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Original article

Dose-dependent *in vivo* effect of *Rhodiola* and *Echinacea* on the mitogen-induced lymphocyte proliferation in mice

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Abstract

Echinacea purpurea (EP) and *Echinacea angustifolia* (EA) are ones of the most important world's herbs with immunotropic activity. They were traditional medicinal plants used by North American Indians for the treatment of various illnesses. Now they are cultivated in many countries and are used mainly to treat respiratory tract infections. *Rhodiola rosea* (RR) and *Rhodiola quadrifida* (RQ) are medicinal plants originated from Asia and used traditionally as adaptogens, antidepressants, and anti-inflammatory remedies. We previously reported, that extracts of underground parts of RR and RQ exhibited immunotropic activity. We have demonstrated in pigs that *in vitro* RR or RQ supplementation of blood lymphocyte cultures stimulated T cell proliferative response to Con A in lower, and inhibited it in higher *Rhodiola* extract concentrations. The aim of this work was to evaluate the *in vivo* effect of these herbal remedies on the *in vitro* proliferative response of mouse splenic lymphocytes to another T-cell mitogen- *Phaseolus vulgaris* haemagglutinin (PHA). We have found significant stimulation of proliferative response, in comparison to the controls, in mice fed lower doses of tested remedies, and inhibition, no effect or lower stimulation, in mice fed higher doses of these drugs.

Key words: *Echinacea purpurea*, *Echinacea angustifolia*, *Rhodiola rosea*, *Rhodiola quadrifida*, mice, lymphocytes, PHA

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Introduction

Native to the North American prairies, *Echinacea purpurea* and *Echinacea angustifolia* (*Asteraceae* or *Compositae*) may be used, both internally and externally, to treat respiratory infections, various skin infections, and to promote wound healing. *Echinacea* species has a long history of medicinal use for a wide variety of conditions. American Indians used *Echinacea* externally for wounds, burns and insect bites, for toothache and throat infections, and internally for pain, coughs, stomach cramps and snake bites. *Echinacea* was known as an "anti-infective" agent, and was used in bacterial, fungal and viral infections. Early white settlers adopted the therapeutic uses of *Echinacea* root, and it has been introduced as an herbal remedy in the United States. In 1762, it was used as a treatment for saddle sores on horses. Dr. H.C.F. Meyer learned of the uses of *Echinacea* from the native Indians of Nebraska around 1870, and later introduced it in Europe (Ernst 2002, Hostettmann 2003, Skopińska-Różewska et al. 2008b).

Echinacea purpurea and *Echinacea angustifolia* contain many compounds with immunomodulatory, antioxidant and anti-inflammatory activities. These are: alkylamides (mainly isobutylamides), caffeic acid glycosides, among them echinacoside characteristic to *E. angustifolia*, caffeic acid esters of quinic acid (chlorogenic acid and others), caftaric acid, cichoric acid (being a major component of *E. purpurea* roots and aerial parts), cynarin, polysaccharides, polyenes and volatile oils. (Sioley et al 2001, Skopińska-Różewska and Wojtasik 2002)

Enhancement of natural killer and antibody-dependent cell cytotoxicity, and enhancement of macrophage function (production of cytokines, phagocytosis) has been documented for various preparations of *Echinacea in vitro* and *in vivo*. (Schimmel and Werner 1981, Burger et al. 1997, See et al. 1997, Awang 1999, Clifford 2002, Goel et al. 2002). Various biological effects of *Echinacea purpurea* on animal and human lymphocytes were also described (Skopińska-Różewska et al. 2003, Bany et al. 2003, Mierzwińska-Nastalska et al. 2005, Rogala et al. 2008, Skopińska-Różewska et al. 2010). Dong et al. (2006) described immunosuppressive effect of cynarin through blocking the CD28 signaling pathway in T cells. Cynarin effectively blocked the binding between CD80 of antigen presenting cells and CD28 of T cells (Dong et al. 2009). It was also reported that *Echinacea* alkylamides modulate TNF-alpha gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways (Gertsch et al. 2004) and inhibit interleukin 2 secretion (Spelman et al. 2009). In another study (Joksic et al. 2009) performed in radiation workers who were identified as carrying dicentric chromosomes in their lymphocytes, administration of *Echinacea purpurea* tablets for 2 weeks resulted in significant lowering of the frequency of lymphocyte chromosome aberrations. It was also reported, that isolated from *Echinacea* cichoric acid inhibits human immunodeficiency virus type 1 (HIV 1) integrase (Robinson 1998, Reinke et al. 2004). *Echinacea* may have some importance in veterinary medicine. A double-blind, placebo-controlled study investigated the effects of *Echinacea angustifolia* extract on horses.

Horses treated with *Echinacea* presented increased phagocytosis of blood granulocytes, increased peripheral lymphocyte counts, stimulated neutrophil granulocytes migration from peripheral circulation into the tissues and increased hemoglobin level and erythrocyte number (O'Neill et al. 2002).

Committee for Veterinary Medicinal Products approved *Echinacea* use in veterinary homeopathy in all food-producing animal species (EMEA 2000).

Rhodiola rosea (RR) and *Rhodiola quadrifida* (RQ) are traditional natural drugs originated from Asia and used mainly as adaptogens, antidepressants and anti-inflammatory remedies (Blomkvist et al. 2009, Hung et al. 2010, Panossian et al. 2010). *Rhodiola rosea* root extract is a potent inhibitor of HIV-1 protease (Min et al. 1999). *In vitro* influenza anti-viral activity (neuraminidase inhibition) of flavonols isolated from *Rhodiola rosea* roots was also reported (Jeong et al. 2009). Other compounds from this group isolated from underground parts of *Rhodiola rosea* exhibited anti-bacterial (*Staphylococcus aureus*) and anti-tumor (prostate cancer cell line) activity (Ming et al. 2005). On the other hand, *R. rosea* preparations stimulate growth of the estrogen receptor- positive cell lines, and should be contraindicated to the patients with breast cancer (Kim et al. 2005). Chemical constituents of RR and RQ are comparable. The main group of chemical substances are phenolic glycosides (rosavin characteristic for *R. rosea*, mongroside isolated from *R. quadrifida* and salidroside characteristic for both species) flavonols and phenolic acids, mainly gallic and chlorogenic (Mielcarek et al. 2005, Ming et al. 2005, Wiedenfeld et al. 2007, Blomkvist et al. 2009 Jeong et al. 2009). Information about immunotropic activity of *Rhodiolas* is scarce. We previously reported that extracts of *R. rosea* and *R. quadrifida* influenced *in vitro* various parameters of specific and non-specific cellular immunity, among them, proliferative response of rat and porcine lymphocytes to T cell mitogen, Con A. *Rhodiola* extracts *in vitro* enhanced these responses in lower concentrations and were ineffective or suppressed them in higher ones. (Siwicki et al. 2007, Skopińska-Różewska et al. 2008a, Wójcik et al. 2008a,b, Skopińska-Różewska 2009). The aim of this work was to evaluate the *in vivo* effect of presented herbal remedies on the *in vitro* proliferative response of mouse splenic lymphocytes to T – cell mitogen PHA.

Materials and Methods

1. *Echinacea* containing remedies (commercial).
   Echinacea purpurea (Ratiopharm, batch 886018)
   Echinacea angustifolia (Triarco, batch XP2162)
2. *Rhodiola* extracts

Rhodiola rosea (Crassulaceae) roots and rhizomes were cultivated, collected and identified in the Research Institute of Medicinal Plants (RIMP), Poznań. The cultivation was established by vegetative propagation. Voucher specimen is kept in the herbarium of Department of Botany and Agronomy Medicinal Plants of Institute of Natural Fibres and Medicinal Plants in Plewiska near Poznań.

Rhodiola quadrifida (Crassullaceae) rhizomes were collected in Altai mountain in Mongolia, thanks to dr H. Wiedenfeld. The Mongolian plant material was identified; voucher specimen was deposited at the herbarium of the Institute of Botany of Mongolian Academy of Science in Ulaanbatar.

Preparation and chemical analysis of Rhodiola extracts.

Finely powdered roots and rhizomes were extracted with ethanol/water solution (1/1, v/v) in the ratio raw material/solvent 1/10 by the percolation method. Then the percolates were lyophylized which was preceded by distilling off the ethanol at the temperature 40-45°C. Dry extracts were stored under silica gel in the exsiccator at the room temperature.

HPLC analysis was performed (with the samples diluted with methanol) on Agilent 1100 HPLC system, equipped with photodiode array detector. For all separations a Lichrospher 100 RP18 column (250.0 × 4.0 mm, 5 µm) from Merck was used. The mobile phase consisted of 0.05% phosphoric acid in water (A) and acetonitrile (B), applied in the following gradient elution: from 95A/5B to 80A/20B for 30 min. then from 80A/20B to 20A/80B for 5 min and an isocratic elution for 15 min. Each run was followed by an equilibration period of 10 min. The flow rate was adjusted to 1 ml/min, the detection wavelength set to DAD at λ=205 nm, 220 nm, 254 nm, 330 nm and 20 µl of samples was injected. All separations were performed at a temperature of 25°C. Peaks were identified by spiking the samples with standard compounds and comparison of the UV-spectra and retention times.

Animals

The study was performed on 8-10- weeks old female inbred Balb/c mice, 20-22 g of body mass, delivered from the Polish Academy of Sciences breeding colony.

Herbal extracts were administered to the groups of 6 to 12 Balb/c mice *per os* in daily doses of 0.01, 0.02, 0.05, 0.15, and 0.3 mg (*Echinacea angustifolia*, EA); 0.04 and 0.2 mg (*Rhodiola rosea* (RR) and *Rhodiola quadrifida* (RQ); 0.005, 0.025, 0.25, and 2.5 mg (*Echinacea purpurea*, EP). Control groups consisted of 6-12 mice. Mice received drugs by Eppendorff pipette, in 40 µl of 10% ethyl alcohol (RR and RQ), in 40 µl of 15% ethyl alcohol(EP) or in 40 µl of distilled water (EA), for 7 days. Control mice were fed 40 µl of 10% ethyl alcohol for RR and RQ experiments, 40 µl of 15% ethyl alcohol for EP experiments, or 40 µl of distilled water for EA experiments. On the eight day mice were bled in anaesthesia from retro-orbital plexus and sacrificed with Morbital. Splenocytes were isolated under sterile conditions from mice by straining spleens through steel sieve and centrifugation on Histopaque 1077 (8 min 440 g) to remove erythrocytes.

For all experiments animals were handled according to the Polish law on the protection of animals and NIH (National Institutes of Health) standards. All experiments were accepted by the local Ethical Committee (nr 1/N/WDP-1/19.01.2006).

Mitogen-induced (PHA) splenocytes proliferation assay

Before establishing cultures, splenocytes from 2-3 mice were pooled. Spleen cells cultures (in multiple repetitions) were incubated in Costar 96 well microplates (10^5 cells in 0,2 ml RPMI-1640 medium, Biomed Lublin, with 2mM L-glutamine, 10% FCs and antibiotics) with or without mitogen PHA (Murex, G. B.) at a concentration of 0.5, 1 and 2 µg/ml, in a humidified atmosphere, at 37°C, with 5% CO_2. After 48 h of incubation 10 µl of tritiated thymidine (3HTdR, 0.2 mCi/ml, specific act. 2 Ci/mM) was added. After further 24 hours cells were harvested (Skatron) and incorporation of tritiated thymidine was measured using

Table 1. Analysis of hydro-alcoholic extracts of *Rhodiola* samples (values in [%]).

Extracts	Gallic acid	Rosarin	Rosavin	Rosin	Salidroside	Tyrosol	Chlorogenic acid	Tannins
R. rosea	0.30	0.17	0.27	0.14	0.73	0.12	0.11	8.37
R. quadrifida	1.37	–	–	–	2.39	2.04	0.30	16.21

β-scintillation counter (Rack Beta 1218, LKB Wallac). The arithmetical mean of quadruplicate count was calculated and expressed as counts per minute (CPM).

Statistical analysis

The results were verified statistically by two-way ANOVA analysis of variance, and the significance of differences between the groups was verified with a Bonferroni post-test (Graph Pad Prism software package)

Results

Figure 1 presents the influence of different doses of *Echinacea angustifolia* (EA), fed to mice for 7 days, on the response *in vitro* of their splenocytes to suboptimal (1 μg/ml) and optimal (2 μg/ml) PHA concentrations (total number of cultures 108). Each of the five doses of EA increased response of splenocytes to suboptimal dose of PHA, if compared to the control values. When splenocyte cultures were stimulated by optimal dose of PHA the statistically significant increase of proliferative response was observed after feeding mice with lower doses of EA (0.01, 0.02 and 0.05 mg) whereas the higher doses (0.15 and 0.3 mg) resulted in significant decrease of proliferative response to mitogen when compared to the control values. According to the statistical analysis (two-way ANOVA with Bonferroni post-test) the highest stimulatory effect ($p<0.001$) was observed under influence of the smallest administered dose of EA (0.01 mg) and the strongest inhibitory effect on proliferative response ($p<0.001$) resulted from administration of the highest dose of EA (0.3 mg).

Figure 2 presents the influence of feeding mice with hydro-alcoholic (A) extracts of *Rhodiola rosea* (RRA, 0.04 and 0.2 mg) or *Rhodiola quafrifida* (RQA 0.04 and 0.2 mg) on the *in vitro* response of their splenocytes to PHA 1 μg/ml and 2 μg/ml (total number of *in vitro* cultures 100). Both remedies in both administered doses increased the response of splenocytes up to the optimal (2 μg/ml) level of mitogen. Nevertheless, the lower doses of RRA and RQA (0.04 mg) most efficiently stimulated response to optimal dose

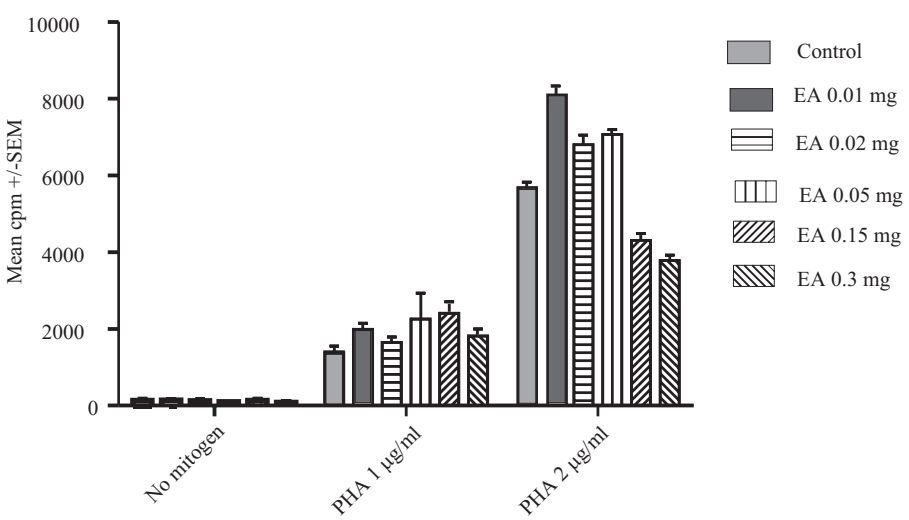

Fig. 1. The effect of feeding mice for seven days with *Echinacea angustifolia* (EA) hydro-alcoholic extract on splenocytes response to PHA

Table 2. Two-way ANOVA (experiments performed with *Echinacea angustifolia).*

Source of Variation	% of total variation	P value
Interaction	7.94	< 0.0001
Drug	3.90	< 0.0001
PHA concentration	85.02	< 0.0001
Source of Variation	**P value summary**	**Significant?**
Interaction	***	Yes
Drug	***	Yes
PHA concentration	***	Yes

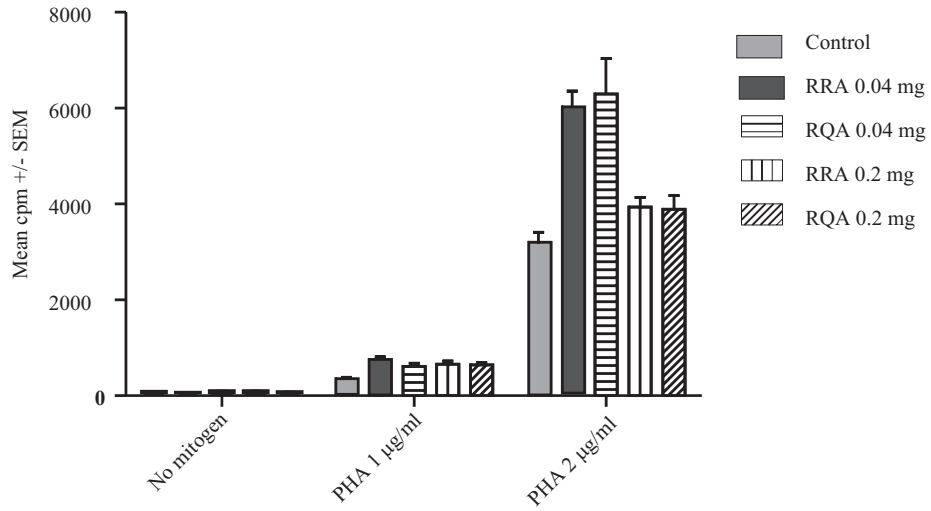

Fig. 2. The effect of feeding mice for seven days with *Rhodiola rosea* (RR) or *Rhodiola quadrifida* (RQ) hydro-alcoholic (A) extracts on splenocytes response to PHA.

Table 3. Statistical analysis of experiments performed with *Rhodiola*.

Two-way ANOVA

Source of Variation	% of total variation	P value
Interaction	6.39	< 0.0001
Drug	3.88	< 0.0001
PHA concentration	83.14	< 0.0001
Source of Variation	**P value summary**	**Significant?**
Interaction	***	Yes
Drug	***	Yes
PHA concentration	***	Yes

of 2 µg/ml PHA ($p<0.001$), whereas the higher doses of the plant remedies (0.2 mg) also increased the response to mitogen but to the lower extent – $p<0.05$ for RRA, $p<0.1$ (difference on the border of statistical significance) for RQA.

Figure 3 presents the average results of three consecutive and identical experiments (total number of *in vitro* cultures 584) performed with splenocytes collected from mice fed different doses of *Echinacea purpurea* (EP 0.005, 0.025, 0.25 and 2.5 mg). *Echinacea purpurea* appeared to be a stronger stimulator of splenocyte response to PHA than *Echinacea augustifolia, Rhodiola rosea* and *Rhodiola quadrifida* (see Fig 1 and 2). It has resulted in the lower dose of PHA (1 µg/ml) which appeared to be the optimal dose. For this reason three doses of PHA (0.5, 1 and 2 µg/ml) were used in this experiment. The dose 0.025 mg of EP significantly increased the response of splenocytes to 1 µg/ml ($p< 0.0001$) and to 2 µg/ml of PHA ($p<0.01$). *Echinacea* dose of 0.25 mg has stimulated the response in the presence of 0.5 and 1 µg/ml of PHA only ($p<0.05$ and $p<0.01$, respectively). In contrast to that, the highest dose of EP (2.5 mg) appeared to exert an inhibitory influence on the response of splenocytes to 1 and 2 µg/ml doses of PHA.

Discussion

One of the best models for studying the influence of various natural and synthetic substances on mammalian cell growth and proliferation, is the response of T lymphocytes to polyclonal activators – mitogenic agents, which stimulate DNA synthesis and cells division. There are two well known mitogens which stimulate T cells: concanavalin A (Con A) and phytohemagglutinin (PHA). The spectrum of T lymphocytes reacting to these two mitogens is partially overlapping, with a thymocytes and naive peripheral T cells vigorously responding to Con A, whereas subpopulation of mature T lymphocytes more actively respond to stimulation with PHA (plant lectin extracted from *Phaseolus vulgaris*). The response to PHA is also more resistant than response to Con A to several blocking agents including calcium (Ca^{2+}) blockers (Ba^{2+}), antibody to

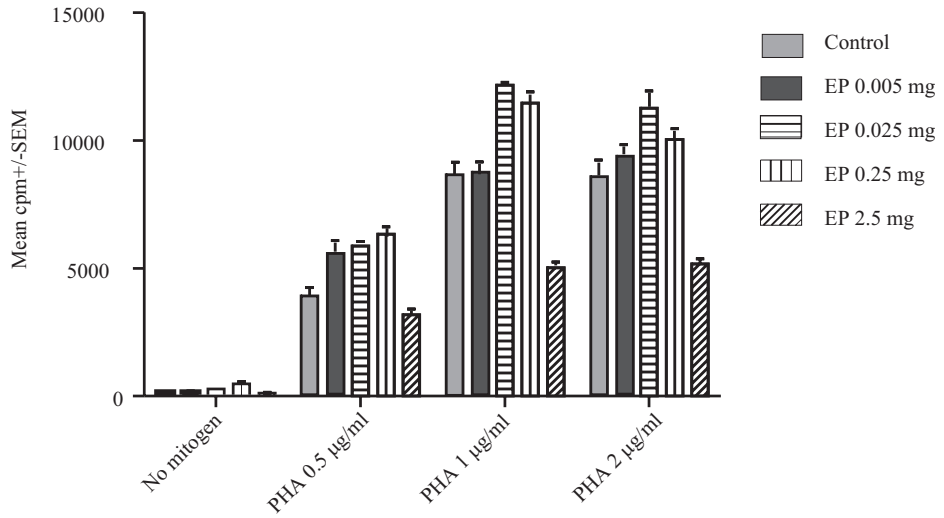

Fig. 3. The effect of feeding mice for seven days with *Echinacea purpurea* on the response of splenocytes to PHA.

Table 4. Statistical analysis of experiments performed with *Echinacea purpurea*

Two-way ANOVA

Source of Variation	% of total variation	P value
Interaction	3.17	0.0002
Treatment	6.82	< 0.0001
PHA concentration	43.36	< 0.0001
Source of Variation	**P value summary**	**Significant?**
Interaction	***	Yes
Treatment	***	Yes
PHA concentration	***	Yes

IL-2 and IL-4, cyclosporine A (CsA) and corticosteroids (Pecanha et al. 1989).

The PHA activates cells remaining in G^0 phase of cell cycle to initiate metabolic activity of G^1 phase following by DNA replication (S phase when ^{3}HTdR can be incorporated), short G^2 phase (preparation of cell to division) and final mitosis. On the way to direct the cell to active cell cycle under influence of PHA two monocyte products (monokines), namely interleukin-1 (IL-1) and antagonist of IL-1 receptor (IL-1ra), play important role. The former is a potent stimulator and the later strongly inhibits the T cell response to PHA (Dąbrowski et al. 2001). It is therefore obvious that functional state of monocytes determines the magnitude of lymphocyte response to PHA. It is also conceivable that some herbal remedies like *Echinacea*, *Rhodiola rosea* and *Rhodiola quadrifida*, possessing the dose -dependent immunostimulatory (lower doses) and anti-inflammatory (higher doses) properties, may affect the T cell response to PHA by pivotal influence on monocyte function stimulating the IL-1 production at lower doses or production of IL-1 receptor antagonist (IL-1ra) at higher doses. The supposition is worth further testing in separate experiments.

It was reported by Neveu et al. 1986, that T cell mitogenesis is modulated and controlled by cerebral neocortex. In mice, lesioning of the left fronto-parietal cortex resulted in depression of mitogenic response of lymphocytes to PHA by 50%. In contrast, in animals with right lesions, proliferative response was enhanced by 140% as compared with the controls and by more than 200% as compared to that observed in left lesioned animals (Neveu et al 1986). Recently, it was reported by Kovaru et al. (2010) that early event in PHA-induced lymphocyte activation (stimulation of Na+/K+ ATPase activity) is a more general response in different animal species and functionally different cells .

In vivo experiments, performed by us previously in mice, with plant extracts of *Eleutherococcus senticosus*, *Centella asiatica*, *Lithospermum canescens, Echinacea purpurea, Rhodiola rosea and Rhodiola quadrifida,* revealed stimulatory effect of lower doses and no effect or inhibitory influence of higher doses (in the case of *Rhodiola rosea* corresponding to the doses recommended by producers) on *ex vivo – in vitro* mobility in tissue culture of splenocytes collected from mice previously fed plant extracts.

In experiments with *Rhodiolas,* mice were fed for 7 days *Rhodiola* extract in daily doses of 0.04 or 0.2 mg. The chemokinetic activity of splenocytes was determined in 24-hour cell cultures in capillary tubes. Both

extracts stimulated splenocytes mobility in lower dose. In cell cultures established from spleens of mice fed higher dose (0.2 mg) of *Rhodiola rosea* extract no stimulation was observed. (Skopińska-Różewska et al. 2009). Similarly, in our previous experiments in mice, feeding cell donors with 0.4 mg daily doses of *R. rosea* extract resulted in suppression of splenic lymphocytes angiogenic activity, what may suggest the presence of suppressor cells or production of suppressory cytokines. Unfortunately, this dose corresponds to the doses recommended by producers of some dietary supplements which contain *Rhodiola rosea* (Siwicki et al. 2007).

In conclusion: we suppose, that the observed dose-dependent modulation of various aspects of immune cells activity (independently of underlying mechanisms) might represent a general phenomenon connected with immunotropic effects of some plant-derived compounds (probably polyphenols, but it would be elucidated in the course of further experiments). It should be stressed that the doses of plant extracts suppressive for mice lymphocytes activity after calculation taking into account differences in body area to body mass ratios between man and mouse, correspond to the doses recommended by producers of various herbal remedies. Evidently, the human doses, as well as the doses recommended to other animal species, should be lowered.

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