The *in vitro* influence of *Rhodiola quadrifida* extracts on non-specific cellular immunity in pigs

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Abstract

Roots and rhizomes of herbs belonging to the genus Rhodiola (Crassulaceae) are traditionally used in Asia as a tonic, adaptogen, anti-microbial and anti-inflammatory drugs. The aim of this work was to study the in vitro immunomodulatory activity of aquaeous and hydro-alcoholic extracts of underground parts of Rhodiola quadrifida in blood leukocyte cultures of pigs. Both extracts in concentration up to 10 µg/ml stimulated granulocyte potential killing (PKA) and respiratory burst (RBA) activity. In higher concentrations the suppression of these reactions were seen.

Key words: *Rhodiola quadrifida, in vitro, pigs, granulocytes.*

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Introduction

In the previous studies performed in rats we have found that extracts from *R. rosea* and *R. quadrifida* (traditional medicines from Asia, known for adaptogenic and antioxidant properties), when present in culture medium in concentration up to 10 µg/ml, enhanced blood leukocytes non-specific and specific immunologic activity *in vitro* [1, 2]. Chemical composition of extracts prepared from these two *Rhodiola* species are different. Compounds common for them are gallic and chlorogenic acids, tyrosol, and salidroside [3, 4]. The aim of this work was to study the in vitro nonspecific immunomodulatory activity of aquaeous and hydro-alcoholic extracts of under-ground parts of Rhodiola quadrifida in leukocyte cultures established from pig's blood.

Material and Methods

Rhizomes and roots of *R. quadrifida* were collected in Altai mountain in Mongolia, thanks to dr H. Wiedenfeld, and further processed in Research Institute of Medicinal Plants (RIMP) in Poznań. The raw material was washed, cut into thick slices and dried in natural conditions.

Preparation and chemical analysis of *Rhodiola* 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. Hydroalcoholic 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. 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, equ-

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ipped with photodiode array detector. For all separation a Lichrospher 100 RP18 column (250.0 × 4.0 mm, 5 μm) from Merck was used. The mobile phase consisted 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 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 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 four PWZ piglets, 4-5 month old, 40-50 kg body mass, females. Experiments were approved by Local Ethical Committee.

Immunological studies

Leucocytes were isolated from blood by centrifugation at 2000g for 30 min at 4˚C on the Gradisol G gradient (Aqua-Medica, Poland), washed three time in PBS and resuspended in RPMI 1640 medium (Sigma) supplemented with 10% of FCS (Foetal Calf Serum, Gibco-BRL) at a stock concentration of 2×10^6 cells/ml of medium. Viability of cells was checked by supravital staining with 0.1% w/v trypan blue. For RBA and PKA tests cells were preincubated for 2 hours at 37˚C in the presence of *R. quadrifida* extracts, in concentrations 1, 5, 10, 20 and 50 μg/ml of culture medium.

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 and adapted by Siwicki et al. [5]. The isolated cells were resuspended in RPMI-1640 medium (Sigma) at 10^6 cells/ml. On 96-well U-shaped microplates 100 μl of isolated blood leukocytes were mixed with of 100 μl of 0.2% nitro blue tetrazolium (NBT, Sigma) solution in 0.2 M phosphate buffer at pH 7.2 and added 1 μl 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 after incubation with 2 M KOH and DMSO (dimethylsulfoxide, Sigma) was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate.

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. [6]. On 96-well U-shaped microplates 100 μl of leucocytes were mixed with 100 μl of 0.2% NBT in phosphate buffer at pH 7.2 and 10 μl of killed Staphylococcus aureus strain 209P (containing 10^6 bacteria). The mixture was incubated 1h at 37˚C and the supermatant was removed. The cell pellet was washed with absolute ethanol and three times with 70% ethanol and dried at room temperature. This was followed by the addition of 2M KOH and DMSO to each well. The amount of extracted reduced NBT was measured at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate.

Statistical analysis

The results of experiments performed on the leukocytes collected from the blood of 4 pigs were analysed statistically by a one-way ANOVA and the significance of differences between groups was verified with a Tukey's Multiple Comparisons Test (GraphPadPrism software package).

Results

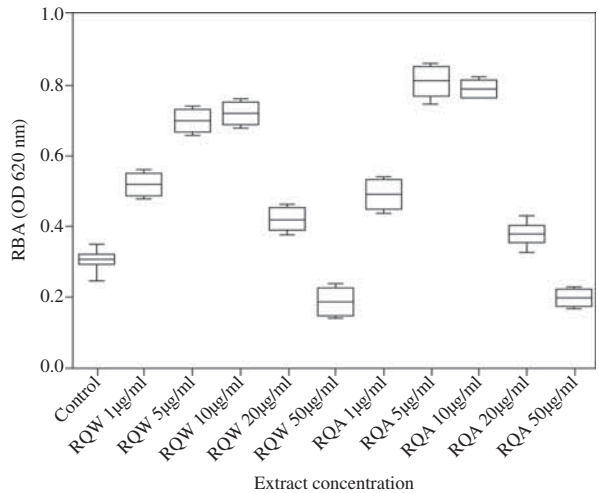
In *in vitro* studies both extracts were non-toxic at concentrations 50, 100, 200, 400, 800, and 1000 μg/ml after 24, 48, 72 and 94 hours of cells cultures.

The results of RBA test are presented on the Fig. 1. Significant, dose-dependent increase of activity was observed up to the concentration of 10 μg/ml of culture medium, and in higher concentrations of both types of extracts, significant, dose-dependent decrease of activity was seen. Similar results were obtained in PKA test (Fig. 2).

Discussion

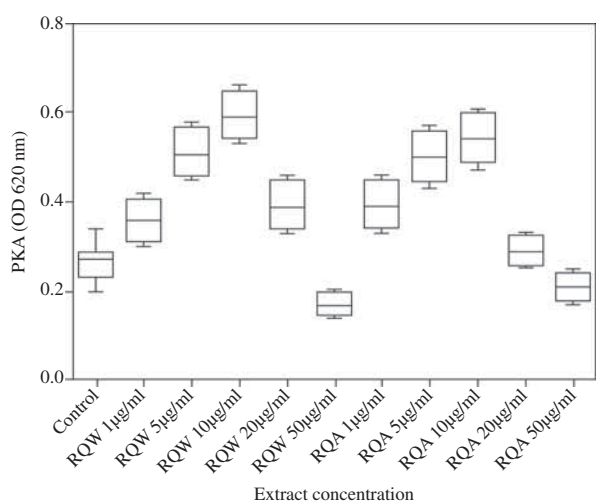
The production of free oxygen radicals is a critical component of the killing process of bacteria by granulocytes. Present findings obtained *in vitro* in pigs confirmed our earlier results obtained *in vitro* in rats for *Rhodiola quadrifida*, and in rats and pigs for *Rhodiola Rosea* [1, 7]. Both extracts stimulated granulocytes activity in lower doses, and were inhibitory in the highest (50 μg/ml) dose applied. As we have not observed cytotoxic effects up to the high 1000 μg/ml concentration, this inhibition might be connected with anti-oxidant activity of higher than 10 μg/ml concentrations [8].

Information about immunotropic activity of *Rhodiolas* is almost absent. *Rhodiolas* are known as adaptogens and antidepressants, increasing resistance to the harmfull effects of various stressors [9, 10]. The beneficial effects of adaptogens are associated with the hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenal-sys-



Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? p<0.05?	Summary
Control vs RQW 1µg/ml	-0,2150	15,16	Yes	***
Control vs RQW 5µg/ml	-0,3950	27,86	Yes	***
Control vs RQW 10µg/ml	-0,4150	29,27	Yes	***
Control vs RQW 20µg/ml	-0,1150	8,110	Yes	***
Control vs RQW 50µg/ml	0,1150	8,110	Yes	***
Control vs RQA 1µg/ml	-0,1850	13,05	Yes	***
Control vs RQA 5µg/ml	-0,5025	35,44	Yes	***
Control vs RQA 10µg/ml	-0,4850	34,20	Yes	***
Control vs RQA 20µg/ml	-0,0750	5,289	Yes	*
Control vs RQA 50µg/ml	0,1050	7,405	Yes	***

Fig. 1. The *in vitro* effect of *Rhodolia quadrifida* water (RQW) and hydro-alcoholic (RQA) extracts on blood leukocytes RBA activity



Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? p<0.05?	Summary
Control vs RQW 1µg/ml	-0,0925	4,553	No	ns
Control vs RQW 5µg/ml	-0,2450	12,06	Yes	***
Control vs RQW 10µg/ml	-0,3250	16,00	Yes	***
Control vs RQW 20µg/ml	-0,1250	6,153	Yes	**
Control vs RQW 50µg/ml	0,0975	4,799	No	ns
Control vs RQA 1µg/ml	-0,1250	6,153	Yes	**
Control vs RQA 5µg/ml	-0,2325	11,44	Yes	***
Control vs RQA 10µg/ml	-0,2725	13,41	Yes	***
Control vs RQA 20µg/ml	-0,0225	1,107	No	ns
Control vs RQA 50µg/ml	0,0575	2,830	No	ns

Fig. 2. The *in vitro* effect of *Rhodolia quadrifida* extracts on blood leukocytes PKA activity

tem (SAS), both of them may influence immunological reactions. The immunomodulatory effects of antidepressants were also described [11]. More experimental work is needed for elucidate the mechanism of immunomodulatory effect of *Rhodiolas* on granulocytes *in vitro* and *in vivo* activity.

References

1. 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.
2. 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 (Suppl 1): 170-171.
3. Mielcarek S, Mścisz A, Buchwald W et al. (2005): Phytochemical investigation of Rhodiola sp. root extracts. Herba Polonica 51 (Suppl 1): 159-160.
4. Wiedenfeld H, Dumaa M, Malinowski M, Furmanowa M, Narantuya S (2007): Phytochemical and analytical studies of extracts from Rhodiola rosea and Rhodiola quadrifida. Pharmazie 62: 308-311.
5. Siwicki AK, Skopińska-Różewska E, Nartowska J, Małaczewska J et al. (2004): Effect of IMUNOSTM PLUS – a standardized fixed combination of Schizandra chinensis with Eleutherococcus extracts on granulocyte activity and tumor angiogenesis in mice. Bull Vet Inst Pulawy 48: 489-492.
6. Rook GA, Steele J, Umar S, Dockrell HM (1985): A simple method for the solubilization of reduced NBT and its use as a colometric assay for activation of human macrophages by gamma interferon. J Immunol Meth 82: 161-167.
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. 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.
9. Panossian A, Nikoyan N, Ohanyan N et al. (2008): Comparative study of Rhodiola preparations on behavioral despair of rats. Phytomedicine 15: 84-91.
10. Panossian A, Wagner H (2005): Stimulating effect of adaptogents: an overview with particular reference to their efficacy following single dose administration. Phytother Res 19: 819-838.
11. Maes M (2001): The immunoregulatory effects of antidepressants. Hum Psychopharmacol Clin Exp 16: 95-103.