

The effect of *Rhodiola kirilowii* extracts on pigs' blood leukocytes metabolic (RBA) and proliferative (LPS) activity, and on the bacterial infection and blood leukocytes number in mice

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Abstract

Rhodiola kirilowii roots and rhizomes extracts are used in Asiatic medicine independently of their adaptogenic properties also as antimicrobial and anti-inflammatory drugs. The aim of this work was to study the *in vitro* modulatory potential of aqueous and hydro-alcoholic extracts of under-ground parts of *Rhodiola kirilowii* (RK) on respiratory burst activity (RBA) and on the proliferative response to lipopolysaccharide (LPS) in blood leukocyte cultures of pigs. Both extracts in concentration up to 10 µg/ml stimulated this parameter. Higher concentrations of RK extracts were ineffective (20 µg/ml) or inhibitory (50 µg/ml).

Further, the effect of feeding mice with *Rhodiola kirilowii* aqueous (RKW) and 50% hydro-alcoholic (RKA) 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* i.p. inoculation, 0.4 mg of RKA or RKW extract. Accordingly, feeding mice with both extracts for 7 days, in 0.1 and 0.2 mg (RKW) and 0.1 mg (RKA) daily doses, increased blood leukocytes (both lymphocytes and granulocytes) number.

Key words: *Rhodiola kirilowii*, mice, pigs, leukocytes, RBA, LPS, bacterial infection.

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Introduction

As a part of our continuing searching for non-toxic natural immunostimulatory remedies, we screened a large panel of immunotropic activities of aqueous and hydro-alcoholic extracts of *Rhodiola rosea* (RR) and *Rhodiola quadrifida* (RQ). After performing experiments on mice, rats and pigs, we obtained evidence of their *in vivo* and

in vitro immunomodulatory activity [1-10]. *Rhodiola* extracts have stimulated both non-specific and specific cellular immunity. *In vivo*, they increased number of blood leukocytes and their metabolic activity, improved the motility of splenic lymphocytes, and lowered the intensity of bacterial infection. *In vitro*, RR and RQ extracts potentiated respiratory burst (RBA) and potential killing (PKA) activity of blood leukocytes. As well, as specific immunity was con-

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cerned, RR and RQ extracts have stimulated *in vitro* and *in vivo* mitogen-induced lymphocyte proliferation and local graft-versus-host reaction (GVH) performed against semi-allogeneic and xenogeneic histocompatibility barrier. The anti-oxidative influence of RR extracts and their immunological action via CD4+ T cells were established [1, 2].

We also performed some experiments with *Rhodiola kirilowii* (RK) extracts [9, 11-13] confirming the results obtained *in vitro* for RR and RQ species. *In vivo*, RKW and RKA extracts enhanced the ability of lymphocytes derived from parental strain mice (fed with these extracts) to induce local GVH reaction in F1 hybrids and have stimulated metabolic activity of mice blood granulocytes (chemiluminescence induced by Zymosan) collected from mice fed with RKW or RKA for 7 days [11-13]. The aim of this study was to evaluate: firstly, the *in vitro* effect of RKW and RKA extracts on respiratory burst activity (RBA) and LPS-induced mitogenic response of leukocytes isolated from the blood of pigs; and, secondly, the *in vivo* effect of these extracts on the intensity of bacterial infection and the number of blood leukocytes in mice fed with RKW or RKA extract for 7 days.

Material and methods

Preparation and chemical analysis of extracts

Rhodiola kirilowii (*Crassulaceae*) roots and rhizomes were cultivated, collected and identified in the Research Institute of Medicinal Plants (RIMP), presently Institute of Natural Fibres and Medicinal Plants, Poznań. The cultivation was established by vegetative propagation. The plant growth was controlled: the reaction and mineral components of the soil, the air temperature, the average sum of humidity, and rain as well as the sun periods were monitored permanently. Voucher specimen is kept in the herbarium of Department of Botany, Breeding and Agriculture in Plewiska near Poznań.

Sample extractions were prepared by the methods as described below. Aqueous extract: 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 lyophilized. Hydro-alcoholic extract: 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 lyophilized which was preceded by the distilling off the ethanol in the temperature 40-45°C. DER values of extracts were: 5.09/1 for RKW and 3.27/1 for RKA. Extracts were stored at -70°C until used.

The content of phenolic compounds was determined by means of two methods: ultra performance liquid chromatography (UPLC) and high-performance liquid chromatography (HPLC).

Ultra performance liquid chromatography

An ultra performance liquid chromatograph connected to a tandem mass spectrometer (UPLC-MS/MS; Waters) was used for chemical analyses. The separation of analytes was performed on an Acquity UPLC BEH C18 column, 1.7 µm 2.1 × 50 mm (Waters). Mobile phase: phase A: 0.1% (v/v) HCOOH solution in water, phase B: 0.1% (v/v) HCOOH solution in acetonitrile. Mobile phase flow rate was: 0.20 ml/min. The assay was performed in gradient elution: 0.0 min – 97% of phase A, 7.5 min – 68% of phase A, 9.0 min – 97% of phase A. Column temperature was 25°C; ion source temperature: 120°C; desolvation temperature: 350°C. Gas flow rate: desolvation gas: 700 l/h; cone gas: 10 l/h. Peaks were identified by spiking the samples with standard compounds.

High-performance liquid chromatography

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 × 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.

Spectrophotometric analysis of tannins was performed according to the methods of European Pharmacopoeia on UV-Visible Spectrometer Cintra 20 GBC 9. (Determination of tannins in herbal drugs. European Pharmacopoeia, 6 ed. 2008; 1: 255).

Animals

Mice

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.

Pigs

Blood for *in vitro* immunological experiments was collected from the vena cava cranialis of four PWZ piglets, 4-5 month old, 40-50 kg body mass, females.

Leukocyte subpopulations

RK extracts were administered to the groups of 8 mice each, *per os*, in daily doses of 50, 100, 200 or 400 µg. These

doses corresponded to about 25, 50, 100 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 a mean number of leukocytes in 1 mm^3 of blood \pm standard error of the mean (SEM).

Bacterial infection

Mice were fed RK extracts 400 μg daily, in 10 % ethyl alcohol, or 0.04 ml of 10% alcohol as a control, for 7 days. On the day 8-ght mice were infected intraperitoneally (i.p.) with *Pseudomonas aeruginosa* strain ATCC (27853). Four hours after administration of 0.1 ml of bacteria 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].

In vitro studies

Leukocytes were isolated from the blood by centrifugation at 2000xg 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 FCS(Gibco), at a stock concentration of 2×10^6 cells/ml of medium. For RBA test, cells were preincubated for 2 hours at 37°C in the presence of RK extracts in concentrations 1, 5, 10, 20 and 50 μg per ml.

Respiratory burst activity test

The metabolic activity of blood phagocytic cells was determined based on the measurement of intracellular respiratory burst activity (RBA) after stimulation with PMA (Phorbol Myristate Acetate, Sigma [3]). The isolated cells were resuspended in RPMI-1640 medium (Sigma) at 10^6 cells/ml. On 96-well U-shaped microplates, 100 μl of the isolated blood leukocytes was mixed with 100 μl of a 0.2% nitro blue tetrazolium (NBT, Sigma) solution in 0.2 M phosphate buffer at pH 7.2, and 1 μl of PMA at a concentration of 1 mg/ml in ethanol was added. After 30 min of incubation at 37°C, the supernatant was removed from each well. The cell pellet was washed with absolute ethanol and, three times, with 70% ethanol and it was dried at room temperature. The amount of extracted reduced NBT after incubation with 2 M KOH and DMSO (dimethylsulfoxide, Sigma) was measured colorimetrically at 620 nm in a microplate reader (Tecan Sunrise). All samples were tested in triplicate, and the results are presented as mean values \pm SD.

Proliferative response to lipopolysaccharide

Proliferative response of pigs blood leukocytes after stimulation with mitogen lipopolysaccharide (LPS), was

determined by MTT spectrophotometry (OD 570 nm) using (3-[4,5- dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide – 3-[4,5-dimethyl-2-thiazol]-2,5-diphenyl-2H-tetrazolium bromide), as described previously [3].

MTT (Sigma) was dissolved in PBS at a concentration of 5 mg/ml and filtered. On 96-well culture plates (Sarstedt, 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) was mixed with 100 μl of RPMI 1640 containing lipopolysaccharide (LPS, 20 μg /ml). After 72 h incubation at 37°C in a 5% carbon dioxide atmosphere (Memmert Incubator), 50 μl of MTT solution was added into each well, and plates were incubated for 4 h at 37°C. After incubation the plates were centrifuged (1,400 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 microplate reader (Tecan Sunrise). All samples were tested in triplicate, and the results are presented as mean values. *Rhodiola* extracts were present in culture medium during whole cultivation period (72 hours) in concentrations 1, 10, and 50 μg /ml.

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).

Statistical evaluation of the results

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

Results and discussion

In experiments with pigs leukocytes in cell culture (Fig.1 and Fig. 2), both extracts in concentration 5 and 10 μg /ml stimulated their metabolic (respiratory burst) and in concentration 10 μg /ml proliferative (LPS) activity ($p < 0.001$). In the presence of 1 μg /ml only RKA extract was effective ($p < 0.05$ for RBA and $p < 0.01$ for LPS). High concentrations of RK extracts were ineffective (20 μg /ml) or inhibitory (50 μg /ml). LPS, commonly used as B-cell mitogen, can stimulate also other cells taking part in innate immunity (monocytes, macrophages, dendritic cells) by Toll-like receptors [14]. The Toll-like receptors recognize a few highly conservative structures existing in prevailing microorganisms [15]. Previous *in vitro* study performed on rats and pigs blood leukocytes did not revealed cytotoxic effects of RK extracts up to the 1000 μg /ml con-

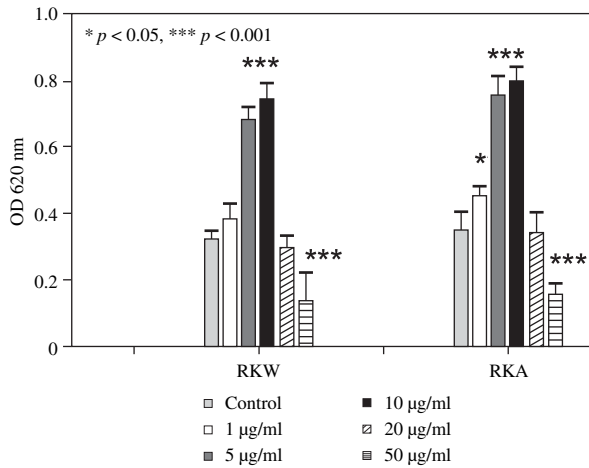


Fig. 1. The *in vitro* effect of *Rhodiola kirilowii* water (RKW) and hydro-alcoholic (RKA) extract on 4 pigs blood leukocytes RBA activity (mean ± SD)

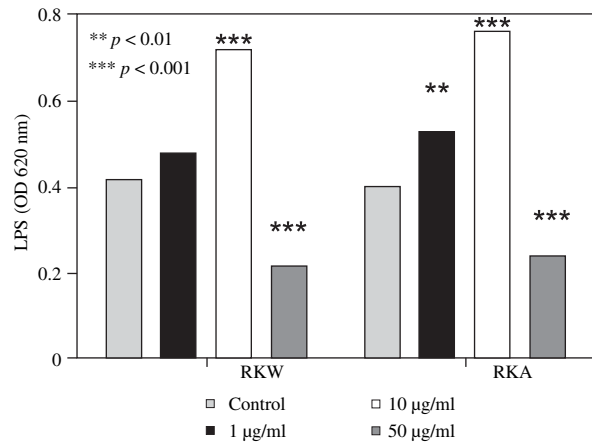
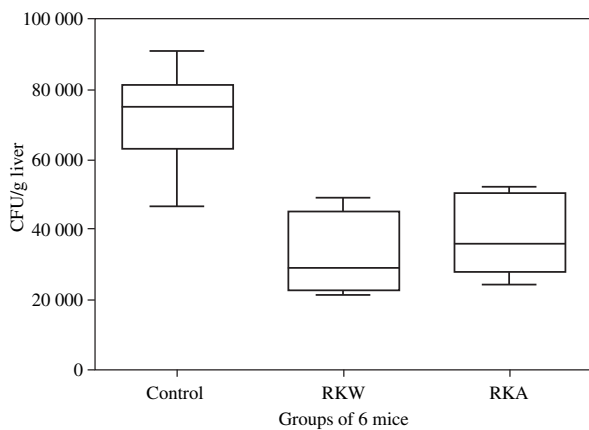


Fig. 2. The *in vitro* effect of *Rhodiola kirilowii* (RKW) and hydro-alcoholic (RKA) extract on pigs blood leukocytes response to LPS (mean OD)

Table 1. Chemical analysis of aqueous and hydro-alcoholic extracts of *Rhodiola kirilowii* (values in [%])

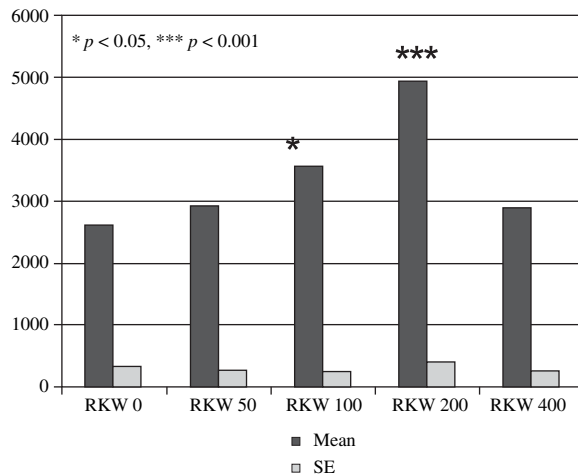
Extract	Compound	Epica- techin	Epigalloca- techin	Epica- techin gallate	Epigalloca- techin gallate	Tyrosol	Salidroside	Tannins
Aqueous (RKW)		0.00086	0.031	0.0051	0.311	0.023	0.00013	2.90
50% hydro-alcoholic (RKA)		0.0019	0.109	0.0042	0.273	0.019	0.00005	7.47



Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs. RKW	39430	5.390	Yes	***
Control vs. RKA	34430	4.706	Yes	***

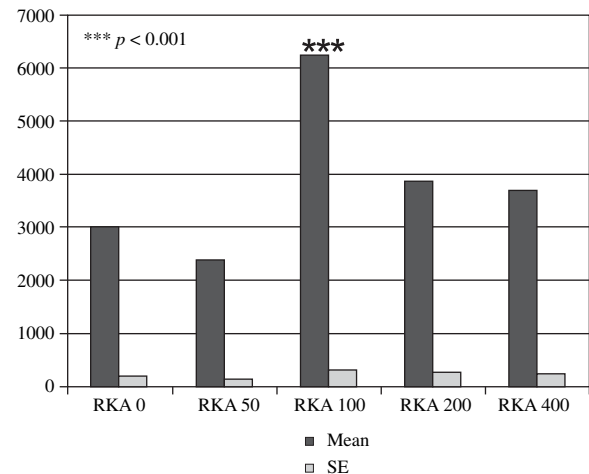
Fig. 3. Number of bacteria in livers of infected mice fed *Rhodiola kirilowii* (mean ± SD and range of results)

centration. Our present results are in agreement with our previously performed studies, where similar modulatory effect on leukocytes RBA, PKA and proliferative activity was observed after leukocytes incubation in extracts of other *Rhodiola* species – *Rhodiola rosea* and *Rhodiola quadri-fida* [3-5]. In these previous experiments RBA and PKA stimulatory activity of hydro-alcoholic extracts of RR and RQ constantly was more pronounced than the activity of corresponding water extracts. One would expect such modulatory effect, because *Rhodiola* extracts contain various phenolic compounds, some of them with antioxidant activity [2, 16, 17]. Anti-oxidant activity of epigallocatechin gallate (EGCG), compound abundant in green tea, but also present in RK extracts (see Table 1) is well documented (reviewed in [13]). *In vivo* experiments performed in mice showed beneficial effect of feeding mice with *Rhodiola kirilowii* aqueous (RKW) or 50% hydro-alcoholic (RKA) extracts on *Pseudomonas aeruginosa* infection (Fig. 3). It was found that the infection intensity was highly significantly lower, in comparison to the control, when mice obtained daily, for 7 days before *P. aeruginosa* i.p. inoculation, 0.4 mg of RKA or RKW extract ($p < 0.001$). Accordingly, both extracts significantly increased blood leukocytes (both lymphocytes and granulocytes) number in mice, at 0.1 and 0.2 mg (RKW) and 0.1 mg (RKA) daily dose



Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs. RKW (200 µg)	39430	5.390	Yes	***

Fig. 4a. The effect of feeding mice for 7 days with various RKW extract doses (µg)



Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs. RKA (100 µg)	34430	4.706	Yes	***

Fig. 4b. The effect of feeding mice for 7 days with various daily doses of RKA extract (µg) on the number of leukocytes in their blood

(Fig. 4a and 4b). Again, in 0.1 mg dose stimulatory effect was more pronounced in mice fed with RKA than in mice fed with RKW extract (0.001 vs.0.05), but disappeared in mice fed with 0.2 mg RKA extract daily doses.

There are some papers about direct *in vitro* anti-viral and anti-bacterial activity of *Rhodiola* extracts and their compounds [18-21]. However, we rather think that in our present *in vivo* study, antibacterial effect of RK extracts was indirect, mediated by activated leukocytes migrating to the peritoneal cavity .

From the results presented in this study, as well as from all our previous reports on immunotropic activity of *Rhodiola*s, one may conclude, that extracts of plants belonging to various *Rhodiola* species (*R. rosea*, *R. quadrifida*, *R. kirilowii*) may represent valuable source of substances with immunostimulatory potential. However, one should remember, that many herbal remedies (among them extracts of *Rhodiola*), being stimulatory in lower doses, in higher doses presented inhibitory effects, affecting proliferation, metabolic activity and mobility of lymphocytes. So, establishing of proper dose is extremely important [22].

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