Modulatory effects of feeding pregnant and lactating mice *Rhodiola kirilowii* extracts on the immune system of offspring

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Abstract. Plants of *Rhodiola* genus are medicinal herbs that have a number of therapeutic properties, including anti-inflammatory and immunomodulatory activity. The present study aimed to determine whether the use *Rhodiola kirilowii* as an immunostimulant during pregnancy has an adverse effect on the development of the offspring immune system. Following mating, pregnant mice were placed in three groups that were fed during pregnancy and lactation with *R. kirilowii* aqueous extract (RKW; 20 mg/kg), *R. kirilowii* 50% hydro-alcoholic extract (RKW-A; 20 mg/kg) or water (control group), receiving water. Following birth, offspring were given six weeks to develop prior to evaluation of their immune system. Morphometric and morphological examination of the spleen did not reveal any abnormalities or differences between the experimental and control groups. However, both RKW and RKW-A splenic lymphocytes presented a diminished proliferative response to concanavalin A. RKW spleen lymphocytes demonstrated increased metabolic activity following phytohaemagglutinin (PHA) stimulation, which was associated with a higher percentage of cluster of differentiation 4 positive spleen cells and lower interleukin-17a (IL-17a) serum concentration. The RKW-A group exhibited a diminished proliferative response of spleen lymphocytes to PHA and lipopolysaccharide (LPS), and increased serum concentrations of IL-10 and tumor necrosis factor-α (TNF-α). The progeny of mice fed with RKW-A extract demonstrated a significantly lower level of anti-SRBC antibody following immunization compared with progeny of the control (P=0.0305) and RKW (P=0.0331) groups. In conclusion, caution is recommended in the use of RKW and RKW-A extracts as immunostimulants in pregnancy.

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Introduction

The genus *Rhodiola* consists of numerous species. The best known is *R. rosea* (1-3). Less reported in Europe are other members of this genus, *R. quadrifida* and *R. kirilowii*. Plants of *Rhodiola* genus, a member of the *Crassulaceae* family, are traditionally used in Asiatic medicine for their adaptogenic and anti-inflammatory properties, and have been recognized as immunomodulators in the past few decades (4,5). In addition, these plants exhibit inhibitory effects against a number of pathogens without the involvement of an immune system. It has been reported that extracts from *R. rosea* inhibit *Clostridium perfringens* and influenza virus neuraminidases *in vitro* (6). Moreover, *R. rosea* extract inhibits the replication of dengue virus, vesicular stomatitis virus and coxsackievirus B3 (7-9). Plants from *Rhodiola* genus are useful in bacterial infections, particularly those displaying antibiotic resistance. Cybulska *et al* (10) demonstrated that addition of *R. rosea* extract to *Neisseria ghonorrhea* culture inhibits its growth *in vitro*. Based upon these findings, it is possible that *Rhodiola*-based therapeutic agents may be useful adjuvants or alternatives to antibiotics in the treatment of viral/bacterial infections during pregnancy.

Previous studies have investigated the immunotropic activity of alcoholic and aqueous extracts of roots and rhizomes of *Rhodiola* plants in mice, rats and pigs. During the initial investigation (11), not a large quantity of information was available about the immunotropic activity of *Rhodiolas*. Further studies showed that the majority of *Rhodiola* extracts stimulated immunity (12,13). The present study investigates the effects of aqueous *Rhodiola kirilowii* (RKW) or 50% hydro-alcoholic (RKW-A) extracts, administered to pregnant and lactating mice, on the immune system of the resulting six-week old progeny. This experimental model is used by the present study to evaluate whether the use of *R. kirilowii* extract as an immunostimulant in pregnancy is safe for the developing immune system of the progeny (14).

Materials and methods

Plant cultivation. R. kirilowii roots and rhizomes were cultivated, identified and collected in the Research Institute of Medicinal Plants, now the Institute of Natural Fibers and Medicinal Plants (Poznań, Poland). The voucher specimen was kept in the herbarium of Department of Botany, Breeding and Agriculture (Plewiska, Poland).

Preparation and chemical analysis of extracts. Extracts were prepared as previously described (12). Briefly, to produce RKW extract, finely powdered *R. kirilowii* roots were extracted twice with water (first for 2 h and then for 1 h) in a raw material: Solvent ratio of 1:5, at between 40 and 45˚C by the water extraction method. Resulting supernatants were mixed together, centrifuged (15 min, room temperature, 2000 x *g*) and lyophilized. To produce RKW-A extract: Finely powdered *R. kirilowii* roots were extracted with a 1:1 ethanol: Water solution, in a raw material: Solvent ratio of 1:10 using the percolation method. Then, percolates were lyophilized following distillation at between 40 and 45˚C. Dry extract ratio were 5.09:1 for RKW and 3.27:1 for RKW-A. Extracts were stored at -70˚C until required.

Chemical analysis of extracts. Chemical analysis of extracts was performed as previously described (15), according to the methods proposed by Hertog *et al* (16). Briefly, the polyphenol concentration of the extracts was assayed using high-performance liquid chromatography (HPLC; Dionex system, pump P680, autosampler ASI100; Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a CoulArray Coloumetric Array Electrochemical Detector (ECD; Thermo Fisher Scientific, Inc.).

Experimental animals. Experiments were performed on 202 6-week old progeny (80 from control, 59 from RKW and 63 from RKW-A mothers) of 70 adult inbred female BALB/c mice (8-9 weeks old; ~20 g; Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland), which were mated with adult males of the same strain. Between the appearance of a copulatory plug and 28 days following delivery, females were fed daily with lyophilized RKW (n=20) or RKW-A (n=19) extract. The extracts were given at a dosage of 20 mg/kg (7 mg/m^2), to mice weighing ~20 g with a body surface area of 0.007 m^2, which corresponds to a dose of 100 mg (1.6 mg/kg) given to a person weighing 60 kg with a body surface area of 1.6 m^2 (1.6 mg/kg) (17). The control group (n=31) received distilled water instead of extract. The substances given were applied on a corn crisp and served to the mice in a Petri dish.

Experimental animals were handled in accordance with the Polish regulations concerning the wellness of laboratory animals (Polish National Institute of Health, Warsaw, Poland). All experiments were accepted by and conducted in accordance with the ethical guidelines of the Local Ethics Committee (IV) on Animal Testing, National Medicines Institute, Warsaw, Poland (permission no. 73/2011). Mice were maintained under typical conditions (22.5-23.0˚C, relative humidity 50-70%, 12 h day/night cycle) with *ad libitum* access to breeding rodent feed (Labofeed H; Factory of Fodder, Kcynia, Poland) and water. Female and male progeny were housed separately. Pups were withdrawn from mothers 24 days following delivery.

Sera and spleen isolation. Mice were retro-orbitally bled under anesthesia [intraperitoneal injection of ketamine (120 mg/kg) and xylazine (12 mg/kg); Polypharm S.A., Warsaw, Poland]. Serum was separated by clotting for 1 h at room temperature, followed by centrifugation at 2000 x *g* for 20 min at 4˚C and then stored at -70˚C until required. Following bleeding, mice were sacrificed by anesthetic overdose (pentobarbital, 400 mg/kg; Polypharm S.A, Warsaw, Poland) and spleens were isolated under aseptic conditions (laminar flow cabinet), for immediate use.

Morphometric evaluation of spleens. Histological evaluation and quantitative microscopic analysis of lymphatic nodules was performed on hematoxylin and eosin stained paraffin sections of the spleens obtained from the progeny (n=10 from each group). The histotechnical criteria applied for quantitative analysis were as follows: i) A thickness of between 3 and 5 µm; ii) complete transversal section of the spleen including white and red pulp structures; and iii) no evidence of traumatic artifacts within the sample, such as fragmentation or hemorrhage. The diameter of splenic nodules and the number of lymphatic nodules per microscopic field were measured. The surface of the section of spleen was then analyzed in regards to the following: i) The total area and number of white pulp lymphatic nodules, with the results expressed as the number per microscopic field (5.5 mm^2); and ii) lymphatic nodule diameters measured in consecutive nodules. Images were acquired and processed using ToupView software (version 3.7; ToupTek Photonics Co., Ltd., Hangzhou, China).

Light microscopy examination, using an Evolution 100 Trino optical microscope (Delta Optical; Mińsk Mazowiecki, Poland) connected to a photometric color CCD camera (UCMOS05100KPA; Hangzhou ToupTek Photonics Co., Ltd.), of the red and white pulp (sites of hematopoiesis) was performed. In the red pulp attention was paid to splenic cords and venous sinuses. In the white pulp, the follicles, germinal centers, periarteriolar lymphoid sheath (PALS) and marginal zone were assessed. Fixed spleen preparations were examined using a panchromatic lens (numerical aperture, 0.25) at x100 magnification.

Preparation of splenocyte suspension. Spleens were gently pressed through a sterile nylon strainer (40 µm) into a 50 ml Flacon tube with 20 ml of culture medium [Roswell Park Memorial Institute (RPMI)-1640 medium with GlutaMAX; Thermo Fisher Scientific, Inc., Warsaw, Poland], supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Warsaw, Poland) and antibiotics (50 IU/ml penicillin; 50 µg/ml streptomycin; Thermo Fisher Scientific, Inc.). Strainers were rinsed twice with media to remove remaining cells. Then, cells were centrifuged (500 x *g*, 5 min, room temperature), the resulting pellet resuspended in media and cells counted in a hematological analyzer (Exigo veterinary hematological system; Boule Medical AB, Stockholm, Sweden). A cell suspension of $1x10^6$ cells/ml was used to evaluate response to mitogens. Splenocyte viability was determined using the trypan blue exclusion test and amounted

to >95% cell viability. Following preparation, cells were immediately used.

Phenotypic determination of splenocytes. Spleen cell suspensions (1x10^6 cell/ml) were washed twice with phosphate buffered saline (PBS) and centrifuged (500 x *g*, 5 min, room temperature). Cell pellets were resuspended in 1 ml of PBS and 100 μl of the suspension was labeled by cell-surface marker staining with the following fluorochrome-coniugated anti-mouse monoclonal antibodies: Mouse T lymphocyte Subset Antibody Cocktail with Isotype Control [hamster anti-mouse phycoerythrin (PE)-Cyanine 7 cluster of differentiation (CD) 3e, rat anti-mouse PE CD4 and rat anti-mouse allophycocyanin (APC) CD8a; cat. no 558431; ready-to-use], Mouse B Lymphocyte Activation Antibody Cocktail with Isotype Control (rat anti-mouse PE-Cy7 CD25, hamster anti-mouse PE CD69 and rat anti-mouse APC CD19; BD Biosciences, Warsaw, Poland; cat. no. 558063; ready-to-use) and PE rat anti-mouse CD335 (natural killer cell p46-related protein; cat. no. 560757) (all BD Biosciences, Warsaw, Poland), according to the manufacturer's instructions (20 min incubation at room temperature). Red blood cells from splenocyte suspensions were lysed (10 min, Lysing Solution 10X Concentrate; BD Biosciences). Phenotypic analysis was then performed using flow cytometry (FACSCalibur; BD Biosciences). Results of this analysis are presented as the mean % of splenocytes of a particular phenotype ± the standard error of the mean.

Response of splenocytes to mitogens. Response to mitogens was measured using two tests; the alamarBlue assay and [^{3}H] thymidine incorporation assay. For the alamarBlue assay, splenocytes were seeded into 96-well plates (1x10^5 cell/well), incubated for 1 h under standard condition (5% CO_2, 95% humidity, 37˚C) and then a mitogen was added to each well: Lipopolysaccharide (LPS; 20 μg/ml), Concanavalin A (ConA; 5 μg/ml) or phytohaemagglutinin (PHA; 2 μg/ml) (all purchased from Sigma-Aldrich, Poznań, Poland). Following 24 h of incubation, alamarBlue (1:10, v/v; Thermo Fisher Scientific, Inc., Warsaw, Poland) was added to the wells. Cells were incubated for 24 h at standard conditions (37˚C, 5% CO_2, 95% relative humidity). Then, alamarBlue fluorescence (excitation 544 nm, emission 590 nm) of the wells was measured using a FLUOstar Omega Microplate Reader (BMG Labtech GmbH, Ortenberg, Germany) as previously described (18). For the [^{3}H] thymidine incorporation assay (19), cells were cultured and treated with mitogens as described above. Then, the cultures were incubated for 48 h prior to being pulsed with thymidine (^{3}HTdR, 2 Ci/mM, 0,4 μC/20 μl/culture) and cultured for a further 24 h. Following culture, cells were transferred onto Whatman filter paper discs (Labo Plus, Warsaw Poland), extracted with 30% trichloroacetic acid, dehydrated using alcohol and ether, and transferred to glass scintillation vessels filled with liquid scintillation mixture (cat no: 327123; Sigma-Aldrich; Thermo Fisher Scientific, Inc.). The measurements were taken using a Packard Tri-Carb 2100TR scintillation counter (PerkinElmer, Inc., Waltham, MA, USA). Tests were performed in triplicate and an unstimulated control was included.

Cytokine determination. Flow cytometry determination of the concentration of selected cytokines [interleukin (IL)-2, -4, -6, -10 and -17A, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)] in the sera was evaluated using the Mouse Th1/Th2/Th17 Cytokine Kit (cat. no. 560485, BD Biosciences), according to the manufacturer's protocol.

Anti-sheep red blood cell (SRBC) CD2 antibody production. Progeny mice (6-weeks old) were immunized with 5% SRBC (0.2 ml intraperitoneal injection; Graso Biotech, Gdański, Poland) 7 days prior to being bled under anesthesia from the retro-orbital plexus. Anti-SRBC antibody levels were evaluated by performing a hemagglutination assay, as previously described (20), on a series sera dilutions. Briefly, following heat inactivation of the sera (56˚C, 30 min), 1% SRBC was added and the mixture was incubated for 60 min at 37˚C, incubated for 18 h at 4˚C, and centrifuged (10 min, 150 x *g*, 4˚C) and shaken. The hemagglutination titer was defined, using light microscopy, as the highest dilution in which ≥3 cell conglomerates were present in ≥3 consecutive fields at an objective magnification of x20. For the purposes of statistical analysis, results were transformed into logarithm inversions of the titers.

Statistical analysis. Statistical evaluation of the results obtained, from the control and experimental groups, was performed using unpaired *t*-tests and one- or two-way analysis of the variance, followed by the Tukey test or Bonferroni correction (in the case of a normal distribution) or non-parametric Kruskal-Wallis and Mann-Whitney U tests (in the case of abnormal distribution). Assessment of the distribution of the data was evaluated using the Shapiro-Wilk test. GraphPad Prism software was used to carry out these tests (version 5; GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Chemical analysis of extracts. RKW and RKW-A extracts were found to contain phenyloethanoid salidroside and thyrozol, four phenolic acids (chlorogenic, ferulic, ellagic and p-coumaric), and flavonoids [fisetin, naringenin, kaempferol, epicatechin, luteolin, quercetin, epigallocatechin and (+)-catechin]. HPLC-ECD analysis revealed a significant difference in the content of biologically active compounds between RKW and RKW-A extracts ($P<0.0001$). Typically, RKW-A extract presented a higher concentration of the identified compounds than RKW. The total concentration of polyphenols amounted to 16.16 μg/mg in RKW and 23.75 μg/mg in RKW-A.

Spleen morphology. No macroscopic abnormalities were identified in the anatomy of spleens from the experimental and control groups. Similarly, no differences in the relative weight, cellularity (Fig. 1) and morphological picture of spleens were observed between the groups. Splenic lymphatic nodules were large, with well-developed germinal centers. In addition, the PALS and marginal zone were found to be normal in all groups. The red pulp of the spleen was moderately plethoric. Morphometric evaluation did not reveal differences between the experimental and control groups in regards to the number of lymphatic nodules per microscopic field and their diameter (Table I).

Table I. Morphometric analysis of the spleen in control and experimental animals.

Group	Number of images analyzed	Number of lymphatic nodules/field ± SEM	Number of lymphatic nodules analyzed	Mean diameter of nodule (mm) ± SEM
Control	34	9.23±0.49	316	0.323±0.0074
RKW	41	10.34±0.52	414	0.318±0.0059
RKW-A	32	9.62±0.59	308	0.334±0.0062

No significant differences were found (statistical analysis: Shapiro-Wilk normality test, one-way analysis of the variance, Tukey comparison test; α=0.05). RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract; SEM, standard error of the mean.

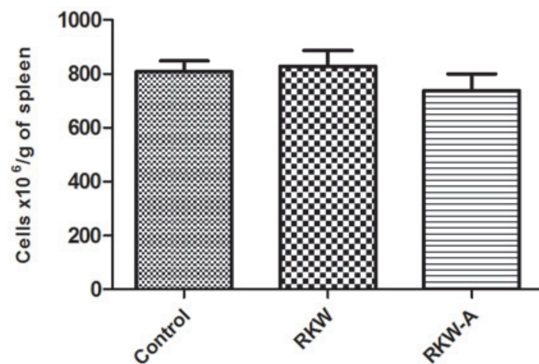

Figure 1. Cellularity of progeny spleens. Results presented are the mean of cells calculated per gram of spleen ± standard error of the mean. Number of spleens tested, 87 (control, 41; RKW, 24; RKW-A, 22). RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract.

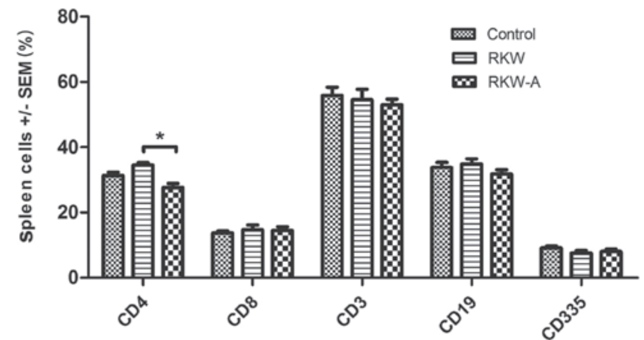

Figure 2. Phenotypes of spleen lymphocytes. Phenotypic analysis was performed by flow cytometry [number of spleens tested, 46 (control, 15; RKW, 13; RKW-A, 18)]. Results are presented as the mean percentage of spleen cells ± SEM. Statistical analysis performed: Unpaired *t*-test, Shapiro-Wilk normality test, two-way analysis of the variance and Bonferroni correction. SEM, standard error of the mean; CD, cluster of differentiation; RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract. *P=0.0004.

Phenotype of splenocytes. The percentage of $CD4^+$ cells was highest in spleens collected from the progeny of mice fed RKW extract during pregnancy, and amounted to 63% of all $CD3^+$ splenocytes from the group. In comparison, in splenocytes from the progeny of RKW-A extract-fed mothers and control mothers, corresponding values were 52 and 55%, respectively. The $CD4^+$:$CD8^+$ ratio was 2.35±0.2 in the RKW group, in comparison to 1.88±0.1 in the RKW-A group and 2.41±0.2 in the control group. The results described are presented in Fig. 2.

Splenocytic response to mitogens. The alamarBlue assay identified that offspring of mice fed with RKW extract during pregnancy and lactation showed a significantly higher level of metabolic activity following addition of the mitogen PHA compared with the offspring of mice fed with RKW-A (P=0.0496), which did not affect metabolic activity and slightly decreased proliferation following PHA stimulation (Fig. 3A). However, no significant differences between groups in response to PHA were found by the [^{3}H] thymidine incorporation assay (Fig. 3B). In cells from ConA-stimulated spleens the alamarBlue assay found no differences in proliferation between the study and control groups in ConA stimulated cells (control, 156±3.9, n=31; RKW, 153±5.2, n=16; RKW-A, 151±4.5, n=12) (Fig. 4A), while the [^{3}H] thymidine incorporation assay identified significantly lower [^{3}H] thymidine incorporation in cells collected from experimental groups in comparison to the control (RKW, P=0.0306; RKW-A, P=0.0356; Fig. 4B). In LPS-stimulated cells the alamarBlue assays found no significant difference between groups (Fig. 5A), while the [^{3}H] thymidine incorporation assays found significantly lower cell proliferation in the RKW-A group compared with the control (P=0.0175; Fig. 5B).

Cytokine concentrations in mice sera. The serum concentrations of selected cytokines (IL-2, -4, -6, -10 and -17a, TNF-α, and IFN-γ) were evaluated by flow cytometry. No statistically significant differences were found in the serum concentrations of IL -2, -4 and -6, and IFN-γ between groups (Fig. 6A-D). The serum concentration of TNF-α was significantly higher in the RKW-A group compared with the control (P=0.0123; Fig. 6E). Serum IL-17a concentration was significantly lower in mice whose mothers were fed during pregnancy and lactation with RKW extract compared with the control (P=0.0347; Fig. 7A). However, there were no differences between mice of RKW-A and control mothers (Fig. 7A). In addition, IL-10 serum concentration was higher (P=0.0581) in the RKW-A group compared with the control (Fig. 7B).

Anti-SRBC antibody production. The mean of log titer of anti-SRBC antibody production was significantly lower in progeny of mice fed RKW-A extract (P=0.0305) compared with those of mice fed water (Fig. 8). There was no difference

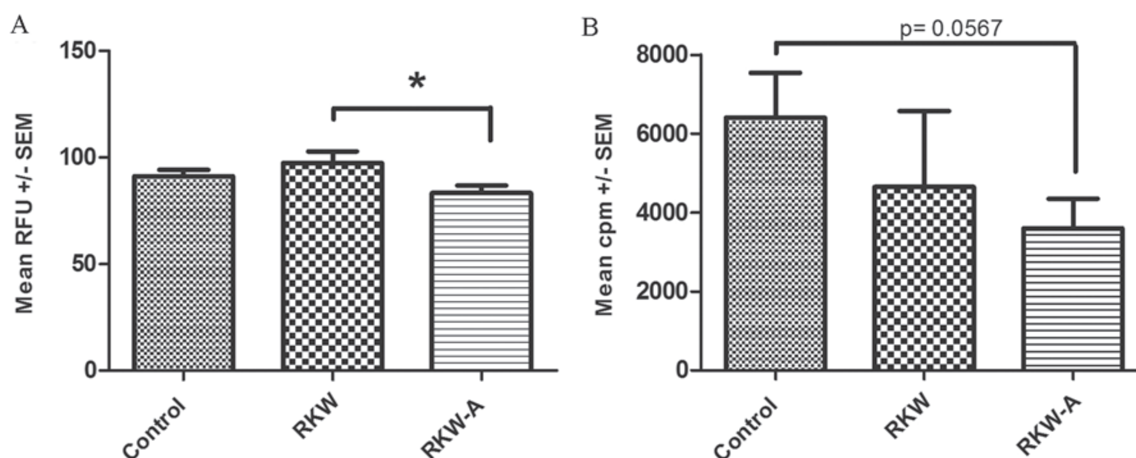

Figure 3. Proliferation index of splenocytes following phytohemagglutinin (PHA) stimulation. Results are presented as the mean RFU or cpm ± SEM, measured by the (A) alamarBlue assay [number of spleens tested, 59 (control, 31; RKW, 16; RKW-A, 12)] or (B) [^{3}H] thymidine incorporation assay following PHA (2 μg/ml) stimulation [number of spleens tested, 27 (control, 9; RKW, 9; RKW-A, 9)]. Statistical analysis performed: Unpaired *t*-test, Shapiro-Wilk normality test, one-way analysis of the variance and the Tukey comparison test. RFU, relative fluorescence units; SEM, standard error of the mean; cpm, counts per minute; RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract. *P=0.0496.

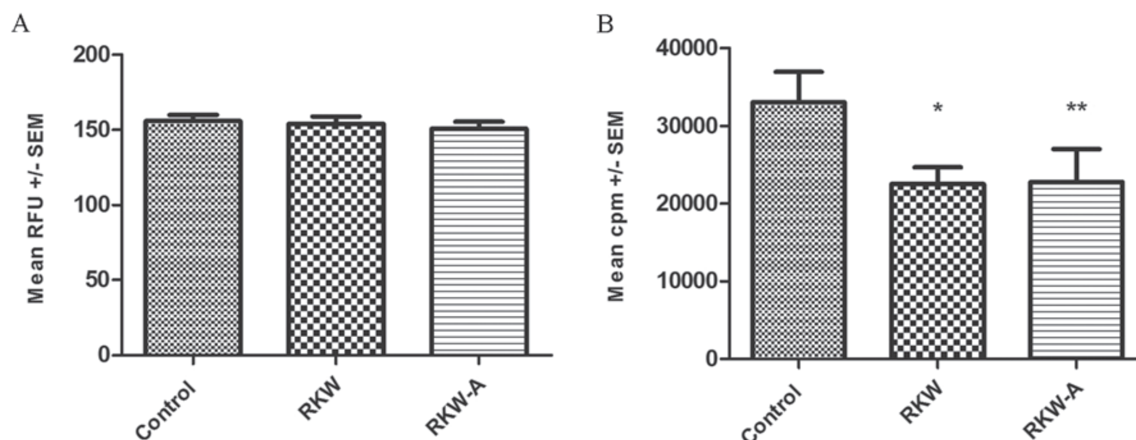

Figure 4. Proliferation indexes of splenocytes following Concanavalin A (ConA) stimulation. Results are presented as the mean RFU or cpm ± SEM, measured by the (A) alamarBlue assay [number of spleens tested, 59 (control, 31; RKW, 16; RKW-A, 12)] or (B) [^{3}H] thymidine incorporation assay following ConA (5 μg/ml) stimulation [number of spleens tested, 27 (control, 9; RKW, 9; RKW-A, 9)]. Statistical analysis performed: Unpaired *t*-test, Shapiro-Wilk normality test, one-way analysis of the variance and the Tukey comparison test. RFU, relative fluorescence units; SEM, standard error of the mean; cpm, counts per minute; RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract. *P=0.0306 vs. control; **P=0.0356 vs. control.

between progeny of mice fed with RKW and those of mice fed water control (Fig. 8).

Discussion

To best of our knowledge, the present study is the first to investigate the influence of *R. kirilowii* extract, administered to pregnant and lactating mice, on the immune system of their progeny. The immune system is crucial for the survival of complex organisms, such as mice and humans, because of its function in defense against diseases and in regulation of homeostasis. The primary cells of the immune system are lymphocytes, therefore, the present study investigated their proliferative activity and expression of cytokines.

The results of the present study determined that the spleen lymphocytes of mice whose mothers were fed during pregnancy and lactation with RKW extract were more able to metabolize resazurin when stimulated with PHA, compared with splenocytes from mice whose mothers were fed RKW-A extract. Differences in the results obtained from the alamarBlue and [^{3}H] thymidine incorporation assays are a result of the test specificity. Tge alamarBlue assay measures the metabolic activity of the cells, whereas [^{3}H] thymidine incorporation is based on *de novo* DNA synthesis during proliferation.

Lymphocyte activation is usually typically associated with the expression of IL-2, -4, -6, -10 and -17, and TNF-α, (21,22). IL-2 promotes proliferation of and cytokine production by T-cells, and serves an important role in the maintenance of the functional properties of B cells (23). IL-4 is a key regulator of the immune response and promotes the differentiation of naive T cells into T helper (Th) 2 cells (24). IL-6 has been shown to influence inflammatory action and the antigen-specific immune response (25). In addition, IL-6 serves an important role in cellular defense mechanisms trough regulating hema-

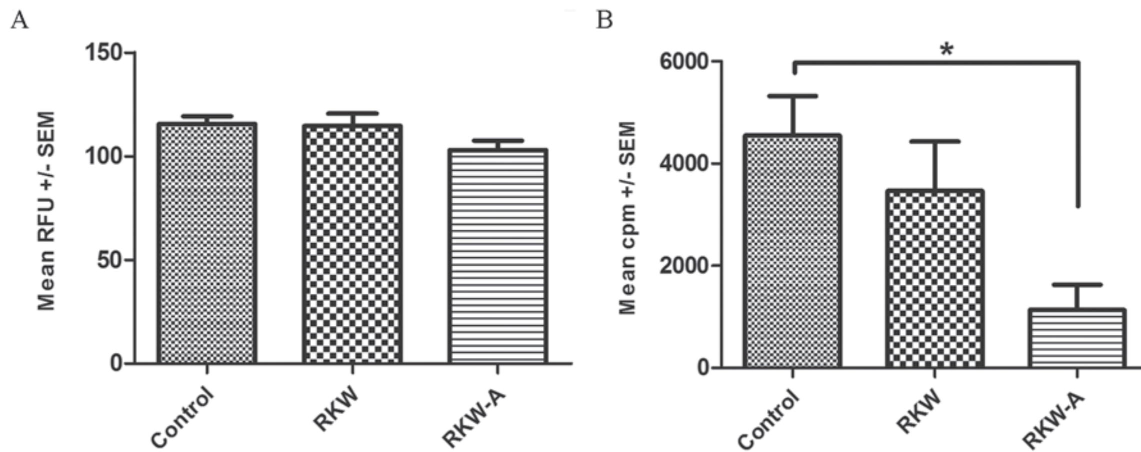

Figure 5. Proliferation index of splenocytes following lipopolysaccharide (LPS) stimulation. Results are presented as the mean RFU or cpm ± SEM, measured by the (A) alamarBlue assay [number of spleens tested, 59 (control, 31; RKW, 16; RKW-A, 12)] or (B) [^{3}H] thymidine incorporation assay following LPS (20 μg/ml) stimulation [number of spleens, 27 (control, 9; RKW, 9; RKW-A, 9)]. Statistical analysis performed: Unpaired *t*-test, Shapiro-Wilk normality test, one-way analysis of the variance and the Tukey comparison test. RFU, relative fluorescence units; SEM, standard error of the mean; cpm, counts per minute; RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract. *P=0.0175.

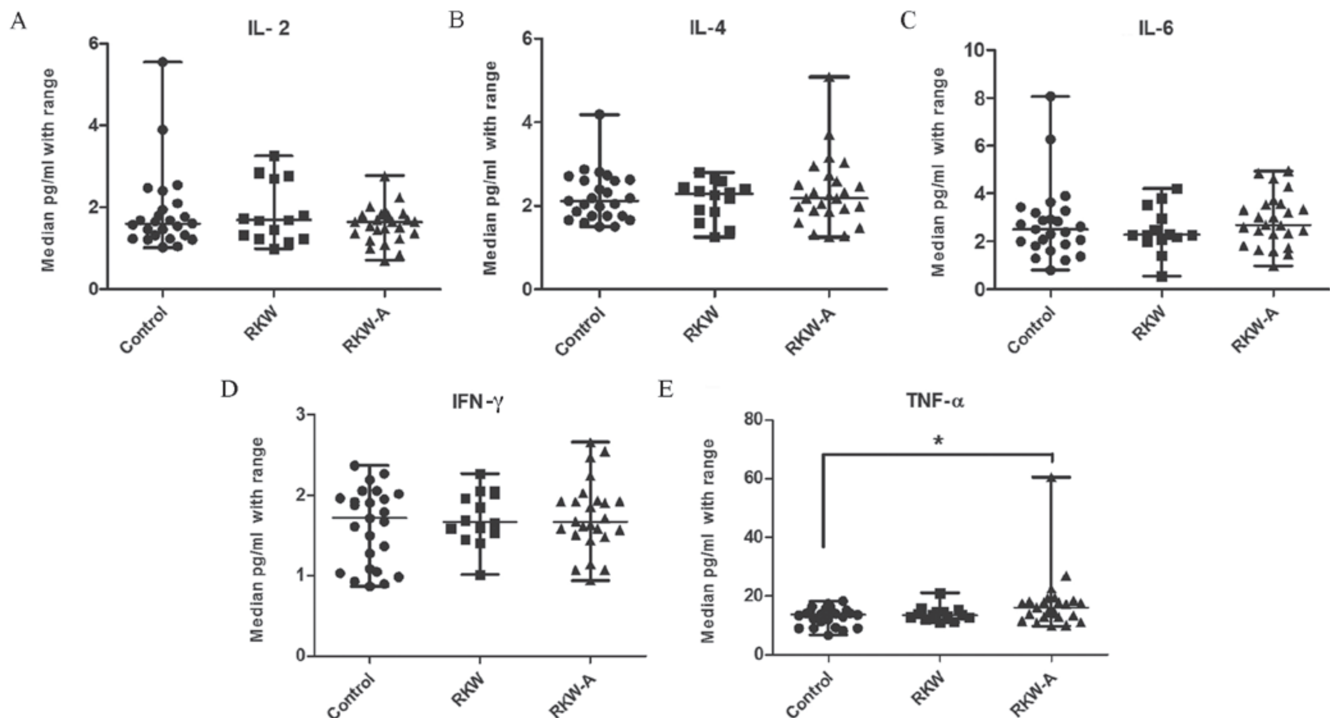

Figure 6. Concentration of selected cytokines (A) IL-2, (B) IL-4, (C) IL-6, (D) IFN-γ and (E) TNF-α in the sera. n=54 mice (control, 20; RKW, 18; RKW-A, 16). Results presented are the median ± the range of the cytokine in pg/ml. Statistical analysis performed: Unpaired *t*-test, Shapiro-Wilk normality test, Kruskal-Wallis test and Dunn's test. RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract. *P=0.0123 from Mann Whitney test.

topoiesis and the immune response (26). Primarily produced by Th2 lymphocytes, IL-10 generates and promotes T cell tolerance, via downregulation of IFN-γ, IL-2 and -5 production, proinflammatory cytokines, and eosinophil function and activity (27). TNF-α functions in the initiation of cellular and humoral immune responses (28).

In the present study, a minor increase was observed in the production of immunoregulatory IL-10 in mice whose mothers were fed with RKW-A extract, with a simultaneous increase in sera TNF-α concentration. This may be the reason for the altered spleen cell metabolism observed earlier, confirming the results of the alamarBlue and [^{3}H] thymidine incorporation assays. TNF-α directly promotes the growth and differentiation of neutrophils, macrophages and B cells.

IFN-γ, synthesized by natural killer cells and T lymphocytes, serves a role in the immune response against pathogens. IFN-γ activates macrophages and promotes differentiation of CD4$^+$ T lymphocytes into Th1 cells. However, in the present study this was not the case, because there were no differences in sera IFN-γ concentrations between the progeny of mice

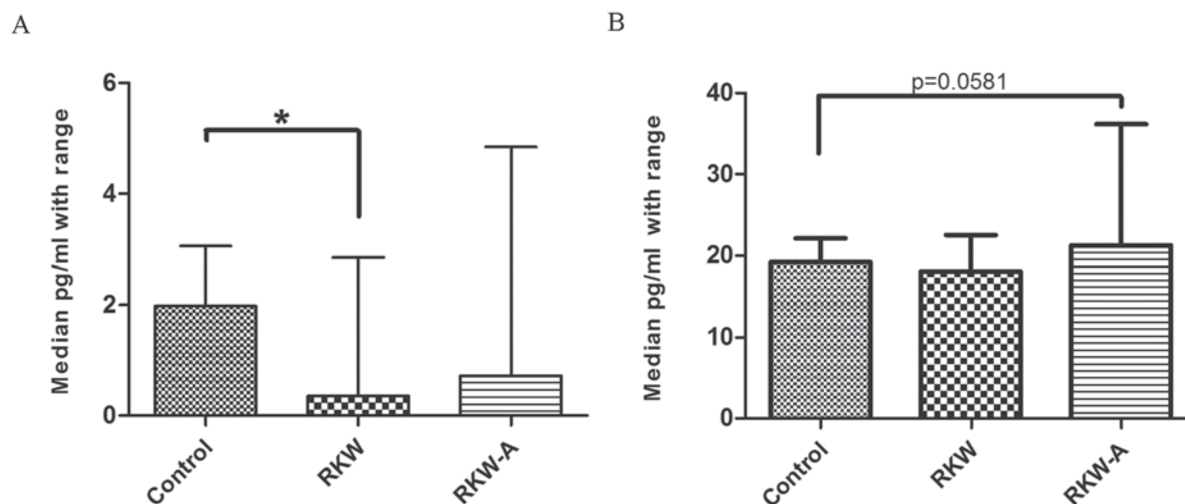

Figure 7. Concentration of (A) IL-17a and (B) IL-10 cytokines in the sera. n=54 mice (control, 20; RKW, 18; RKW-A, 16). Results present the median ± the range of the cytokine in pg/ml. Statistical analysis performed: Unpaired *t*-test, Shapiro-Wilk normality test and the Kruskal-Wallis test. RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract. *P=0.0347.

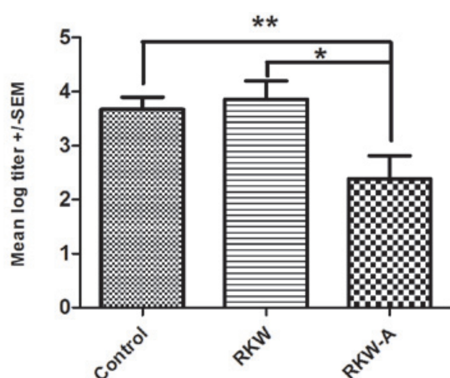

Figure 8. Levels of anti-sheep red blood cell (SRBC) antibody production. Results are presented as the mean log titer ± SEM of anti-SRBC antibody production in progeny whose mothers were fed RKW and RKW-A extracts during pregnancy and lactation. n=29 mice (control, 9; RKW, 7; RKW-A, 13). Statistical analysis performed: Unpaired *t*-test, Shapiro-Wilk normality test, one-way analysis of the variance and the Tukey comparison test. SEM, standard error of the mean; RKW, *Rhodiola kirilowii* water extract; RKW-A, *Rhodiola kirilowii* hydro-alcoholic extract. *P=0.0331, **P=0.0305.

fed *Rhodiola* extracts and the progeny of mice fed water (control).

Th17 cells primarily produce IL-17a, which is important in inducing and mediating the proinflammatory responses. In addition, IL-17a induces and promotes the production of the cytokines IL-6, TNF-α, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-1β, TGF-β cytokines and the chemokines IL-8, growth related oncogene-a and monocyte chemoattractant protein-1 (29,30). A previous study found that IL-17a can down-regulate Th1 differentiation (31).

The present study identified that RKW and RKW-A extracts did not change progeny sera expression of IFN-γ, IL-2, -4 or -6 compared with the control. However, TNF-α and IL-10 expression were increased in the progeny of mice fed with RKW-A extract. In addition, mice whose mothers were fed with RKW-A had lower antibody titers following immunization with SRBC, which may be connected with the increased serum concentration of IL-10 and decreased splenocyte response to LPS (a B cell mitogen) identified in the current study.

Interestingly, a significantly lower concentration of IL-17a in the sera and a significantly higher metabolic response to PHA was observed in the splenocytes of mice born to RKW-fed mothers, compared with mice born to mothers fed with RKW-A. This is consistent with the results of a previous study in a mouse model of acute graft-vs.-host disease, in which the authors determined that the absence of Th17 cells lead to augmented Th1 differentiation (31). These results suggest that PHA-responsive spleen cells are T helper lymphocytes, members of the Th1 population.

A previous study (14), in addition to the present study, observed a higher number of pups in the litters delivered by mothers that were fed RKW-A extracts. This is in agreement with a number of pre-clinical and clinical observations on the beneficial reproductive effects of other *Rhodiola* species, such as *R. rosea* (32,33).

Antibody production by B-cells is the primary component of the adaptive immunity response. Disorders in antibody production impair the ability of an organism to defend against microbial infections. In the present study, administration of RKW-A extract to mothers significantly decreased SRBC antibody production in comparison with RKW extract and the control (water). A previous study found water and hydro-alcoholic extracts of *R. quadrifida* had no effect on anti- SRBC antibody production (34). In contrast, Mishra *et al* reported that *R. imbricata* aqueous extract significantly enhanced tetanus toxoid-specific immunoglobulin levels and ovalbumin-induced antibody responses in a rat model (35). Differences in antibody production following supplementation of water or hydro-alcoholic extracts may be the result of the various *Rhodiola* species used in studies.

A recent study, performed in the same model, found that thymuses obtained from the progeny of mice treated with both types of *R. kirilowii* extract showed significantly lower total

apoptotic cell counts compared with the progeny of control mice, and that such treatment of mothers had no significant influence upon IL-7 expression of progeny thymocytes (36). The study concluded that *R. kirilowii* extracts may help to preserve thymus function in the progeny of treated animals.

The question of what causes the differences in the observed effects of RKW and RKW-A extracts arose from the results of the present study. In the present study, analysis of the content of selected polyphenolic compounds in RKW and RKW-A showed quantitative differences only, for which there are two possible explanations. Firstly, RKW-A extract may contain substances other than the analyzed polyphenolic compounds, which in RKW are absent or present in very low concentrations. Secondly, the different influences of RKW and RKW-A extracts on developing fetuses may be a result of having different concentrations of the analyzed polyphenols. Thirdly, there may differences in the bioavailability and biodistribution of selected polyphenols in serum and milk of mice-mothers.

In conclusion, progeny of RKW-A mothers differ from the control and RKW offspring in lower antibody production, lower response of splenocytes to LPS and ConA, and higher serum concentrations of TNF-α and IL-10. Spleens of RKW progeny were found to contain more $CD4^+$ cells compared with the spleens of control and RKW-A progeny. In addition, spleen cells collected from the progeny of RKW mice responded more to PHA (significantly in the almarBlue assay, on the borderline of significance in the [^{3}H] thymidine incorporation assay) compared with corresponding cells from RKW-A progeny. However, RKW and RKW-A offspring splenocytes presented significantly lower responses to ConA in the [^{3}H] thymidine incorporation assay compared with control group. Therefore, caution is recommended in the use of RKW and RKW- extracts, particularly long-term, as immunostimulants in pregnancy prior to further research.

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