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Original article

Interdependence of productive effort and *in vitro* vegetal extract treatment on specific cell-mediated immunity in horses

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Abstract

The research aimed at identifying and rendering alcoholic extracts of various plants based on their *in vitro* potential in stimulating cell mediated immunity and in alleviating stress effects subsequent to workout type in horses.

Experimental horses were selected from different working environments: (a) draft, agricultural works – n = 16, average age 8 years, (b) inconstant effort, leisure, n = 15, average age 7.5 years and (c) constant training, endurance, average age 3.5 years. Blood samples were collected in heparinized vials (50 UI/ml) before and after the workout. Alcoholic extracts (2 µl/well, duplicate) of *Taraxacum officinale* (L) Weber, *Symphytum officinale* L., *Equisetum palustre* L., *Viola tricolor* L., *Avena sativa* L., *Capsella bursa pastoris* (L) Medik., *Hypericum perforatum* L., *Chelidonium majus* L. were investigated by blast transformation test and statistically interpreted (Student's t test).

In vitro responses were the highest to all tested extracts in endurance horses and the lowest in draft horses (p<0.01-0.001). *C. bursa pastoris*. was the most efficient in draft and endurance categories before the workout (draft 45.13 ± 8.05, in leisure 48.09 ± 24.13 and 53.44 ± 9.32 in endurance horses), but not after the workout, where *C. majus* performed better (41.86 ± 22.47 in leisure and 48.1 ± 6.82 in endurance horses), probably due to its complex, protein-rich latex structure. The effects of plant extracts depended on the type of effort and the level of constant training rather than on the taxonomy of the plant.

Key words: Alcoholic plant extracts, blast transformation, effort stress, horses, leukocytes

1. Introduction

Equine represent important partners of humans as help for work, participant to sporting activities or simply as companions. As one of the most sensitive indicators of health, the immune system, provides information on well-being and disease resistance in these animals. The use of horses for various types of exercise could result in stress induced conditions that directly or indirectly prejudice their working performance (Wong *et al*., 1992; Bartolome *et al*., 2016). Within this framework, the study of immune effectors' variations under differentiated workout conditions can be a measure of the predictability of individual welfare and health (Buschmann *et al*., 1991). The economy of horse keeping is subject to a negative balance due to disease control costs, thus preventive, immune enhancing treatments, especially based on natural and highly bioavailabe products, could be an asset.

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As in human medicine, in veterinary therapy immunologically active compounds are used for which plants could represent an inexhaustiblere source. Scientific data support the healing activity of plants (Pearson *et al*., 2012; Subramoniam, 2014; Biradar, 2015). Due to their feeding peculiarities, equine are exposed to grazing and/or hay consumption and to highly variable nutritional value of the consumed plants. Amongst those, some plants from spontaneous flora also benefit of medicinal qualities and consumed by horses can positively influence their immunity to diseases.

The aim of the research was to render several alcoholic vegetal extracts of various sources in alleviating stress effects subsequent to effort on cell mediated immunity in horses, while monitoring the potential differences in response to these extracts between workout groups of horses.

2. Material and Methods

2.1 Animals

Experimental horses were selected from different working environments: (a) draft, semi-constant effort, agricultural works – n=16, average age 8 years, (b) inconstant effort, leisure, n=15, average age 7.5 years and (c) constant training, endurance, n=10, average age 3.5 years. Blood was sampled on heparine (50 UI/ml) before and after the workout specific for each category, with the same given duration. The blood samples were collected within the context of the national surveillance program for equine infectious anemia, thus being subject to serological testing approved by the national competent authority (National Veterinary and Food Saftery Authority) and also with the approval of the owners.

2.2 Plant extracts

To monitor the potential immune modulating effects of various plant extracts on the adaptive cell-mediated immune response, several species of plants were chosen; some of them as such (grass, hay or grain) entetring the horses' regular diet. Thus, commercial alcoholic extracts for human use, obtained from *Taraxacum officinale* (L) Weber, *Symphytum officinale* L., *Equisetum palustre* L., *Viola tricolor* L., *Avena sativa* L., *Capsella bursa pastoris* (L) Medik., *Hypericum perforatum* L., *Chelidonium majus* L. were used. The extracts were prepared by percolation method by Plant extract, Romania, according to the provisions of German Homeophatic Pharmacopeia from plants originating in Romania. Their content in active principles was standardised by the producer.

2.3 Methods

2.3.1 Neutrophile/lymphocyte (N/L index) evaluation

Blood smears were performed according to the usual technique and stained with Dia Quick Panoptic. Leukocyte subpopulations were counted and N/L ratios were evaluated as an indicator of the stress level caused by the workout type (Davis *et al*., 2008).

2.3.2 Leukocyte blast transformation test

The leukocyte blast transformation test measures the *in vitro* reactivity of lymphocytes and monocytes to sensitizing antigens. The blood samples were diluted (1:4) with RPMI 1640 (with 5% FCS and antibiotics, at pH 7.4) (Sigma-Aldrich, USA) and distributed in duplicate in sterile 96-well plates (200 µl per well). Twelve *in vitro* experimental variants were tested for each individual animal, namely; (1) untreated control culture, (2) phytohaemagglutinin-M (PHA) (1µ per well), (3) LPS, (4) 70°C alcohol and (5-12) commercial alcoholic extracts of *T. officinale, S. officinale, E. palustre, V. tricolor, A. sativa, C. bursa pastoris, H. perforatum , C. majus* (Plant extract, Romania) (1.5 µl/well). Subsequent to an incubation of 60 h at 37°C in a 5% CO_2 atmosphere, glucose consumption was evaluated. For this, 12.5 µl of the cultural supernatant was transferred to 0.5 ml of orto-toluidine reagent, boiled for 8 min and read in a spectrophotometer at 610 nm wavelength (SUMAL PE2, Karl Zeiss, Jena, Germany). The stimulation/inhibition index (SI) was calculated as: SI % = [(IG–GR)/IG]×100, where SI =blast transformation index, IG = the initial glucose concentration in the culture medium and GR = glucose residue in the sample after incubation (Spinu *et al*., 2016).

2.3.3 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.

3. Results and Discussion

Phytomedicine, in spite of being known for millennia, is nowadays gaining more importance due to the scientific recognition of the therapeutic power of natural molecules from plants with increased bioavailability and lesser side effects than synthetic compounds (Pushpangadan, 2013). Veterinary medical use of plant extracts represents one of the broad windows opened to scientific research due to numerous potential subject species and also different perception of the active plant compounds by each and every one of those.

The medicinal plants that represented the subject of the present study were chosen due to their high prevalence, thus the high likeliness to be ingested by the horses on the pasture. Nevertheless, researches indicated immunological effects for all including anti-inflammatory effects (*C. bursa pastoris*, *C. majus*) (Choi *et al*., 2014; Nawrot *et al*., 2017), inhibition of activated lymphocyte proliferation (*E. palustre, V. tricolor*) (Gründemann *et al*., 2014; Hellinger *et al*., 2014), anticomplementary activity (*S. officinale*) (Staiger, 2012), modulation of antibody levels (*A. sativa*, *T. officinale*) (Yoon, 2008; Chatuevedi *et al*., 2011).

Antimicrobial defence in mammals is mainly based on adaptive immunity, involving the tri-celular cooperation among antigen presenting cells, T and B lymphocytes. These mechanisms are subject to external influence including stress of various origins. Several studies indicated that the type of exercise is important in conditioning the types of changes that occur at the immune system level, including an augmentation of lymphokine activated killer (LAK) cell function (Horohov *et al*., 1996).

It has been shown that during a single strenuous exercise, in spite of the significant decrease in chemotactic index and peak chemo-luminescence, one day after exercise, both IgA levels and the mitogen-induced blastogenesis of lymphocytes stayed unchanged (Wong *et al*., 1992; Souza *et al*., 2010). Similarly, a prolonged bout of exercise lead to a long-term suppression of the non-specific immune response which was thought to partially stand for certain infectious episodes in trained horses (Robson *et al*., 2003).

Regular, moderate stress was reported as beneficial for enhancing the function of neutrophils (Korhonen *et al*., 2000). The N/L was increased in horses after transportation, due to increased stress (Davies *et al*., 2018). As indicated in recent studies (Krumrych *et al*., 2018), and also supported by the N/L index in this study (Figure 1); the stress levels in various workout categories of horses investigated differed by effort type. It depended less on the constant effort, such as in draft or endurance horses, than on the interrupted, inconstant exercise as in leisure equine.

The higher values of the N/L ratio in the majority of the working horses might indicate their constant exposure to acute bouts of stress and, most probably, also to subliminary chronic stress. The N/L ratio calculated for leisure horses was the highest in this experiment, the animals being subject to inconstant, different intensity workout and, therefore, more prone to workout stress. Some authors consider a N/L ratio around 3 indicating an impairment of the health status that can not be restored without substantial changes in the working schedule or management system. In draft horses, the animals showed the lowest levels of stress, probably because the workout had a relatively constant pase.

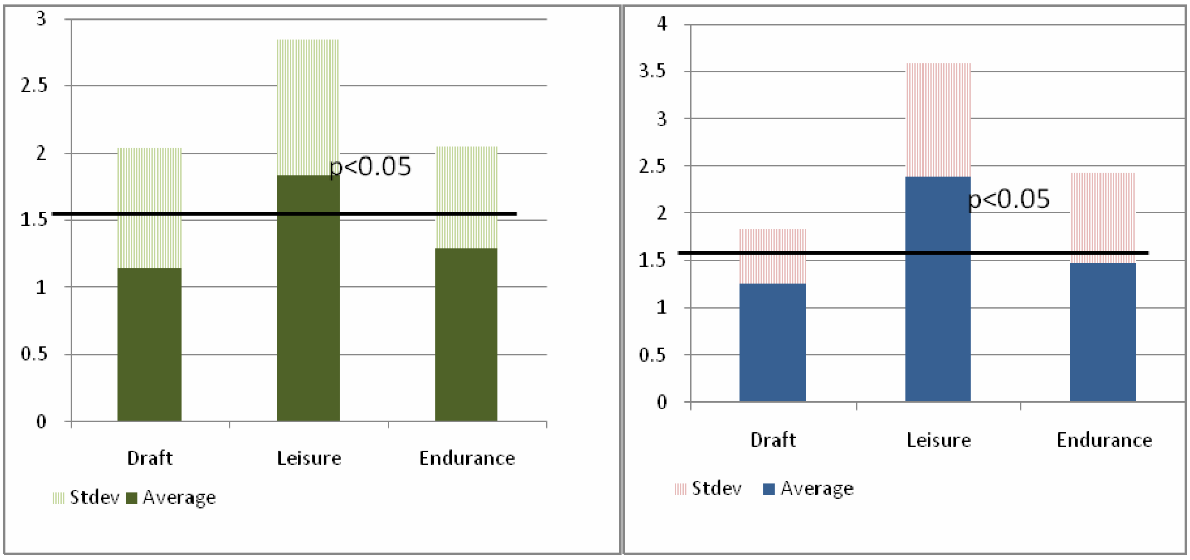
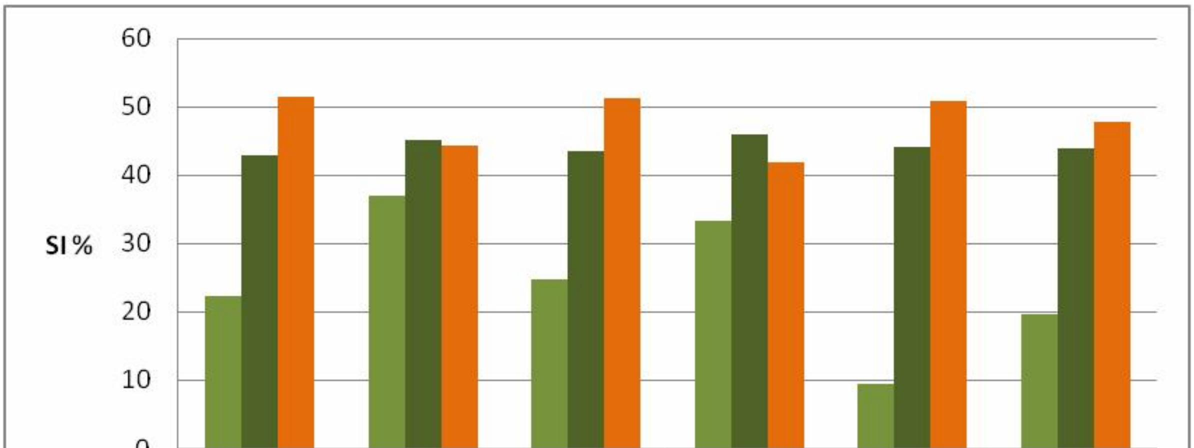


Figure 1: Changes in N/L ratios before and after workout depending on exploitation category (draft, leisure, endurance). The black line indicates the average N/L value for adults of the species.



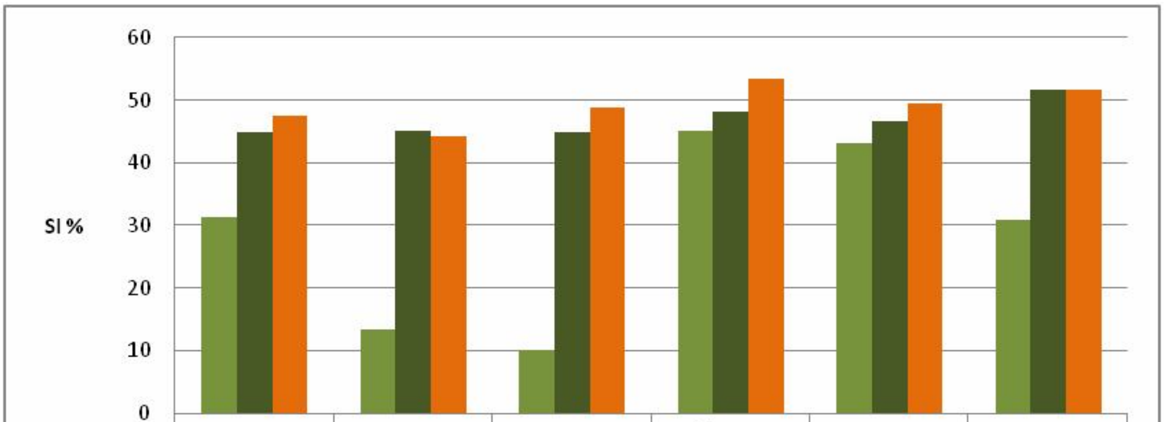
	Control	LPS	PHA	Alcohol	Taraxacum	Symphitum
Draft	22.21	36.99	24.7	33.33	9.37	19.69
Leisure	42.8	45.03	43.5	45.97	44.09	43.85
Edurance	51.33	44.31	51.27	41.77	50.9	47.69

Figure 2(a): *In vitro* stimulation indices (%) in horses differentiated before their workout (draft-leisure, endurance) (unstimulated control, alcohol and mitogen controls, *Taraxacum* and *Symphitum* alcoholic extracts).

Equine athletes are exposed to various levels of effort which might impact on their health status and, therefore, susceptibility to various infectious agents, including ported ones (Hines*et al*., 1996); a previous study indicated that subsequent to endurance ride, the number of CD4 (helper) T cells decreased, while the number of suppressor cells increased (Heines *et al*., 1994), decreasing the antimicrobial adaptive immune potential. A decrease in proliferative responses of lymphocytes appears as soon as 12 to 16 hours after a horse participates in a race (Nesse *et al*., 2002).

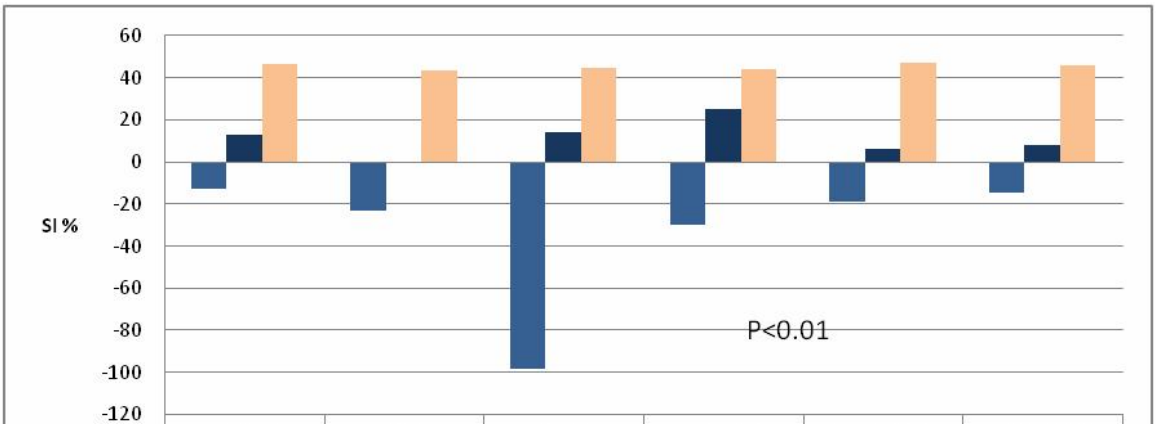
Different extracts of plant origin were tested for their therapeutic and disease preventing potential in both humans and animals (Pearson *et al*., 2012; Ansari, 2016). As products with increased bioavailability and little or no side effects, plant extractscould intervene in restoring the immune response and improving the welfare and the antimicrobial protective level. In this study, the immune enhancing capacity of some alcoholic plant extracts was tested, while the main cell groups were exposed to these extracts *in vitro*, in whole blood cultures from horses exposed to various effort levels.

The highest values of stimulation indices were observed in endurance horses, both before and after the workout, associated with intermediate stress levels (Figures 2a and 2b). Although, the encountered SIs were low compared to other species (Spinu *et al.*, 2016), they were always in the positive zone. *C. majus* was the most efficient in all categories (draft 30.973 ± 22.578, in leisure 51.63 ± 25.19 and 41.86 ± 22.48 and endurance 51.59 ± 4.83 and 48.06 ± 6.82, before and after the workout, respectively), probably due to its complex, protein-rich latex structure.



	E. palustre	V.tricolor	A. sativa	C.bursa pastoris	H.perforatum	Ch. Majus
Draft	31.41	13.45	10.03	45.13	43.06	30.967
Leisure	44.8	45.03	44.8	48.09	46.56	51.62
Edurance	47.53	44.15	48.79	53.44	49.53	51.59

Figure 2(b): *In vitro* stimulation indices (%) in horses differentiated before their workout (draft, leisure, endurance) (alcoholic plant extracts, continued).



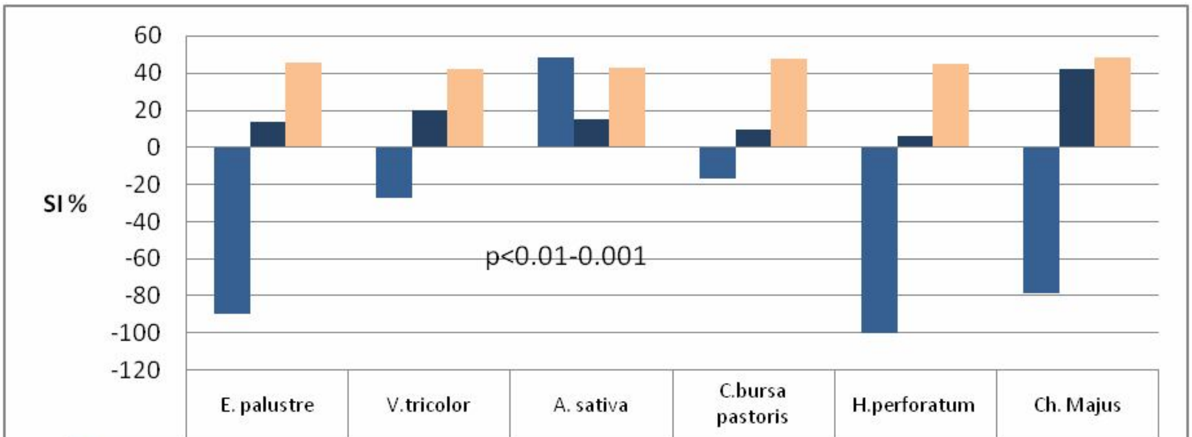
	Control	LPS	PHA	Alcohol	Taraxacum	Symphitum
Draft	-12.59	-23.27	-98.19	-29.5	-18.82	-14.37
Leisure	12.79	-0.39	14.2	25.15	6.08	8.08
Edurance	46.47	43.36	44.68	43.94	47	45.68

Figure 3(a): *In vitro* stimulation indices (%) in horses differentiated after their workout (draft-leisure, endurance) (unstimulated control, alcohol and mitogen controls, *Taraxacum* and *Symphitum* alcoholic extracts).

Of the other vegetal extracts, *C. bursa pastoris* was the most active in the same workout category, before the effort, suggesting an appropriate mean to boost the adaptive response of the mononuclear equine cells during the workout. In Romania, it represents a very handy cure, the plant being ubiquitous on almost all pastures. *C. majus* performed better after the workout (41.86 ± 22.47 in leisure and 48.1 ± 6.82 in endurance horses), probably due to its complex, protein-rich latex structure (Nawrot *et al*., 2017).

The SIs values were close for the leisure and endurance horses, with lower figures recorded for the draft group before the exercise, but highly variable after the animals were subject to the workout. Thus, all indices were in the negative zone for the draft horse group after the effort, except for the *A. sativa* (common oats) extract, which proved to be the strongest stimulating extract for this group (Figure 3b).

When compared to the SI for the same extract before the workout (Figure 2b), which were the lowest for the group, we could conclude that the active principles of the plant had modulating effects. *T. officinale*, *S. officinale* and *V. tricolor* extracts had stimulating effects when compared with the alcohol but not with the untreated control.



	E. palustre	V.tricolor	A. sativa	C.bursa pastoris	H.perforatum	Ch. Majus
Draft	-89.8	-27.02	48.1	-16.55	-99.68	-78.13
Leisure	13.38	20.09	15.26	9.49	6.08	41.86
Edurance	45.26	42.15	42.46	47.53	45.1	48.1

Figure 3(b): *In vitro* stimulation indices (%) in horses differentiated after their workout (draft, leisure, endurance) (alcoholic plant extracts, continued).

At the dosage used in this protocol, none of the extracts were stimulating in leisure horses. In connection to the highest stress levels in this category, either the used plants or the dosage could act as variables in restoring the workout induced stress.

Only *V. tricolor* and *A. sativa* extracts were inhibiting in the endurance horses, while all the others acted stimulating. Thus, intermediate stress and connected adaptive immune response induced by constant exercise, seemed to be more easy to restore by use of medicinal plants than that induced by the semi-constant or inconstant effort.

The results of the Student t-test indicated significance of the differences between draft versus leisure/endurance horses (p< 0.010 -p<0.001), for most of the variants, between the initial and after workout measurements.

4. Conclusion

The effects of various plant extracts depended mostly on the type of effort/degree of stress and the level of constant training rather than on the taxonomy of the plant. Nevertheless, the experiment allowed rendering some plant extracts that could help in alleviating the stress subsequent to various levels of effort in horses.

Conflict of interest

We declare that we have no conflict of interest.

References

Ansari, S.H. (2016). Globalization of herbal drugs. Ann. Phytomed., **5**(2):1-5.

Bartolome, E. and Cockram, M.S. (2016). Potential effects of stress on the performance of sport horses. Journal of Equine Veterinary Science, **40**:84-93.

Biradar, D.P. (2015). Medicinal plants and phytomedicine. Ann. Phytomed., **4**(1):1-5.

Buschmann, H. and Baumann, M. (1991). Alterations of cellular immune response during intensive training of event horses. Zentralbl. Veterinarmed. B., **38**(2):90-94.

Chatuevedi, N.; Yadav, S. and Shukla, K. (2011). Diversified therapeutic potential of *Avena sativa*: An exhaustive review. Asian Journal of Plant Science and Research, **1**(3):103-114.

Choi, W.J.; Kim, S.K.; Park, H.K.; Sohn, U.D. and Kim, W. (2014). Anti-inflammatory and anti-superbacterial properties of sulforaphane from shepherd's purse. Korean J. Physiol. Pharmacol., **18:**33-39.

Davis, A. K.; Maney, D. L. and Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: A review for ecologists. Functional Ecology, **22**:760-772.

Gründemann, C.; Lengen, K.; Sauer, B.; Garcia-Käufer, M.; Zehl, M. and Huber, R. (2014). *Equisetum arvense* (common horsetail) modulates the function of inflammatory immunocompetent cells. BMC Complementary and Alternative Medicine, **14**:283.

Hellinger, R.; Koehbach, J.; Fedchuk, H.; Sauer, B.; Huber R.; Gruber C.W. and Gründemann, C. (2014). Immuno suppressive activity of an aqueous *Viola tricolor* herbal extract. J. Ethnopharmacol., 1**51**(1):299-306.

Hines, M.T.; Leroux, A.J. and Schott, H.C. (1994). Changes in lymphocyte subpopulations following prolonged exercise in horses. Proceedings of the 12th Annual Veterinary Medical Forum, San Francisco, CA. 1016.

Hines, M.T.; Schott, I.I.H. C.; Bayly, W.M. and Leroux, A. J. (1996). Exercise and Immunity: A review with emphasis on the horse. Journal of Veterinary Internal Medicine, **10**(5):280-289.

Horohov, D.W.; Keadle, T.L.; Pourciau, S.S.; Littlefield-Chabaud, M.A.; Kamerling, S.G.; Keowen, M.L.; French, D.D. and Melrose, P.A. (1996). Mechanism of exercise-induced augmentation of lymphokine activated killer (LAK) cell activity in the horse. Vet. Immunol. Immunopathol., **53**(3-4):221-233.

Korhonen, P.A.; Lilius, E.M.; Hyyppä, S.; Räsänen, L.A. and Pösö, A.R. (2000). Production of reactive oxygen species in neutrophils after repeated bouts of exercise in standard bred trotters. J. Vet. Med. A., **47**: 565-573.

Krumrych, W.; Golda R.; Golyñski M.; Markiewicz H. and Buzala M. (2018). Effect of physical exercise on cortisol concentration and neutrophil oxygen metabolism in peripheral blood of horses. Ann. Anim. Sci., **18**(1):53-68.

Nawrot, R.; Lippmann, R.; Matros, A.; Musidlak, O.; Nowicki, G. and Mock, H.P. (2017). Proteomic comparison of *Chelidonium majus* L. latex in different phases of plant development. Plant Physiol. Biochem., **112**:312-325.

Nesse, L.L.; Johansen, G.I. and Blom, A.K. (2002). Effects of racing on lymphocyte proliferation in horses. Am. J. Vet. Res., **63**(4):528-530.

Pearson, W.; Fletcher, R.S. and Kott, L.S. (2012). Oral rosmarinic acid-enhanced *Mentha spicata* modulates synovial fluid biomarkers of inflammation in horses challenged with intra-articular LPS.J Vet Pharmacol. Ther., **35**(5):495-502.

Pushpangadan, P. (2013). Ethnopharmacology and phytomedicine. Ann. Phytomed., **2**(2):1-4.

Robson, P.J.; Alston, T.D. and Myburgh, K.H. (2003). Prolonged suppression of the innate immune system in the horse following an 80 km endurance race. Equine. Vet. J., **35**(2):133-137.

Souza, C.M.; Miotto, B.A.; Bonin, C.P. and Camargo, M.M. (2010). Lower serum IgA levels in horses kept under intensive sanitary management and physical training. Animal, **4**(12):2080-2083.

Spinu, M.; Niculae, M.; Pastiu, A. I.; Sandru, C.D.; Pall, E. and Vasiu, A. (2016). Vegetal extracts influence *in vitro* on the cell-mediated immunity in carnivores depending on health status, target species and plant taxonomy, Industrial Crops and Products, **88**:44-47.

Subramoniam, A. (2014). Phytomedicines for healthcare. Ann. Phytomed., **3**(1):1-3.

Staiger, C. (2012). Comfrey: A clinical overview, Phytother. Res., **26**: 1441-1448.

Wong, C.W.; Smith, S.E.; Thong, Y.H.; Opdebeeck, J.P. and Thornton, J.R. (1992). Effects of exercise stress on various immune functions in horses. Am. J. Vet. Res., **53**(8):1414-1417.

Yoon, T. J. (2008). Effect of water extracts from root of *Taraxacum officinale* on innate and adaptive immune responses in mice. Korean J. Food and Nutr., **21**(3):275-282.