# The *in vitro* influence of *Rhodiola kirilowii* extracts on blood granulocytes potential killing activity (PKA) 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 stimulatory activity of aquaeous and hydro-alcoholic extracts of under-ground parts of Rhodiola kirilowii in blood leukocyte cultures of pigs. Both extracts in concentration up to 10 μg/ml stimulated granulocyte potential killing (PKA) activity. In higher concentrations the suppression of this reaction was seen.*

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

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## Introduction

In the previous studies performed in rats we have found that extracts from *Rhodiola rosea* and *Rhodiola 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 [1-4]. Chemical composition of extracts prepared from these two *Rhodiola* species are different. Compounds common for them are gallic and chlorogenic acids, tyrosol, and salidroside. The main chemical substances present in *Rhodiola kirilowii* roots and rhizoma extracts are salidroside, tyrosol, daucosterol, lotaustralin, sucrose, beta-sitosterol, arbutin, rhodiocyanoside A, epigallogatechin gallate and fructopyrano-(1-4)-glucopyranose [5-7].

*Rhodiola kirilowii* is used in traditional Chinese medicine for many purposes, as a herbal drug protecting people against cardiopulmonary problems when moving to high altitude (astronauts, pilots, mountaineers), as plant adaptogen, as anti-bacterial and anti-viral remedy [8-11]. Information about immunotropic activity of *Rhodiola kirilowii* is very scarce. Recently, we presented for the first time evidence of immunomodulatory activity of *Rhodiola kirilowii* extracts on *in vitro* and *in vivo* experimental models in rats and mice [12]. The aim of this work was to study the *in vitro* stimulatory activity of aquaeous and hydro-alcoholic extracts of under-ground parts of *Rhodiola kirilowii* on the potential killing activity (PKA) of leukocytes in cell cultures established from pig's blood.

## Material and Methods

### Preparation of extracts

*Rhodiola kirilowii* (Reg.) Reg. (Crassulaceae), roots and rhizomes were cultivated, collected in September 2003 and identified in the Research Institute of Medicinal Plants (RIMP), Poznań. Sample extracts and their chemical analysis were performed at the RIMP (Mrozikiewicz PM, Mścisz A, Krajewska-Patan A., Mielcarek S, Buchwald W) and Zych M. from the Warsaw Medical University, as previously described [1, 5]. Briefly: air-dried finely

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powdered rhizomes were extracted two times with water (aqueous extract) or 50% ethanol (hydro-alcoholic extract), at 40-45°C, evaporated to dryness and lyophilized.

All the samples were diluted in methanol. HPLC analysis was performed on Agilent 1100 HPLC system, equipped with photodiode array detector. 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.

### PKA test

Leucocytes were isolated from blood by centrifugation at 2000 g 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 $2 \times 10^6$ cells/ml of medium. Viability of cells was checked by supravital staining with 0.1% w/v trypan blue. For PKA test cells were preincubated for 2 hours at 37°C in the presence of *R. kirilowii* extracts, in concentrations 1, 10, and 50 µg/ml of culture medium.

Potential bactericidal activity of phagocyting cells was determined in isolated blood leukocytes stimulated with killed microorganisms, according to Rook et al. as was described previously [13]. 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 1 h at 37°C and the supernatant 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. The mean value of 4 experiments ± SEM served as the result.

### Statistical analysis

The results were verified statistically by a one-way ANOVA and the significance of differences between groups was verified with a Bonferroni'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 hours of cell cultures.

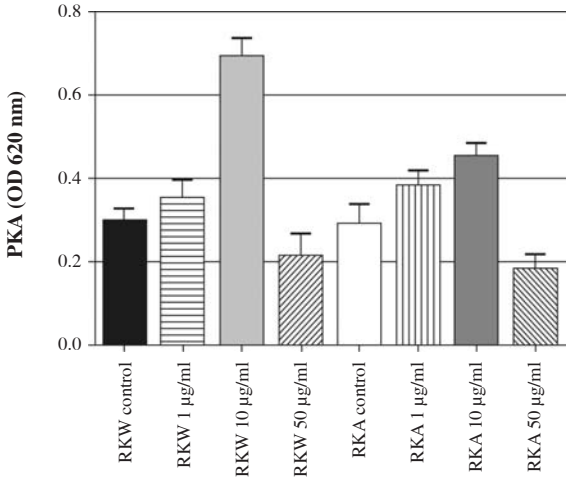


**Fig. 1.** *In vitro* effect of *Rhodiola kirilowii* water (RKW) and hydro-alcoholic (RKA) extracts on bactericidal activity of blood phagocyting cells in pigs (PKA test, means ± SEM).

The results of PKA test are presented on the Figure 1. On the Table 1 the results of statistical analysis are presented. Significant increase of granulocyte killing activity was observed at the concentration of 10 µg/ml of culture medium. Stimulatory effect of water extract was significantly higher than the effect of alcoholic extract. In higher concentration of both types of extracts, significant decrease of activity was seen.

## 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 kirilowii*, and in rats and pigs for *Rhodiola rosea* and *Rhodiola quadrifida* [1-4, 12]. The difference between water and alcoholic extract observed in the present study at 10 µg/ml concentration was not present in experiments with rat granulocytes cultivated in the presence of *Rhodiola kirilowii* extracts. However, we previously observed better *in vivo* stimulatory effect of *Rhodiola kirilowii* water extract on lymphocyte activity in mice than the effect of hydro-alcoholic extract. This also corresponds to the results obtained for *Rhodiola rosea* aqueous extract which also showed better stimulation of lymphocyte-induced angiogenesis than the hydro-alcoholic extract [1]. Generally, *in vitro*, both types of 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 [14].

**Table 1.** One-way analysis of variance of the *in vitro* effect of *R. kirilowii* extracts on bactericidal activity of blood phagocyting cells in pigs

| **One-way analysis of variance** | | | | |
|---|---|---|---|---|
| P value | < 0.0001 | | | |
| P value summary | *** | | | |
| Are means signif. different? (p < 0.05) | yes | | | |
| Number of groups | 8 | | | |
| F | 49.19 | | | |
| R square | 0.9348 | | | |
| | | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 0.7232 | 7 | 0.1033 | |
| Residual (within columns) | 0.0504 | 24 | 0.0021 | |
| Total | 0.7736 | 31 | | |
| **Bonferroni's Multiple Comparison Test** | **Mean diff.** | **t** | **Significant (p < 0.05)** | **Summary** |
| RKW control vs. RKW 1 ug/ml | -0.05000 | 1.543 | no | ns |
| RKW control vs. RKW 10 ug/ml | -0.3900 | 12.04 | yes | *** |
| RKW control vs. RKW 50 ug/ml | 0.09000 | 2.777 | no | ns |
| RKW control vs. RKA control | 0.01000 | 0.3086 | no | ns |
| RKW control vs. RKA 1 ug/ml | -0.08000 | 2.469 | no | ns |
| RKW control vs. RKA 10 ug/ml | -0.1500 | 4.629 | yes | ** |
| RKW control vs. RKA 50 ug/ml | 0.1200 | 3.703 | yes | * |
| RKW 1 ug/ml vs. RKW 10 ug/ml | -0.3400 | 10.49 | yes | *** |
| RKW 1 ug/ml vs. RKW 50 ug/ml | 0.1400 | 4.320 | yes | ** |
| RKW 1 ug/ml vs. RKA control | 0.0600 | 1.852 | no | ns |
| RKW 1 ug/ml vs. RKA 1 ug/ml | -0.0300 | 0.9258 | no | ns |
| RKW 1 ug/ml vs. RKA 10 ug/ml | -0.1000 | 3.086 | no | ns |
| RKW 1 ug/ml vs. RKA 50 ug/ml | 0.1700 | 5.246 | yes | *** |
| RKW 10 ug/ml vs. RKW 50 ug/ml | 0.4800 | 14.81 | yes | *** |
| RKW 10 ug/ml vs. RKA control | 0.4000 | 12.34 | yes | *** |
| RKW 10 ug/ml vs. RKA 1 ug/ml | 0.3100 | 9.567 | yes | *** |
| RKW 10 ug/ml vs. RKA 10 ug/ml | 0.2400 | 7.407 | yes | *** |
| RKW 10 ug/ml vs. RKA 50 ug/ml | 0.5100 | 15.74 | yes | *** |
| RKW 50 ug/ml vs. RKA control | -0.0800 | 2.469 | no | ns |
| RKW 50 ug/ml vs. RKA 1 ug/ml | -0.1700 | 5.246 | yes | *** |
| RKW 50 ug/ml vs. RKA 10 ug/ml | -0.2400 | 7.407 | yes | *** |
| RKW 50 ug/ml vs. RKA 50 ug/ml | 0.03000 | 0.9258 | no | ns |
| RKA control vs. RKA 1 ug/ml | -0.0900 | 2.777 | no | ns |
| RKA control vs. RKA 10 ug/ml | -0.1600 | 4.938 | yes | ** |
| RKA control vs. RKA 50 ug/ml | 0.1100 | 3.395 | no | ns |
| RKA 1 ug/ml vs. RKA 10 ug/ml | -0.07000 | 2.160 | no | ns |
| RKA 1 ug/ml vs. RKA 50 ug/ml | 0.2000 | 6.172 | yes | *** |
| RKA 10 ug/ml vs. RKA 50 ug/ml | 0.2700 | 8.332 | yes | *** |

Extracts prepared from *Rhodiola kirilowii* contain other spectrum of compounds than extracts of *Rhodiola rosea* and *Rhodiola quadrifida*. Only tyrosol and salidroside are common for these three species. However, in some studies of *Rhodiola kirilowii* extracts, salidroside was not detected [7].

Immunomodulatory activity of *Rhodiola kirilowii* extracts may be, at least partly, connected with their epigallocatechin-gallate (EGCG) content. EGCG was recognized as a modulator of macrophage activity and pro-inflammatory cytokines production [15-17]. High EGCG concentrations exerted inhibitory effects, low concentrations were stimulatory, what corresponds to our results obtained for *R.kirilowii* extracts and granulocytes.

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