The *in vivo* effect of *Rhodiola quadrifida* extracts on the antibody production, on the blood leukocytes subpopulations and on the bacterial infection in mice

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Abstract

The effect of feeding mice Rhodiola quadrifida aqueous (RQW) and 50% hydro-alcoholic (RQA) extracts on Pseudomonas aeruginosa infection was studied. It was found that the infection intensity was significantly lower, in comparison to the control, when mice obtained daily, for 7 days before P. aeruginosa and Pseudomonas inoculation, 0.4 mg of RQA extract. In contrast, no difference was observed between the number of bacteria in livers of mice fed RQW extract and mice belonging to the control group. Both lymphocytes and granulocytes numbers in the blood collected from mice fed RQW extract were significantly lower than in the respective controls. Mice fed higher RQA dose presented more lymphocytes in their blood than their corresponding controls, and no differences in granulocytes level. There were no effect of both extracts on anti-sheep red blood cells (SRBC) antibody production in lower 0.04 dose, in higher dose (0.2 mg) aqueous (RQW) extract diminished anti-SRBC antibody response.

Key words: *Rhodiola quadrifida, mice, bacterial infection, blood leukocytes.*

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Introduction

The genus *Rhodiola* (*Crassulaceae*) consists of many species. These plants originate from arctic regions of Asia, Europe and North America, and are used in traditional medicine of many countries, mainly as adaptogens, anti-depressants, and anti-inflammatory drugs. The most known is *Rhodiola rosea.* Extracts of *Rhodiola rosea* are present on the European market as dietary supplements. Our investigations on the immunotropic effects of extracts obtained from three *Rhodiola* species – *rosea*, *quadrifida* and *kirilowii*, began more than fifteen years ago [1, 2]. Since this time, we published the results of many *in vitro* and *in vivo* experimental studies on the immunotropic and anti-angiogenic effects of extracts prepared from underground parts of *Rhodiola rosea*, *Rhodiola quadrifida* and *Rhodiola kirilowii*. We presented for the first time stimulatory effect of extracts prepared from these three *Rhodiola* species on some parameters of specific and non-specific cellular immunity in mice, rats and pigs [3-13]. We also reported anti-angiogenic activity of these extracts on tumor-induced cutaneous angiogenesis [14-17]. The aim of the present study was to investigate the influence of feeding mice for 7 days *R. quadrifida* aqueous (RQW) or 50% hydro-alcoholic (RQA) extracts on the subsequently induced bacterial

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infection (*Pseudomonas aeruginosa*) in mice, on the leukocyte blood subpopulations in their blood and on the anti-sheep red blood cells (SRBC) antibody production. *Pseudomonas aeruginosa* is one of the most dangerous pathogens for patients during cancer therapy, subjected to immunosuppressive treatment, or people immunocompromised from the other reasons.

Material and methods

Preparation and analysis of extracts

Rhizomes of *R. quadrifida* 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 Ulaanbaatar. Sample extractions were performed as described before [3]. Briefly, aqueous extracts: finely powdered roots were extracted with water two times in the temperature 40-45°C. The supernatants were combined and after centrifugation at 3000 rpm for 15 min, were lyophilized. Hydro alcoholic extracts: finely powdered roots were extracted with ethanol/water solution (1/1, V/V) by the percolation method. The percolates were lyophilized which was preceded by the distilling off the ethanol in the temperature 40-45°C. Extracts were dissolved in 10% ethyl alcohol before administration to the animals.

High-performance liquid chromatography (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 mm × 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.

Animal

Studies were performed on B6C3F1 hybrid mice, males, at the age of 7-9 weeks, 22-27 g of body mass, delivered from own breeding colony (leukocytes and bacterial infection study) and on 7-9 weeks old female Balb/c mice, 20-25 g of body mass, delivered from the Polish Academy of Sciences breeding colony (antibody study).

Study of antibody production

Mice were fed *Rhodiola* extract or 10% alcohol (controls) for 7 days, before intraperitoneal injection of 0.2 ml 10% SRBC suspension. Animals received daily 40 or 200 µg of the extract (feeding with use of Eppendorf pipette). These doses corresponded to about 20 and 100 mg given to 70 kg person (applying the coefficient equal 7 for adjusting differences between mouse and human in relation of the surface to body mass). Mice received drugs by Eppendorf pipette, in 0.04 ml of 10% ethyl alcohol. Control mice were fed 0.04 ml of 10% ethyl alcohol. Each experimental or control group consisted of ten animals. Mice were bled in anesthesia from retro-orbital plexus 7 days after immunization.

The antibody level was evaluated with haemagglutination assay in heat inactivated (56°C, 30 min) sera. After performing a series of sera dilutions, 0.5% SRBC were added and the mixture was incubated for 60 min at room temperature, then centrifuged (10', 150 g) and shaken. The hemagglutination titer was evaluated in a light microscope – as the last dilution in which at least 3 cell conglomerates were present in at least 3 consecutive fields at objective magnification 20×.

Leukocyte subpopulations

Rhodiola quadrifida extracts were administered to the groups of 8 mice each, *per os*, in daily doses of 50 or 400 µg. These doses corresponded to about 25 or 200 mg given to 70 kg person. Mice were bled in anesthesia from retro-orbital plexus 7 days after immunization and sacrificed with Morbital. Counting of leukocytes and blood smears examination were performed by routine methods. The results are presented as inhibitory or stimulatory indices, calculated by dividing number of cells (lymphocytes or granulocytes) in 1 ml of blood of each experimental animal, by mean number of cells/ml of blood of animals belonging to the corresponding control groups.

Bacterial infection

Mice were fed *R. quadrifida* extracts 400 µg daily, in 10% ethyl alcohol, or 0.04 ml of 10% alcohol as a control, for 7 days. On the day eighth mice were infected intraperitoneally (*i.p.*) with *Pseudomonas aeruginosa* strain ATCC (27853). Four hours after administration of 0.1 ml of bac-

Table 1. Analysis of extracts of *Rhodiola quadrifida* (values in [%])

Extracts	Gallic acid	Salidroside	Tyrosol	Chlorogenic acid	Tannins
Aqueous	3.22	1.15	1.00	0.23	5.66
50% hydro-alcoholic	1.37	2.39	2.04	0.30	16.21

teria suspension (3×10^7 CFU) the mice were anaesthetized with barbiturates and killed by spinal dislocation after which the livers were isolated. The livers were homogenized and the number of viable bacteria were estimated by plating after 24 hours growth on Cetrymide agar (Merck) [8].

All experiments were approved by Local Ethical Committee.

Statistical evaluation of the results

The results were verified statistically (GraphPad Prism software package) by two-way ANOVA and Bonferroni post-test (leukocytes and antibody production) and by one-way ANOVA and Dunnett's multiple comparison test (bacterial infection).

Results

The results of the estimation of blood leukocytes number are presented on the Fig. 1. Both lymphocytes and granulocytes numbers in the blood collected from mice fed *R. quadrifida* water extract were significantly lower than in the respective controls. In the case of *R. quadrifida* hydro-alcoholic extract, however, no such differences were observed. Moreover, mice fed higher RQA dose presented more lymphocytes in their blood than their corresponding controls.

The results of the experiments with bacterial infection are presented on the Fig. 2. RQA extract significantly diminished number of bacteria in livers of infected mice. Mice fed aqueous RQW extract presented no differences from mice belonging to the control group.

There were no effect of both extracts on anti-SRBC antibody production in lower 0.04 mg dose, in higher dose (0.2 mg) aqueous (RQW) extract diminished anti-SRBC antibody response ($p < 0.01$).

Discussion

In the previous studies, performed *in vitro*, we observed enhancement of intracellular respiratory burst and potential bactericidal activity in rat blood leukocyte cultures in the presence of *R. rosea*, *R. kirilowii* and *R. quadrifida* extracts [3, 5, 9, 10]. We also reported inhibitory effect of *R. rosea* extracts on bacterial infection in mice [8]. In the present paper we confirmed this last effect for the hydro-alcoholic extract obtained from *R. quadrifida*. Aqueous extract from *R. quadrifida*, however, have not presented inhibitory activity. The reason for this difference is not clear. One might hypothesize that it is connected with different pattern of leukocytosis in mice pretreated with these two types of *R. quadrifida* extracts. In mice fed hydro-alcoholic extract number of blood leukocytes significantly increased, in mice fed aqueous extract number of blood leukocytes significantly decreased (both lymphocytes and granulocytes). Accordingly, antibody production was diminished in mice fed 0.2 mg of aqueous extract. We have not found reports on the influence of *R. quadrifida* on humoral immunity. In our previous study on the influence of *R. rosea* roots on the

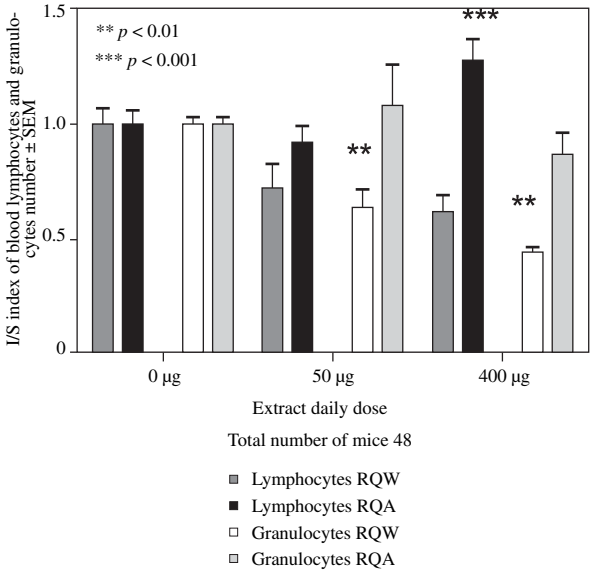
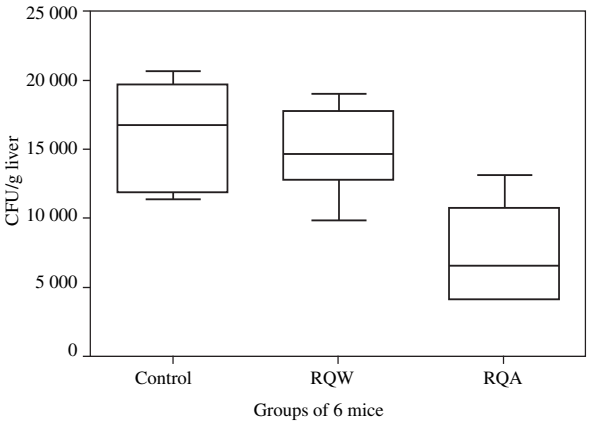


Fig. 1. The effect of feeding mice *Rhodiola quadrifida* water (RQW) or hydro-alcoholic (RQA) extracts for 7 days on lymphocyte and granulocyte number in their blood. Results are presented as inhibition/stimulation indices in respect to the control



Dunnett's Multiple Comparison Test	Mean difference	q	Significant? P < 0.05?	Summary
Control vs. RQW	1234	0.5982	No	NS
Control vs. RQA	8710	4.222	Yes	**

Fig. 2. Number of bacteria in livers of infected mice fed *Rhodiola quadrifida* water (RQW) or 50% alcoholic (RQA) extracts (mean ± SD and range of results)

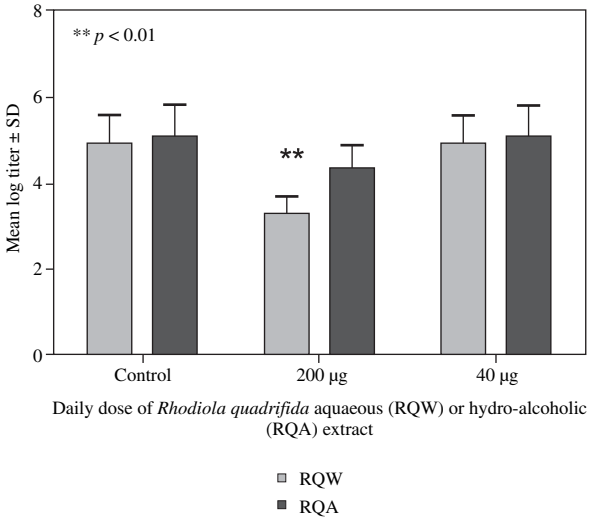


Fig. 3. The effect of feeding mice *Rhodiola quadrifida* water (RQW) or hydro-alcoholic (RQA) extracts for 7 days on antibody production in mice

antibody production we obtained stimulation of this parameter of humoral immunity [1]. Also Guan *et al.* and Mishra *et al.* reported adjuvant effects of extracts and compounds isolated from *R. rosea* and *Rhodiola imbricata* [18, 19]. It remains to elucidate, whether the lack of anti-bacterial effect presented by water extract of *R. quadrifida* might be connected with the presence of factors impairing lympho- and granulopoiesis, or factors toxic for mature leukocytes. Previous *in vitro* study performed on rats and pigs blood leukocytes did not reveal cytotoxic effects up to the 1000 µg/ml concentration. However, concentrations higher than 10 µg/ml diminished respiratory burst and potential killing activity, but without difference between both types of extracts [4, 11]. In other *in vivo* study, we observed again stimulatory effect of *R. quadrifida* hydro-alcoholic extract on cellular immunity in mice (graft-versus-host reaction) and no effect of *R. quadrifida* aqueous extract on this parameter [5]. Mielcarek *et al.* [20] performed phytochemical investigation of *Rhodiola* extracts (prepared from underground parts of *R. rosea* and *R. quadrifida*) using high performance thin layer chromatography (HPTLC), HPLC and spectrophotometric methods. Substantial differences were found in the qualitative and quantitative composition of the extracts. Generally, hydro-alcoholic extracts contained more glycosides and phenolic acids than aqueous extracts, with one exception – the highest concentration of gallic acid was observed in aqueous extract of *R. quadrifida.*

There are some papers about direct *in vitro* anti-viral and anti-bacterial activity of *Rhodiola* extracts and gallic acid [21-24]. We could not exclude such possibility, but we rather think that in our present *in vivo* study, antibacterial effect of *Rhodiola* hydro-alcoholic extract was indirect, mediated by activated leukocytes migrating to the peritoneal cavity from the blood. Lack of effect of aqueous extract may be connected with high content of gallic acid and its negative influence on the level and activity of white blood cells (mononuclears and granulocytes). It was reported, that gallic acid inhibits cell viability and induces apoptosis in human monocytic cell line U937 [25], and induces neuronal cell death through activation of c-Jun *N*-terminal kinase and downregulation of Bcl-2 [26]. Gallic acid derivative, propyl gallate, inhibits the growth of endothelial cells via caspase-independent apoptosis [27]. It was also reported that gallic acid inhibits the growth of HeLa cervical cancer cells via apoptosis and/or necrosis [28]. Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation [29].

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