Taraxacum Officinale Alcoholic Extract Augments the Cell-Mediated Immune Response in Anti-Anthrax Vaccinated Sheep

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RESEARCH ARTICLE

Abstract

Phytotherapy used various preparations in both human and veterinary medicine over time, due to their increased bioavailability and lower costs. *Taraxacum officinale* of *Compositae* family, well-known for its therapeutic effects, abundantly grows on Romanian pastures. The research aimed to investigate its alcoholic extract's effects on cell-mediated immunity in anti-anthrax vaccinated, grazing sheep. Local Turcana ewes kept on pasture from spring to fall were sc vaccinated with R1190 attenuated anthrax strain, and blood was sampled before and 14 days after the vaccination. *In vitro* cell-mediated immunity was monitored employing phagocytosis and blast transformation tests in the presence of *T. officinale* alcoholic extract. The extract increased phagocytosis two weeks after the vaccination ($p < 0.001$), while the blastogenic response also showed significant variations ($p < 0.05$) over time. There was a positive effect of *T. officinale* extract on the cellular immunity, suggesting its stress alleviating effects in anti-anthrax vaccinated sheep.

Keywords: sheep; anti-anthrax vaccination; stress; *T. officinale*.

INTRODUCTION

In the food animal industry, appropriate health is a prerequisite of productivity at optimal levels. Technology must not exert a negative influence on animals welfare and vigor by any of the processes involved (Çokçalışkan et al. 2019). Therapy undertaken in animal communities contributes to protecting against various diseases. Still, the shortage of drugs to control infectious and transmissible diseases in the sector resides in up to 35% loss in breeding stock (Souto et al. 2012). Thus, vaccination remains one of the most important preventive means, mainly against zoonotic diseases such as anthrax (Dutz and Kohout-Dutz 1981; Schneemann and Manchester 2009). This sporadic, non-contagious, disease of mammals, accidental in humans, is caused by *Bacillus anthracis*, a ground born pathogen, persistent for a very long period, reaching even 200 years, due to its resistant spores (Dragon and Rennie 1995; De Vos V 2004). Herbivorous animals are exposed during the grazing period, of which sheep and goats show the highest susceptibility and shortest clinical course of the disease. Anthrax represents a threat in all areas of the world where vaccination procedures are not implemented. Vaccination against anthrax, a consolidated preventive procedure, usually leads to immunity for the entire grazing season. Prevention must be considered, especially in endemic areas. Nevertheless, in emergency situations, 20% of the vaccinated animals remain uncovered (Ezzell and Abshire 1988), and supplementary drawbacks were mentioned (Hahn et al. 2006). Some strains, including the Romanian Stamatin R1190 strain, also cause side effects in some of the vaccinated sheep, such as

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local swelling and pain, or, sometimes, the clinical disease. Thus, immune enhancement is being sought by the use of improved vaccines (Fasanella, Adone, and Hugh-Jones 2014) or immune response amplifiers/herbal enhancers (Yurdakok-Dikmen, Turgut, and Filazi 2018). Nevertheless, before adopting vaccination protocols or therapeutic measures, it is vital to investigate the functional status of various immune effectors involved in protection.

Non-specific and specific immune cell-mediated defense mechanisms are protective barriers at the level of possible pathogens entry points into the susceptible host and responsible for antibody synthesis, respectively. Phagocytosis not only provides immediate defense but by incorporating and processing the aggressors (bacteria, viruses, parasites, fungi) or their fractions, which contributes to activating the specific immune system. This way, a whole chain of reactions is triggered, resulting usually in specific protection for the host. Mechanisms and effectors involved differ fundamentally for innate and acquired sides of the immune response, but cooperation between all those is essential for a complete and entirely protective immunity (Arstila 1996; Chaplin D. D. 2010).

For millennia, plants provided a paragon of active principles to treat various diseases, not only in people but in animals as well (Farnsworth et al. 1985). Traditional medicine relies on ethnobotanical practices in more than 80% of the world's population, located on the rim of illness, and is the most exposed to emerging and re-emerging diseases in endemic areas (Iqbal et al. 2003; Phondani, Maikhuri, and Kala 2010). Plants were also used to improve the efficacy of conventional therapies (Buldain et al. 2018) or procedures such as vaccination (Tamminen, Emanuelson, and Blanco-Penedo 2018).

Compositae (*Asteraceae*) family, distributed all over the world, is one of the largest plant families with over 20000 species, including some genera well known for their biological activity (*Echinacea*, *Calendula*, *Chrysanthemum*, *Taraxacum*, *Helianthus*, *Tagetes*, *Achillea*, *Arnica*, etc) (Koc et al. 2015; Lakshman, Yeasmin, and Gabriel 2014) and showing a very complex chemical composition, based on the species and extraction method (Soković, Skaltsa, and Ferreira 2019).

Taraxacum officinale of *Compositae*, native to Eurasia and North America, with proven antioxidant and anti-inflammatory activities (Sari and Keçeci 2019) abundantly grows on Romanian pastures. The research aimed to investigate the effects of an alcoholic *Taraxacum officinale* extract on cell-mediated immunity in anti-anthrax vaccinated, grazing sheep.

MATERIALS AND METHODS

Animals and sampling. The research was conducted in a flock of 130 Turcana sheep, of which 127 were lactating ewes, and 3 were rams. The breed is a Romanian one, somewhat primitive, well adapted to local conditions. The age of animals ranged between 2 and 5 years. The animals were kept mainly on the pasture from spring to fall, benefitting of a concentrate supplement daily. Antigen priming was carried out with an anti-anthrax live attenuated vaccine, Stamatin 1190 R strain (INMV Pasteur, Bucharest), administered by s.c. injections, during the fall season. Blood was sampled on heparin (50 IU/ml), from the jugular vein of the animals, two weeks prior and two weeks after the vaccination, and both non-specific and adaptive cell-mediated immunity were monitored by means of *in vitro* phagocytosis and blast transformation tests in the presence of *T. officinale* alcoholic extract.

Methods. Phagocytic test. Phagocytic cells engulf inert particles such as carbon due to their defensive capacity. An *in vitro* carbon clearance assay was used to evaluate phagocytic activity in the experimental batches, adapted from the technique described before (Khokhlova et al. 2004). 1.5 ml of venous blood collected on heparin (50 IU/ml) received 6 µl of the supernatant fraction of India ink (Pelikan AG D-3000, Hanover, Germany) that had been centrifuged at 3000 g for 30 min. After mixing, each sample was divided into 3 equal aliquots: a) untreated control, b) treated with 70° alcohol, 1.5 microliters/tube, and c) treated with a *Taraxacum officinale* (1.5 microliters/tube) alcoholic extract, respectively, which were incubated at 37°C for 15 and 30 min each. Volumes of 150 µl of each blood and India ink mixture from each aliquot were added to 2 ml saline following incubation. These diluted samples were centrifuged at 50 g for 4 min, and the supernatant was read spectrophotometrically at 535 nm, with the saline taken as background. There was a decrease in absorbency with time as carbon was phagocytosed. Optical density readings were converted to a log2 scale and the phagocytic index was taken as the negative of the slope of the regression of optical density (log2) on time (h).

Blast transformation test. The blast transformation capacity of leukocytes was tested on whole blood cultures by use of RPMI 1640 (Sigma-Aldrich) culture medium, with 5% fetal calf serum (FCS)(Sigma-Aldrich). The blood samples were diluted 1:4 with the medium, distributed in 96 well plates in 0.2 ml aliquots. PHA M and LPS (Sigma-Aldrich) (1µl/well) were used as a standard mitogen. Alcohol (70°, 1.5µl/well) served as the control for *Taraxacum officinale* alcoholic extract treated variants (1.5µl/well). All variants were performed in duplicate. The plates were incubated at 37°C in a 5% CO2 atmosphere for 63 hours. The cell growth was estimated by measuring the glucose residue in a colorimetric orto-toluidine test (ICCF, Bucharest) and spectrophotometrical (SUMAL PE2, Karl Zeiss, Jena) measurement ($\lambda=610\text{ nm}$, $d=0.5\text{ cm}$) of the results. Blast transformation indices were calculated as percentages of the consumption versus the initial glucose concentration of the RPMI 1640 medium.

The data were statistically interpreted, by calculating mean values and standard deviations as well as the statistical significance of the differences by use of Student's t-test.

RESULTS AND DISCUSSIONS

A continuously growing pool of literature stands for the ever-increasing importance of traditional knowledge in healing with the help of plants, which provided the foundation and continuous support to sustainable development in small communities (Wanzala et al. 2005). Medicinal plants with cholagogic/choleretic properties included chamomile (*Chamomilla recutita*), elecampain (*Inula helenium*), dandelion (*Taraxacum officinale*), St. John's wort (*Hypericum perforatum*), Artemisia sp, yarrow (*Achillea millefolium*), rosemary (*Rosmarinus officinalis*), chelidonium (*Chelidonium majus*). Essential oils of liquorice, coriander, turmeric, black pepper, red chili, cumin, onion, peppermint also have choleretic properties (Yurdakok-Dikmen, Turgut, and Filazi 2018). Phytotherapy also included various preparations used as bactericidal (Cerioli et al. 2018), antiviral (Kalvatchev, Walder, and Garzaro 1997), immune-stimulating (Bauer 2002), anti-inflammatory, antiparasitic, neurotropic (Attele, Wu, and Yuan 1999), cardio-angiotropic, pneumotropic agents along time (Bullitta, Piluzza, and Viegi 2007; Eshetu et al. 2015), due to increased bioavailability, easy access and lower cost of plant preparations (Raman 2017).

Integration of ethno-veterinary practices into official health care systems still needs scientific support (Wanzala et al. 2005). As prerequisites to improve the results and reproducibility of phytotherapy, the upgrade of clinical trials and real estimates of cost/benefit ratio was also some of the concerns expressed by specialists (Blanco-Penedo et al. 2018).

The *in vitro* use of the alcoholic *T. officinale* extract highly significantly ($p < 0.001$) increased the phagocytosis two weeks after the vaccination (from -0.00267 to 0.022903 units), while the specific cell-mediated response also showed significant variations ($p < 0.05$) over the tested period (from 36.01 ± 17.4 to $55.17 \pm 9.30\%$).

The cells involved in the immediate immune response, those with undifferentiated activity against all aggressors, bacteria, viruses, or fungi, intervene through accumulation in large numbers at the site of aggression as well as by activating the functions they perform, especially phagocytosis. Nevertheless, its intervention in vaccinated animals, while protecting against disease is unclear (Tchalla et al. 2020). Whole blood phagocytes have the ability to ingest not only bacteria but also inert particles (carbon, silicon, etc.). The reduction in the number of carbon particles in a mixture of whole blood and Chinese ink, evaluated spectrophotometrically, provides clues to the functional capacity of circulating phagocytes. *In vitro* testing of the functional potential of *in vitro* phagocytes offers information about their ability to recognize and subsequently embed "non-self" particles, not only inert but also biotic (microbial aggressors).

By monitoring the ability to absorb inert carbon particles *in vitro*, it was noticed that the level at which phagocytes were stimulated differed both by the time of harvest and by the *in vitro* treatment applied. The immune-stimulating effect exerted by plant extracts on the mechanisms of *in vitro* phagocytosis would contribute to better uptake and presentation of vaccine antigens, augmenting antibody production. The study of the extent to which this treatment increased phagocytosis would therefore provide data for the assessment of the possible adjuvant value of these types of compounds.

Literature data mention the positive influence of natural feed additives such as freshwater algae (*Chlorella vulgaris*) on phagocytosis and lymphoid tissue development (Kwak et al. 2012). Significant effects are found in humans after the administration of *Echinacea* extract, amplifying phagocytic activity in the infused rat liver (Roesler et al. 1991).

The level of phagocytosis in the control variant was decreased subsequent to two weeks after the vaccination. The alcoholic dandelion extract used *in vitro* had a weak stimulating effect on phagocytosis when compared to controls, notable results being recorded only after 2 weeks, for the first measurement period of 15 min, after which it decreased (Table 1).

Table 1. Phagocytosis during the experiment (units)

Sampling	Control		Alcohol		<i>Taraxacum officinale</i>	
	In0-In15/15	In15-In30/15	In0-In15/15	In15-In30/15	In0-In15/15	In15-In30/15
I	0.00915 $\pm$ 0.0174	-1.18162 $\pm$ 0.7591	0.001049 $\pm$ 0.0071	0.000213 $\pm$ 0.0063	0.001766 $\pm$ 0.0144	-0.00193 $\pm$ 0.0149
II	-0.00321 $\pm$ 0.0104	0.003313 $\pm$ 0.0239	0.007885 $\pm$ 0.0288	-0.00773 $\pm$ 0.0287	0.003077 $\pm$ 0.0151	-0.00267 $\pm$ 0.0081

The plant extracts used *in vitro* have different immunomodulatory effects depending on the time at which the blood was collected.

The blastogenesis of mononuclear cells can be monitored by the *in vitro* blast transformation test (Lacetera and Ronchi 2004). This technique allows the final calculation of the blast index, a parameter that indicates the stimulation or inhibition of cell growth. The *in vitro* supplementation of cultures with classical mitogens allowed an estimate of the virtual response to microbial aggression of mononuclear and B lymphocytes.

Table 2. Blast transformation indices in treated cultures (%, IInd sampling)

Experimental variant	Sampling I	Sampling II
Control culture	18.77 $\pm$ 27.96	35.70 $\pm$ 8.71
LPS	22.22 $\pm$ 28.70	38.61 $\pm$ 7.12
PHA	23.45 $\pm$ 23.92	37.81 $\pm$ 8.40
Alcohol	29.34 $\pm$ 23.21	43.95 $\pm$ 10.20
<i>Taraxacum officinale</i>	23.58 $\pm$ 24.29	37.58 $\pm$ 9.77

All values obtained in the blast transformation test were higher after 14 days post-vaccination. When compared to the pre-vaccination results, the latter suggested that the vaccination-induced very low growth, indicating the stress induced by both vaccination and handling, the animals were subjected to.

Although the solvent control induced the highest effect, the level of stimulation induced by the alcoholic dandelion extract was very promising, similar to that of PHA M, both at the initial sampling and significantly higher at the second sampling ($p < 0.05$). These values were increased when compared to the untreated controls at both readings.

Stimulation indices showed very clearly that animals exposed to dandelion on the pastures during their grazing period of more than 6 months, depending on the climate, could have improved both non-specific and specific immune responses. These could prove efficient not only versus vaccine antigens administered to the animals but also in providing them protection against other aggressors. These data are consistent with some similar citations in the literature regarding the potentiating effects of some other plant extracts used as alcoholic extracts to improve immunity over time (Farnsworth et al. 1985; Iqbal et al. 2003; Khokhlova et al. 2004; Roesler et al. 1991; Yurdakok-Dikmen, Turgut, and Filazi 2018).

CONCLUSIONS

The results of the present experiment supported the positive effect of *T. officinale* active principles on the innate and adaptive cell-mediated immunity, indicating the potential uses as immune enhancer/adjuvant in alleviating the stressful effects of anti-anthrax vaccination in sheep.

Author Contributions: M.S. and I.S.G. conceived and designed the analysis; S.Z, A.V. and D.O. Collected the data; C.C. Contributed data or analysis tools; P.P. and S.P. Performed the analysis; P.P. and MS wrote the original draft.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest

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