The *in vivo* effect of *Rhodiola kirilowii* extracts on blood granulocytes metabolic activity in mice

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Abstract

Rhodiola kirilowii roots and rhizomes are traditionally used in China as a tonic, adaptogen, antimicrobial and anti-inflammatory drug. The plant is also used in the Chinese medicine for the enhancement of the ability of anti-anoxia, moreover it shows anticoagulative properties and decreases the level of blood sugar. R. kirilowii also protect people against cardiopulmonary function problems when moving to high altitude (4500 m).

The aim of this work was to study the in vivo effect of aqueous and 50% hydro-alcoholic extracts of R. kirilowii roots on metabolic activity of blood granulocytes in mice. Hydro-alcoholic extract of R. kirilowii exerted stimulatory effect in the lowest and in the highest dose applied. No difference from the control was observed in the group fed intermediate doses. In the case of aqueous extract intermediate doses presented inhibition of granulocytes chemiluminescence.

Key words: *mice, Rhodiola kirilowii, granulocytes, chemiluminescence.*

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Introduction

Roots and rhizomes of plants belonging to various *Rhodiola* species are traditional natural drugs used in Asia as adaptogens and antidepressants. *Rhodiola kirilowii* is a plant used in the Chinese traditional medicine for the enhancement of the ability of anti-anoxia, and for its decreasing blood sugar and anticoagulative properties. Extracts of this plant has anti- inflammatory, bacterio- and fungo-static properties. Their *in vitro* activity against chronic hepatitis C virus and against *Mycobacterium tuberculosis* was also reported [1-3].

Information about immunotropic activity of *Rhodiola* species is scarce. We previously reported for the first time that extracts of *R. rosea*, *R. quadrifida* and *R. kirilowii* influenced some parameters of specific and non-specific cellular immunity and angiogenesis in mice, rats and pigs [4-12].

The aim of this work was to study the *in vivo* effect of aqueous (RKW) and 50% hydro-alcoholic (RKA) extracts of *R. kirilowii* roots on the metabolic activity of blood granulocytes in mice.

Material and methods

Plant

Rhodiola kirilowii (Reg.) Reg. (*Crassulaceae*) roots were collected in September and identified in the Research Institute of Medicinal Plants (RIMP), Libelta 27,

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61-707 Poznań. 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.

Preparation of extracts

Sample extractions and their chemical analysis were performed by the scientists from RIMP (P.M. Mrozikiewicz, A. Mścisz, A. Krajewska-Patan, S. Mielcarek, W. Buchwald) and M. Zych from Warsaw Medical University, as described before [6, 13]. Aqueous extract (RKW): 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 (RKA): 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. Dry extracts were stored under silica gel in the exsiccator at the room temperature.

Chemical analysis of extracts

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´4.0 mm, 5 mm) 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 in 30 min to 80A/20B then from 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 l = 205 nm, 220 nm, 254 nm, 330 nm and 20 ml 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.

Content of tannins and gallic acid in *R. kirilowii* extracts was compared to the content of these substances in extracts obtained previously from the roots of two other *Rhodiola* species – *R. rosea* and *R. quadrifida.* As presented in Table 1, content of tannins in hydro-alcoholic extract of R.kirilowii roots was substantially higher than in the aqueous extract. However, the situation with gallic acid was reverse- aqueous extracts contained three times more of this substance than alcoholic ones. It concerned extracts obtained from *R. kirilowii*, *R. rosea* and *R. quadrifida*. As presented in Table 2, other compounds identified in extracts from *R. rosea* and *R. quadrifida* were not found in *R. kirilowii* extracts.

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

Rhodiola kirilowii extracts were administered to the groups of 8 Balb/c mice each, *per os*, in daily doses of 0.05, 0.1, 0.2 or 0.4 mg. These doses corresponded to 25, 50, 100 or 200 mg given to 70 kg person (applying the coefficient equal 7 for adjusting differences between mouse and human

Table 1. Content of tannins and gallic acid in *Rhodiola* sp. extracts

Tannins [%]			
Extract	***R. rosea***	***R. kirilowii***	***R. quadrifida***
Hydro-alcoholic	8.37	7.47	16.21
Aqueous	3.27	2.90	5.66
Gallic acid [%]			
Extract	***R. rosea***	***R. kirilowii***	***R. quadrifida***
Hydro-alcoholic	0.30	0.93	1.37
Aqueous	1.03	3.06	3.22

Table 2. Analysis of different extracts of *Rhodiola* samples (values in [%])

Extracts	Rosarin	Rosavin	Rosin	Salidroside	Tyrosol	Chlorogenic acid
R. rosea	0.23* (0.17)	0.003* (0.277)	0.16* (0.14)	0.46* (0.73)	0.07* (0.12)	0.07* (0.11)
R. kirilowii	–	–	–	–	–	–
R. quadrifida	–	–	–	1.15* (2.39)	1.00* (2.04)	0.23* (0.30)

** aqueous extract, ( ) hydro-alcoholic extract*

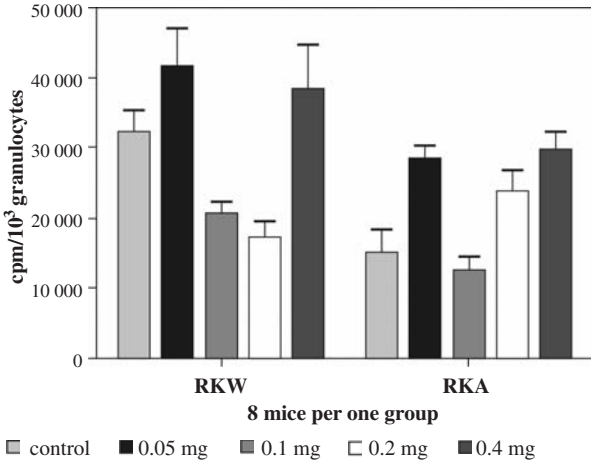

Fig. 1. The effect of feeding mice *Rhodiola kirilowii* extracts for 7 days on blood granulocytes chemiluminescence (mean ± SEM)

in relation of the surface to body mass). Mice received drugs by Eppendorff pipette, in 40 µl of 10% ethyl alcohol, for 7 days. Controls mice were fed 40 µl of 10% ethyl alcohol. On the day 8th the mice were bled in anaesthesia from retro-orbital plexus and sacrificed with Morbital.

Chemiluminescence test (CL)

Chemiluminescence test was measured using the method of Easmon and Cole with some modifications [14, 15] at room temperature, in scintillation counter (Rack-Beta 1218, LKB, Sweden). Briefly: samples of 0.05 ml heparinised blood were diluted 1 : 4 with PBS (Biomed Lublin, Poland) supplemented with 0.1% BSA (Sigma-Aldrich, USA) and 0.1% glucose (Polfa, Poland). Next, 0.05 ml of this diluted blood was mixed with 0.2 ml of luminol (Sigma-Aldrich, USA) solution (10-5 M) in PBS and placed in a scintillation counter in the "out of coincidence" mode for background chemiluminescence measurement. Then, the cells were activated by addition of 0.02 ml solution of opsonised zymosan (10 mg/ml) and chemiluminescence activity was measured for the next 15 min. Counting of leukocytes and blood smears examination were performed by routine methods and the results were shown as the maximum value of chemiluminescence (cpm) obtained for 10^3 granulocytes.

Statistical analysis

The results were verified statistically by a one-way ANOVA analysis of variance (GraphPad Prism software package), and the significance of differences between the groups was verified with a Tukey-Kramer Multiple Comparisons Test.

Results

One-way ANOVA. Performed analysis of variance revealed, that variation among columns means is significantly greater than expected by chance. The *p*-value is < 0.0001, considered extremely significant.

The results are presented graphically on Figure 1.

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Tukey-Kramer Multiple Comparisons test:

RKA Control vs. RKA 0.05 p < 0.05,

"– " RKA 0.4, $p < 0.01$,

0.05 vs. 0.1, $p < 0.01$,

0.1 vs. 0.2, $p < 0.05$,

0.1 vs. 0.4, $p < 0.001$.

Hydro-alcoholic extract of *R. kirilowii* exerted stimulatory effect in the lowest and in the highest dose applied. No difference from the control was observed in the group fed intermediate doses.

Water (aqueous) extract (RKW).

The *p*-value is 0.0006, considered extremely significant. Variation among column means is significantly greater than expected by chance:

RKW 0.05 vs. RKW 0.1, $p < 0.05$,

"– " vs. RKW 0.2, $p < 0.05$,

RKW 0.1 vs. RKW 0.4, $p < 0.05$,

"–" 0.2 vs. RKW 0.4, $p < 0.01$.

Aqueous extract (RKW) inhibited granulocyte metabolic activity in intermediate doses. In the lowest and the highest dose no statistically significant effect was observed.

Discussion

In this paper we present for the first time *in vivo* modulatory effect of *Rhodiola kirilowii* extracts on mice granulocytes luminol-dependent chemiluminescence, measured in scintillation counter. This test is widely accepted as a method of measuring granulocytes metabolic activity and their oxygen-dependent killing potential.

Previously, we have observed dose-dependent stimulation of chemiluminescent granulocytes activity in mice fed for seven days extracts from another *Rhodiola* species – *Rhodiola quadrifida* [10]. Unexpectedly, in the present study we observed different pattern of response in the case of *Rhodiola kirilowii* extracts. The lowest and the highest dose was stimulatory (in the case of RKA) or without effect (in the case of RKW). Intermediate doses were inhibitory (in mice fed RKW) or without effect (in mice fed RKA). These differences may be connected with different chemical composition of extracts obtained from various *Rhodiola* species. The main group of chemical substances present in *Rhodiola* extracts are phenolic glycosides (rosavin characteristic for *R. rosea*, mongroside characteristic for *quadrifida* and salidroside characteristic for both species).

The following compounds were isolated by Wiedenfeld [16] from root extracts of RK: rhodiocyanoside A, arbutin, epigallocatechin gallate, fructopyrano-(1-4)-glucopyranose and lotaustralin. Lotaustralin, salidroside, daucosterol and tyrosol were already described by other authors.

Wong *et al.*[1] separated and purified twelve compounds: β-sitosterol, tyrosol, trans-hydroxycinnamic acid, geranyl β-glucopyranoside, neryl β-glucopyranoside, hexyl β-glucopyranoside, gallic acid, epigallocatechin-gallate, rhodiolgin, isolariciresinol-9-0-β-glucopyranoside, rhodiooctanoside and sacranoside B. Gallic acid and epigallocatechin gallate exhibited *in vitro* inhibitory and bactericidal activities against *Mycobacterium tuberculosis*.

Immunomodulatory activity of RK extracts may be partly connected with their epigallocatechin-gallate content. The effect of (–)-epigallocatechin 3-gallate (EGCG), a major polyphenol of green tea, on neutrophil migration has been studied by Takano *et al.* They reported, that EGCG inhibited rat neutrophil chemotaxis toward cytokine-induced neutrophil chemoattractant-1 (CINC-1) in a concentration-dependent manner. Oral administration of EGCG (1.0 mg or 1.5 mg/rat) at 1 h before the challenge with FITC-OVA suppressed neutrophil infiltration into the air pouch (inflammatory site) in the air-pouch type FITC-OVA-induced allergic inflammation in rats. Chemokine levels in the pouch fluids, however, were not influenced by EGCG administration. The results suggest that EGCG suppressed neutrophil infiltration by a direct action on neutrophils [17].

Other authors [18] investigate the ability of the major tea polyphenols: (–)-epigallocatechin gallate (EGCG), theaflavins (TF) and gallic acid (GA) to protect *in vitro* human neutrophils from oxidative damage induced by phorbol myristate acetate (PMA), and they estimated the level of reactive oxygen species (ROS) production in patients depending on the red tea Pu-Erh drinking. A decrease in ROS generation after red tea consumption was accompanied by the decrease of ROS in response to tested compounds in normal cells. EGCG and TF showed similar potency in antioxidative activities.

The potential health benefits ascribed to green tea and EGCG include antioxidant effects, cancer chemoprevention, improving cardiovascular health, enhancing weight loss, protecting the skin from the damage caused by ionizing radiation, and others were reported [19].

It was shown that (–)-epigallocatechin-3-gallate (EGCG), strongly inhibits neutrophil elastase, micromolar EGCG represses reactive oxygen species activity and inhibits apoptosis of activated neutrophils, and dramatically inhibits chemokine-induced neutrophil chemotaxis *in vitro*; moreover both oral EGCG and green tea extract block neutrophil-mediated angiogenesis *in vivo* in an inflammatory angiogenesis model [20].

Inhibitory and stimulatory effects observed by us in the present study may depend on the interplay between immunostimulatory and antioxidant factors present in RK extracts.

References

1. Wong YC, Zhao M, Zong YY et al. (2008): Chemical constituents and anti-tuberculosis activity of root of Rhodiola kirilowii. Zhongguo Zhong Yao Za Zhi 33: 1561-1565.
2. Zhang ZH, Feng SH, Hu GD et al. (1989): Effect of Rhodiola kirilowii (Regel) Maxim on preventing high altitude reactions. Zhongguo Zhong Yao Za Zhi 14: 687-690.
3. Zuo G, Li Z, Chen L, Xu X (2007): Activity of compounds from Chinese herbal medicine Rhodiola kirilowii (Regel) Maxim against HCV NS3 serine protease. Antiviral Res 76: 86-92.
4. Furmanowa M, Skopińska-Różewska E, Rogala E, Hartwich M (1988): Rhodiola rosea in vitro culture-phytochemical analysis and anti-oxidant action. Acta Societatis Botanicorum Poloniae 6: 69-73.
5. Skopińska-Różewska E, Furmanowa M, Siwicki AK et al. (2005): The influence of different Rhodiola extracts on cellular imunity in mice and pigs. Herba Polonica 51 (Suppl1): 170-171.
6. Siwicki AK, Skopińska-Różewska E, Hartwich M et al. (2007): The influence of Rhodiola rosea extracts on non-specific and specific cellular immunity in pigs, rats and mice. Centr Eur J Immunol 32: 84-91.
7. Skopińska-Różewska E, Wójcik R, Siwicki AK et al. (2008): The effect of Rhodiola quadrifida extracts on cellular immunity in mice and rats. Pol J Vet Sci 11: 105-111.
8. Skopińska-Różewska M, Malinowski M, Wasiutyński A et al. (2008): The influence of Rhodiola quadrifida 50% hydroalcoholic extract and salidroside on tumor-induced angiogenesis in mice. Pol J Vet Sci 11: 97-104.
9. Skopińska-Różewska E, Hartwich M, Siwicki AK et al. (2008): The influence of Rhodiola rosea extracts and rosavin on cutaneous angiogenesis induced in mice after grafting of syngeneic tumor cells. Centr Eur J Immunol 33: 102-107.
10. Skopińska-Różewska E, Bychawska M, Sommer E, Siwicki AK (2008): The in vivo effect of Rhodiola quadrifida extracts on the metabolic activity of blood granulocytes in mice. Cent Eur J Immunol 33: 179-181.
11. Wójcik R, Siwicki AK, Skopińska-Różewska E et al. (2009): The in vitro effect of Rhodiola quadrifida and Rhodiola kirilowii extracts on pigs blood lymphocyte response to mitogen ConA. Centr Eur J Immunol 34: 166-170.
12. Wójcik R, Siwicki AK, Skopińska-Różewska E et al. (2009): The effect of Chinese medicinal herb Rhodiola kirilowii extracts on cellular immunity in mice and rats. Pol J Vet Sci 12: 399-405.
13. Mielcarek S, Mścisz A, Buchwald W et al. (2005): Phytochemical investigation of Rhodiola sp. root extracts. Herba Polonica 51(suppl 1): 159-160.
14. Easmon CSF, Cole PJ, Williams AJ, Hastings M (1980): The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence. Immunology 41: 67-74.
15. Skopińska-Różewska E, Krotkiewski M, Sommer E et al. (1999): Inhibitory effect of shark liver oil on cutaneous angiogenesis induced in Balb/c mice by syngeneic sarcoma L-1, human urinary bladder and human kidney tumor cells. Oncology Rep 6: 1341-1344.
16. Wiedenfeld H, Zych M, Buchwald W, Furmanowa M (2007): New compounds from Rhodiola kirilowii. Scientia Pharmaceutica 75: 29-34.

17. Takano K, Nakaima K, Nitta MJ et al. (2004): Inhibitory effect of (–)-epigallocatechin 3-gallate, a polyphenol of green tea, on neutrophil chemotaxis in vitro and in vivo. Agric Food Chem 52: 4571-4576.
18. Zielińska-Przyjemska M, Dobrowolska-Zachwieja A (2005): Effect of tea polyphenols on oxidative metabolism of polymorphonuclear neutrophils in healthy and obese people. Pol Merkur Lekarski 19: 41-47.
19. Nagle DG, Ferreira D, Zhou YD (2006): Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. Phytochemistry 67: 1849-1855.
20. Donà M, Dell'Aica I, Calabrese F et al. (2003): Neutrophil restraint by green tea: inhibition of inflammation, associated angiogenesis, and pulmonary fibrosis. J Immunol 170: 4335-4341.