of reproducible results.

2.3 Carbon particle inclusion test

In order to evaluate the phagocytic capacity of cells, 500 µl of heparinised blood was mixed with 2 µl of supernatant of Indian ink. The mixture was incubated at 37°C for a total of 50 min, with aliquots of 150 µl being transferred to 2 ml of PBS (Sigma-Aldrich) immediately after the addition of the Indian ink, after 25 min and at the end of the incubation period. The prepared samples were centrifuged at 1800 rpm and the optical densities of the supernatants were measured using spectrophotometry ($\lambda=535$ nm, d=1 cm). Phagocytic activity index was calculated as the difference between the natural logarithms of the optical densities of the phagocytosis at 0-25 min and 25-50 min (first and second incubation intervals) (Khokhlova *et al.*, 2004).

2.4 Glucose consumption test

In order to assess the blast transformation capacity of mononuclear leukocytes, a glucose consumption test was used, technique as described by Khokhlova *et al.* (2004). The blood samples were diluted with RPMI 1640 (Sigma-Aldrich, USA) culture medium (1:4) supplemented with 5% FCS (Gibco) and penicillin and streptomycin (Sigma-Aldrich), at pH 7.4. The diluted samples were added 96-

well plate, 200 µl/well, in duplicate, 5 variants: (i) untreated control culture, (ii) alcohol treated culture (solvent control) (1.5 µl/well), (3) *S. marianum* treated culture (1.5 µl/ well), (4) *Vaccinium myrtillus* treated culture (1.5 µl/ well) and (5) *Thymus vulgaris* treated culture (1.5 µl/well). The cultures were incubated for 72 h at 37°C and 5% CO₂. Glucose concentrations were measured in the initial medium and in all variants, using Orto-toluidine colorimetric test. An aliquot of 12.5 µl of the cultural medium and each culture variant supernatant was transferred to 0.5 ml of orto-toluidine reagent, boiled for 8 min., cooled suddenly in cold water and read in a spectrophotometer (Sumal PE2, Karl Zeiss, Germany) at 610 nm wavelength, d=0.5 cm, using the reagent as a blank. For transformation index (TI), the following formula was used: $TI\% = [(MG-SG)/MG] \times 100$, where TI, blast transformation index, MG, glucose concentration in the initial culture medium and SG, glucose concentration in the sample after incubation.

2.5 Statistical analysis

Minitab 16.0 was used for statistical interpretation. Results were expressed as Mean \pm standard deviation. The statistical significance of the differences was ranked with letters within the variants in the same group of animals (a- $p < 0.05$, b- $p < 0.01$, c- $p < 0.001$) and with stars between the raising systems (*- $p < 0.05$, **- $p < 0.01$, ***- $p < 0.001$).

3. Results and Discussion

Phagocytic function is one of the important aspects of anti-infectious protection. Functional testing of neutrophil granulocytes correlated with other tests can elucidate the suspected neutrophilic immunodeficiency susceptibility. In order to monitor spontaneous innate phagocytosis, carbon particle inclusion test was used. In this test, the activity being in reverse proportion with the optical density of the supernatants. All phagocytic indices were lower in the extensively raised animals when compared to the semi-intensively raised ones. Statistically significant differences were recorded between the *in vitro* experimental variants in both groups of animals, different by raising technology.

Thus, while the phagocytic index in semi-intensively raised cows was higher in control cultures during the second period ($p < 0.01$), it stayed unchanged after the alcohol and *S. marianum* treatment between the two reading intervals. In extensively raised cows, the differences were significant at higher levels ($p < 0.01-0.001$) between the two readings in all the variants. While incubation length acted negatively on the control variant, it positively influenced the phagocytosis in both alcohol and *S. marianum* extract treated samples, with the plant extract acting strongly stimulating (Table 1).

When comparing the cows raised in different systems, a significant inhibition of the phagocytosis was recorded during the second reading interval in control samples and during the first interval in *S. marianum* treated samples ($p < 0.01$), while the activity of alcohol was also inhibiting but stronger than that of the plant extract ($p < 0.001$).

In the *in vitro* blast transformation test, *S. marianum* acted more stimulating in extensively than in the semi-intensively raised animals ($55.22 \pm 11.10\%$ and 50.56 ± 9.82 , respectively) ($p < 0.05$) (Table 2). All the plant extracts used were inhibiting in the blast transforma-

tion test, but to a lesser extent in semi-intensively raised animals. *V. myrtillus* and *T. vulgaris* proved to act inhibiting, more in the semi-intensively raised than in the extensively raised animals.

Correlation coefficients between the phagocytosis and blast transformation in the presence of *S. marianum* extract were non-significant.

Medicinal herbs, subject to empirical use and scientifically studied resources, represent a tempting and meanwhile very accessible alternative to antibacterial and immunestimulating drugs, with an increased bioavailability and safer than synthetic drugs, ensuring not only human but also animal health and welfare (Ansari, 2016).

Table 1: Mean and standard deviation for spontaneous phagocytic activity

	Semi-intensively raised cows					
	Control		Alcohol		<i>Sylibum marianum</i>	
	Ln0-ln25'	Ln25-ln50'	Ln0-ln25'	Ln25-ln50'	Ln0-ln25'	Ln25-ln50'
Mean	0.065a	0.155**a	0.097***	0.094	0.160**a	0.106
St. Dev.	0.060	0.326	0.070	0.083	0.069	0.128
	Extensively raised cows					
	Control		Alcohol		<i>Sylibum marianum</i>	
	Ln0-ln25'	Ln25-ln50'	Ln0-ln25'	Ln25-ln50'	Ln0-ln25'	Ln25-ln50'
Mean	0.123b	0.042**b	0.08***c	0.054c	0.23**b	0.073b
St. Dev.	0.158	0.021	0.014	0.028	0.195	0.149

Legend: Statistical significance of the differences: Between readings in the same group a- $p < 0.05$, b- $p < 0.01$, c- $p < 0.001$; Between similar variants in different groups *- $p < 0.05$, **- $p < 0.01$, ***- $p < 0.001$

Table 2: Spontaneous blast transformation capacity test results-transformation index

	Semi-intensively raised cows				
	Control	Alcohol	<i>Sylibum marianum</i>	<i>Vaccinium myrtillus</i>	<i>Thymus vulgaris</i>
Mean	56.78	58.96	55.22a	36.68a	35.36a
St. Dev.	17.84	14.75	11.10	10.02	9.33
	Extensively raised cows				
	Control	Alcohol	<i>Sylibum marianum</i>	<i>Vaccinium myrtillus</i>	<i>Thymus vulgaris</i>
Mean	60.28	54.86	50.56	52.53	46.92
St. Dev.	12.27	8.29	9.82	12.85	12.22

a- $p < 0.05$

Immune effects such as anti-inflammatory and cytokine inhibiting ones were tested on dendritic cells for ayurvedic polyherbal oils (Kumar *et al.*, 2014). Their interference with pro-inflammatory cytokines expression and mRNA levels of antioxidant enzymes were also indicated (Taranu *et al.*, 2014; Dharavath *et al.*, 2016).

Functional tests can detect, such as carbon particle inclusions exerted by small phagocytes (neutrophiles, acidophiles, basophiles) in the case of secondary neutrophilic deficits, disturbances occurring in the different stages of phagocytosis:

chemotaxis, interactions with opsonized particles, ingestion, development of respiratory shunt, destruction and digestion of microorganisms. All functions depend on the integrity of membrane receptors, so that their identification by the application of monoclonal antibodies can be extremely useful (Spinu *et al.*, 2018).

The results of monitoring the phagocytic activity indicated a stimulating potential, *S. marianum* alcoholic extract on phagocytic activity recorded in the cows from the semi-intensive raising system during the first period of reading and for the extensively raised

animals, to a lesser extent, for the second period of reading. Nevertheless, this effect proved to be of short duration. This kind of differentiated response could be connected with the different activation status of small phagocytes in the animals from raising systems which certainly differ in their microbiome (de Menezes *et al.*, 2011).

The immune effects of various plant extracts were dependent on cell type (small phagocytes or lymphocytes/monocytes) (Davis *et al.*, 2008) and also on the raising system, allowing the choice of immune modulating compounds in dairy cows.

In the blast transformation test, differentiated effects were observed based on plant genera and species, but at a lesser extent than in the carbon particle inclusion test. These results stood for a differentiated activity of active principles of the same plant, milk thistle, on two different types of immune cells: those representing the immediate line of defense (small phagocytes) and those responsible for the acquired immunity, including the memory cells (lymphocytes). In extensive raising, the more diverse microbiome of the broader habitat acts as aggressor on the immune system and the first line of defense is initially targeted, activated and could be more responsive. In semi-intensive technology, the animals are kept in a more controlled environment, the diversity of the microbiome is lesser but persistent species are present, therefore, the adaptive immune cells are more active and probably more responsive to the milk thistle extract as well.

S. marianum alcoholic extract proved under the experimental circumstances in this study, the complex of choice for increasing both innate and adaptive resistance to potential microbial aggressors from the environment.

4. Conclusion

This experiment carried out on dairy cattle raised extensively and semi-intensively to monitor the functional response of the two different circulating cell types involved in immediate and adaptive immunity which were small phagocytes and mononuclear cells, to various alcoholic plant extracts revealed a more pronounced increase in activity during the phagocytosis than during the blast transformation under plant extract influence. Thus, the *S. marianum* alcoholic extract improved more the phagocytosis and blast transformation in semi-intensively raised cows, than in those raised extensively. When comparing its effects with those of thyme and blueberry, obvious differences were observed depending on the plant and raising technology, which could support further research on selecting the best extract to improve immunity of both types. The obtained data clearly indicated that the immune activity of various plant extracts was also dependent on cell type (small phagocytes-carbon particle inclusion test or lymphocytes/monocytes-blast transformation test), allowing a choice of immune modulating plant extracts in dairy cows.

Conflict of interest

We declare that we have no conflict of interest.

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