The influence of Rhodiola rosea extracts on non-specific and specific cellular immunity in pigs, rats and mice

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Abstract

The genus Rhodiola (Crassulaceae) consists of more than 100 species growing mainly in Asia and Europe. The best known is Rhodiola rosea. The roots of this species are traditionally used as a tonic, adaptogen, antidepressant and anti-inflammatory drugs. The aim of our work was to study in vitro and in vivo the effects of aqueous and 50% hydroalcoholic extracts of Rhodiola rosea on non-specific and specific cellular immunity in pigs, rats and mice. Mice were fed 50, 100, 200, or 400 μg of Rhodiola extracts daily, for 7 days before cellular immunity study (local GVH reaction). Blood leukocytes collected from pigs and rats were cultivated in vitro with PHA or LPS in the presence of 1-50 μg/ml of Rhodiola rosea extracts for 72 hours.

The metabolic activity of blood phagocytes (mostly granulocytes) was determined based on the measurement of intracellular respiratory burst after stimulation by PMA (phorbol myristate acetate), and potential bactericidal activity was determined in isolated blood leukocytes stimulated with microorganisms. Additionally, some in vitro toxicological studies were performed. For these experiments GMK (monkey’s kidney), EPC (fish epithelial cells), and KFC (Koy fins cells) cell lines, as well as lymphocytes and monocytes isolated from the blood of pigs and rats were used.

Both extracts enhanced non-specific and specific cellular immunity to the various degrees; however, in higher doses or concentrations they presented inhibitory effects. In in vitro studies all extracts were non-toxic at concentrations 50, 100, 200, 400, 800, and 1000 μg/ml after 24, 48, and 72 hours of cells cultures.

Key words: pigs, rats, mice, Rhodiola rosea, cellular immunity.

(Centr Eur J Immunol 2007; 32 (2): 84-91)

Introduction

The genus Rhodiola (Crassulaceae) consists of more than 100 species growing mainly in Asia and Europe. The best known is Rhodiola rosea L. This arctic-alpine plant grows in Russia, Mongolia, North China, Korea, Japan, Sakhalin, Kuriles, North America, Greenland and Europe [1]. Rhodiola rosea grows very rarely in Poland mountains [2]. To the less known, but used in traditional medicine in China and Russia belong species: Rhodiola Kirilovii and Rhodiola quadrifida. The roots of these species are traditionally used as a tonic, adaptogen, antidepressant and anti-inflammatory drugs. The rhizomes and roots of the plants contain...
phenylpropanoides: rosavin, rosin, rosarin and their aglycone: cinnamyl alcohol (specific to *Rhodiola rosea*); phenylethanol derivatives: salidroside, tyrosol; flavonoids (catechins and proanthocyanidins), monoterpenes, triterpenes, phenolic acids (gallic, caffeic and chlorogenic acid), volatile oil, aminoaacids and minerals. Most of these compounds posses antioxidant activity [3-5].

The adaptogenic properties and cardiopulmonary protective effects of *Rhodiola rosea* are connected with its ability to influence levels and activity of biogenic monoamines in brain tissue, and also may be attributed to induction of opioid peptide biosynthesis and activation of opioid receptors [3]. *Rhodiola rosea* extracts are antimutagens due to ability to raise the efficiency of the intracellular DNA repair mechanisms. Mitochondrial DNA (mtDNA) represents a critical target for oxidative damage. Once damaged, mtDNA can amplify oxidative stress by decreased expression of critical proteins important for electron transport leading to a vicious cycle of reactive oxygen species (ROS) and organellar dysregulation that eventually trigger apoptosis. The oxidative damage to mtDNA can culminate in cell death and thus represents an important target for therapeutic intervention in a number of human diseases [6]. Extracts of *Rhodiola* have been shown to posses marked cytoprotective and antioxidant activities against tert-butyl hydroperoxide induced injury in U-937 human macrophages [7].

*Rhodiola rosea* root extract was the most potent inhibitor of HIV-1 protease (70.4% inhibition at a concentration of 100 μg/ml) among 92 other Korean medicinal plant extracts. The isolated *Rhodiola rosea* compounds: rosin, rhodiocyanoside A, rosavin, rosin, salidroside, p-tyrosol and lotaustralin alone were not responsible for the inhibitory effect in the studied system [8].

In Poland, the cultivation of *Rhodiola rosea* was begun since 1980 year in the Research Institute of Medicinal Plants in Poznań and is there continued, since 1986, the *in vitro* cultivation of *R. rosea* is also maintained as *in vitro* culture of organs, callus tissue and suspension cultures of this species, in Warsaw Medical University. Informations about immunotrophic activity of *Rhodiola* are very scarce. We previously reported that 50% alcoholic extracts of *R. rosea* influence some parameters of non-specific cellular immunity [9, 10]. Recently, Kormosh et al. showed, that new plant preparation, AdMax, containing *Leuzea carthamoides*, *Rhodiola rosea*, *Eleutherococcus senticosus* and *Schizandra chinensis* increased the mean numbers of various T-cells subclasses and immunoglobulin levels in cancer patients during 4 weeks therapy. Moreover, in any of the patients the side effects of chemotherapy with cisplatin and cyclophosphamide such as fatigue and depressive mood were observed [11]. The aim of the present work was to compare aqueous and 50% hydroalcoholic extracts of this plant on the experimental models of *in vitro* and *in vivo* cell-mediated immunity in pigs, rats and mice.

**Material and methods**

**Cultivation of *Rhodiola rosea* L.**

The cultivation was established by vegetative propagation. The seedlings of *Rhodiola rosea* L. originated from many years’ cultivation of the Research Institute of Medicinal Plants (RIMP) in Poznań. Taxonomic status of plants was confirmed on the base of Flora of China (Vol. 8, 2001). Voucher specimen is kept in the herbarium of Department of Botany, Breeding and Agronomy of RIMP in Plewiska near Poznań.

Field cultivation was established in the fade soil made from boulder clay of ground moraine. Clayey sand fills the upper layer. The thickness of humus was 30 cm. Soil reaction (pH) was about 7. The content of mineral components in the arable layer was: NH4 – 3.5; NO3 – 7.0; P – 102.8; K – 43.2; Ca – 1574; Mg – 117; Cl – 9.3; SO4 – 45.7; Na – 10.0 mg/l.

In autumn 2004 the roots of *Rhodiola rosea* were collected for phytochemical and pharmacological analysis. The raw material was washed, cut into thick slices and dried in natural conditions. The average crop of aerial dry raw material from 1 m² was 150 g. It gives the conversion crop about 1.5 t of dry raw material from 1 ha (spacing 40 x 40 cm). On average, 70% of roots withered.

**Preparation and chemical analysis of *Rhodiola rosea* extracts**

Sample extractions were prepared by the methods as described below. Aqueous extracts: finely powdered roots were extracted two times with water (extraction was performed: first – 2 hour and second – 1 hour long) in the ratio raw material/solvent 1/5, in the temperature 40-45°C. The supernatants were mixed together and after centrifugation at 3000 rpm for 15 min were lyophylized. Hydro-alcoholic extracts: finely powdered roots 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 the distilling off the ethanol in the temperature 40-45°C. Drug/extraction ratio (DER) values of extracts are shown in table 1. Dry extracts were stored under silica gel in the exsiccator in the room temperature.

All the samples were diluted in methanol. HPLC analysis was performed on Agilent 1100 HPLC system, equipped with photodiode array detector. For all separation a Lichrospher 100 RP18 column (250.0 x 4.0 mm, 5 μm) from Merck was used. The mobile phase consisted of 0.05% phosphoric acid in water (A) and acetonitrile (B),

<p>| Table 1. DER values of extracts |</p>
<table>
<thead>
<tr>
<th>Raw material</th>
<th>Solvent</th>
<th>DER</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodiola rosea</em> – aqueous extract</td>
<td>water</td>
<td>3.72/1</td>
</tr>
<tr>
<td><em>Rhodiola rosea</em> – hydroalcoholic extract</td>
<td>50% ethanol</td>
<td>2.78/1</td>
</tr>
</tbody>
</table>
applied in the following gradient elution: from 95A/5B in 30 min to 80A/20B in 5 min to 20A/80B and an isocratic elution in 15 min to the end. Each run was followed by an equilibration period for 10 min. The flow rate was adjusted to 1 ml/min, the detection wavelength set to DAD at \( \lambda = 205 \) nm, 220 nm, 254 nm, 330 nm and 20 \( \mu l \) of samples was injected. All separations were performed at a temperature of 25°C. Peaks were assigned by spiking the samples with standard compounds and comparison of the UV-spectra and retention times.

**Animals**

Blood for immunological experiments was collected from the vena cava cranialis of PWZ piglets, 4-5 month old, 40-50 kg body mass, females, and from retroorbitalis plexus of Lewis rats, 8 weeks old, 180-200 g of body mass, females. Spleen cells were obtained from inbred Balb/c mice, females, 8-10 weeks old, 20-22 g of body mass, and transplanted to F1 hybrids Balb/c x DBA2, females, 8 weeks old, 22-25 g of body mass. Experiments were approved by Local Ethical Committee.

**In vitro studies**

Leucocytes were isolated from the blood by centrifugation at 2000 x g for 30 min at 4°C on the Gradisol L or G gradient (Aqua-Medica, Poland), washed three times in PBS and resuspended in RPMI 1640 medium (Sigma) supplemented with 10% of PCS (Foetal Calf Serum, Gibco-BRL) at a stock concentration of 2x10^5 cells/ml of medium. Viability of cells was checked by supravital staining with 0.1% w/v trypan blue [12]. For RBA and PKA tests cells were preincubated for 2 hours at 37°C, in the presence of *Rhodiola rosea* extracts in concentrations 1, 5 and 10 \( \mu g \) per ml (rats), or 1, 5, 10, 20 and 50 \( \mu g \) per ml (pigs). In the proliferative response tests *Rhodiola* extracts were present in culture medium during whole cultivation period (72 hours).

**RBA test.** The metabolic activity of blood phagocyting cells (mostly granulocytes) was determined based on the measurement of intracellular respiratory burst after stimulation by PMA (phorbol myristate acetate, Sigma), as described by Chung and Secombes [12] and adapted for dogs by Siwicki et al. [13]. The isolated cells were resuspended in RPMI-1640 medium (Sigma) at 10^6 cells/ml. On 96-well U-shaped microplates 100 \( \mu l \) of isolated blood leukocytes were mixed with 100 \( \mu l \) of 0.2% nitro blue tetrazolium (NBT, Sigma) solution in 0.2 M phosphate buffer at pH 7.2 and added 1 \( \mu l \) of PMA at concentration 1 mg/ml in ethanol. After 30 min of incubation at 37°C, the supernatant was removed from each well. The cells pellet was washed with absolute ethanol and than three times in 70% ethanol and dried at room temperature. The amount of extracted reduced NBT was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value served as the result.

**PKA test.** Potential bactericidal activity of phagocyting cells was determined in isolated blood leukocytes stimulated with killed microorganisms, according to the method presented by Rook et al. [14]. On 96-well U-shaped microplates 100 \( \mu l \) of leucocytes were mixed with 100 \( \mu l \) of 0.2% NBT in phosphate buffer at pH 7.2 and added 10 \( \mu l \) of killed *Staphylococcus aureus* strain 209P (containing 10^9 bacteria). The mixture was incubated 1 h at 37°C and the supernatant was removed. The cell pellet was washed with absolute ethanol and three times in 70% ethanol and dried at room temperature. The amount of extracted reduced NBT was measured at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value served as the result.

**The proliferative response of splenic and blood lymphocytes**

Proliferative response of lymphocytes stimulated by mitogen concanavaline A (ConA) or lipopolysaccharide (LPS) were determined by MTT assay [15, 16]. MTT [3-(4,5-Dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide]
The influence of Rhodiola rosea extracts on non-specific and specific cellular immunity in pigs, rats and mice

Rhodiola rosea (Sigma) was dissolved in PBS at concentration of 5 mg/ml and filtered. On 96-well culture plates (Costar, USA) 100 μl of blood lymphocytes in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% hepes buffer, penicillin/streptomycin (100 U/100 μg/ml) were mixed with 100 μl of RPMI 1640 containing mitogens ConA (5 μcg/ml), PHA (10 μcg/ml) or LPS (20 μcg/ml). 3 cultures from each pool of leukocytes were established. After 72 h incubation at 37°C with 5% carbon dioxide atmosphere (Assab Incubator, Sweden), 50 μl of MTT solution were added into each well and plates were incubated for 4 h at 37°C. After incubation the plates were centrifuged (1400 g, 15°C, 5 min). Supernatants were removed and 100 μl of DMSO (Sigma) were added into each well and incubated for 15 min at room temperature. After incubation the solubilized reduced MTT was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). The results from three cultures were pooled. The mean values and standard errors from pooled experiments were used for comparison between the groups by Student’s t-test.

Cytotoxicity assays. Additionally, some in vitro toxicological studies were performed. For these experiments GMK (monkey's kidney), EPC (fish epithelial cells), and KFC (Koy fins cells) cell lines, as well as lymphocytes and monocytes isolated from the blood of pigs and rats were used. Cells GMK, EPC and KFC were cultivated firstly in culture medium (EMEM, Sigma + 10% FCS), supplemented with Rhodiola rosea extracts in concentrations 50, 100, 200, 400, 800, 1000 and 1200 μg/ml, for 72 hours, in optimal temperatures (GMK 37°C, EPC 30°C, KFC 24°C) in 24-well tissue culture plates (Nunclon). The cultures were observed daily in reverse microscope, and after 72 hours stained with trypan blue and number of dead cells were evaluated.

Lymphocytes were cultivated for 48 hours in culture medium(RPMI-1640 + 10% FCS) supplemented with Rhodiola rosea extracts in concentrations 50, 100, 200, 400, 800, 1000 and 1 μg/ml of culture medium. Higher concentration of the extract was ineffective (20 μg/ml) or stimulatory in 5 and 10 μg/ml extract concentrations, again suggested by the authors of the method, at magnification of 6 x, in 1/3 central area of microscopic field. Statistical analysis of the results was performed by Mann-Whitney and Student’s t-test.

Results

In cytotoxicity assays both extracts were non-toxic at concentrations 50-1000 μg/ml.

The results of RBA and PKA tests performed after in vitro culture of leukocytes collected from the blood of pigs, in the presence of various amounts of Rhodiola rosea aqueous extract are presented on the table 4. We observed significant stimulation of RBA and PKA activity of cells in the presence of 5 and 10 μg/ml of culture medium. Higher concentration of the extract was ineffective (20 μg/ml) or inhibitory (50 μg/ml).

Similar situation was observed in leukocyte cultures supplemented with Rhodiola rosea hydroalcoholic extract (table 5). However, in these experiments stimulation of leukocyte activities was more pronounced, and visible in the lowest concentration (1 μg/ml) also.

The results of similar experiments (RBA and PKA tests) performed on leukocytes isolated from the blood of rats are presented on the table 6 (aqueous extract) and table 7 (50% alcoholic extract). In these experiments extracts were present in culture medium in concentrations 1, 5 and 10 μg/ml only. Both water and alcoholic extracts were stimulatory in 5 and 10 μg/ml extract concentrations, again the stimulation was better in cultures containing 50% alcoholic extract than the aqueous one.

<table>
<thead>
<tr>
<th>Extract concentration (μg/ml)</th>
<th>RBA (OD 620 nm) Mean ±SD</th>
<th>PKA (OD 620 nm) Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.35±0.03</td>
<td>0.27±0.04</td>
</tr>
<tr>
<td>1</td>
<td>0.39±0.04</td>
<td>0.30±0.05</td>
</tr>
<tr>
<td>5</td>
<td>0.58±0.05*</td>
<td>0.47±0.03*</td>
</tr>
<tr>
<td>10</td>
<td>0.64±0.04*</td>
<td>0.58±0.05*</td>
</tr>
<tr>
<td>20</td>
<td>0.31±0.05</td>
<td>0.29±0.03</td>
</tr>
<tr>
<td>50</td>
<td>0.15±0.05* ↓</td>
<td>0.20±0.05* ↓</td>
</tr>
</tbody>
</table>

* p<0.05
The results of experiments in tissue cultures with mitogens presented stimulation of lymphocytes T and B proliferative activity in lower, and inhibition in the highest Rhodiola rosea extracts concentrations (tables 8-11). Again, the stimulation was more pronounced in cultures containing 50% alcoholic extract, than the aqueous one.

Contrary to the results obtained from in vitro studies, in in vivo experiments performed in mice we observed better stimulation of the local graft-versus-host response (lymphocyte-induced angiogenesis, LIA) when donors of spleen lymphocytes are fed the aqueous R. rosea extract, than when they are fed the hydroalcoholic one. Optimal dose was 50 μg, what corresponds to 25 mg human dose. Dose 400 μg (200 mg for human) was inhibitory (figures 1 and 2).

Discussion

Our experiments showed immunomodulatory effect exerted by Rhodiola rosea extracts in vitro and in vivo. Both types of extracts stimulated non-specific and specific cellular immunity in lower doses, and were inhibitory in the highest dose applied. However, we have not observed cytotoxic effects of this highest dose, what may suggest stimulation of non-specific suppressor cells in cultures containing 50 μg/ml of the extract. In experiments in mice, feeding cell donors 400 μg daily doses of extracts resulted in suppression of splenic lymphocytes angiogenic activity, what may suggest the presence of non-specific suppressor cells in grafted cell suspension. This dose corresponds to 200 mg human dose, unfortunately being recommended by producers of such dietary supplements as FORMA-REGENERIX-ANTYSTRES (Biofarm, Poznań) and LENTAYA (Hermes Arzneimittel GmbH). So, in our opinion, use of these dietary supplements in recommended doses may be dangerous for non-specific suppressor cells in cultures containing 50 μg/ml of the extract.
The influence of Rhodiola rosea extracts on non-specific and specific cellular immunity in pigs, rats and mice

Table 10. The in vitro effect of Rhodiola rosea aqueous extract on lymphocyte proliferative response in rats. *p<0.05

<table>
<thead>
<tr>
<th>Extract concentration (μg/ml)</th>
<th>Con A (OD 620 nm) Mean ±SD</th>
<th>LPS (OD 620 nm) Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.34±0.03</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>1</td>
<td>0.44±0.05*</td>
<td>0.29±0.05</td>
</tr>
<tr>
<td>5</td>
<td>0.58±0.04*</td>
<td>0.45±0.04*</td>
</tr>
<tr>
<td>10</td>
<td>0.55±0.03*</td>
<td>0.47±0.05*</td>
</tr>
</tbody>
</table>

Table 11. The in vitro effect of Rhodiola rosea hydroalcoholic extract on lymphocyte proliferative response in rats. *p<0.05

<table>
<thead>
<tr>
<th>Extract concentration (μg/ml)</th>
<th>Con A (OD 620 nm) Mean ±SD</th>
<th>LPS (OD 620 nm) Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.32±0.04</td>
<td>0.28±0.05</td>
</tr>
<tr>
<td>1</td>
<td>0.53±0.05*</td>
<td>0.36±0.06*</td>
</tr>
<tr>
<td>5</td>
<td>0.58±0.04*</td>
<td>0.50±0.03*</td>
</tr>
<tr>
<td>10</td>
<td>0.59±0.05*</td>
<td>0.48±0.04*</td>
</tr>
</tbody>
</table>

human immune system, leading to the suppression of some specific and, possibly, some non-specific cellular immune responses. However, in daily doses of 25 and 50 mg R. rosea extracts may mildly stimulate cellular immunity. Some recent studies provide evidence of the efficacy of R. rosea extracts after a single administration to mice, in 200 μg dose, to exert antidepressant-like and anxiolytic-like activities [19].

It is possible, that the fact of better in vitro stimulatory activity of our hydroalcoholic extract than the aqueous one, is connected with higher content of salidroside, rosavin and chlorogenic acid. These compounds are also active in other test systems. Salidroside has effect on antineuronal apoptosis [20], exerted also protective effects on hydrogen peroxide-induced apoptosis in human neuroblastoma cells [21] and produced significant sedative-hypnotic effect [22]. In high concentration (250 μg/ml) aqueous extract of Rhodiola imbricate rhizome was found to stimulate the production of proinflammatory mediators in human blood mononuclear cells and mouse macrophage cell line RAW 264 [23]. Rhodiola improved angiogenesis by regulating the expression of receptors for VEGF and angiopoietin [24].

Since 1975, the R. rosea extract (40% EtOH) was produced in a large-scale and was registered in the Pharmacopoeia of Soviet Union (1989) with the name “Rhodiola Extract liquid” as a medicine and a tonic. The Extract is standardized up-to-date on salidroside and rosavin. In Sweden, R. rosea was recognized as an Herbal Medicinal Product “Arctic Root” in 1985 and has been described as an antifatigue agent and saled by Swedish Herbal Institute. In Germany, a dietary supplement of R. rosea “Lentaya” (Hermes) is in the sale in drugstores and via Internet (among many other international R. rosea number of newly-formed blood vessels.

Fig. 1. The effect of feeding mice hydroalcoholic extract of Rhodiola rosea on the activity of their lymphocytes in local GVH reaction.
products) and in Poland – a product Antistress-Forma. Many, if not most, cancer patients will try such remedies in the course of their disease, often without the knowledge of their surgeon or oncologist. 

*R. rosea* has a very low level of toxicity. However, should be taken early in the day because it can interfere with sleep or cause vivid dreams. Because *R. rosea* has an activating antidepressant effect, it should not be used in individuals with bipolar disorder who are vulnerable to becoming manic when given antidepressants or stimulants.

Recently, risks of the complementary use of *Rhodiola rosea* preparations with antiestrogens Tamoxifen and Faslodex in breast cancer *in vitro* studies were noted. *R. rosea* may be effective and potent growth stimulator of the estrogen receptor-positive cell lines [25]. Consumption of *Rhodiola* could, therefore, pose a significant hazard to the patients with breast cancer who have ER-positive tumors and who are being treated with antiestrogens.

**Fig. 2.** The effect of feeding mice aqueous extract of *Rhodiola rosea* on the activity of their lymphocytes in local GVH reaction

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**References**

The influence of *Rhodiola rosea* extracts on non-specific and specific cellular immunity in pigs, rats and mice


