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Original article

Spleen content of selected polyphenols, splenocytes morphology and function in mice fed *Rhodiola kirilowii* extracts during pregnancy and lactation

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Abstract

The genus *Rhodiola (Crassulaceae*) consists of many species, growing mainly in Asia and traditionally used as adaptogens and anti-inflammatory drugs. In order to elaborate herbal immunostimulator which could be safely given to pregnant women, we performed a study on immunotropic effects of feeding pregnant and lactating mice *Rhodiola kirilowii* extracts. This paper presents the results of the first part of our study – spleen content of selected polyphenols, spleen cellularity, splenocytes phenotype and their response to mitogens. Experiments were performed on adult inbred females of Balb/c strain, mated with adult males. Females, since copulatory plug was noted, up to the 28-th day after delivery were fed daily with 20 mg/kg b.m. water (RKW) or hydro-alcoholic (RKW-A) extracts of *Rhodiola kirilowii*. Results: 1. Significantly lower proportion of pregnant mice in experimental groups than in the control. 2. Cellularity of spleen and flavonol quercetin spleen concentration were significantly lower in experimental groups in comparison to the controls. 3. Flavanols ((+)-catechin and epicatechin) levels were significantly higher in the spleens of experimental mice than in the controls. 4. Positive correlation between spleen cellularity and quercetin, and negative correlation between spleen cellularity and epicatechin content were observed. 5. Spleen mass and spleen lymphocytes phenotype and proliferation in RKW and RKW-A fed mice did not differ from the control. These results, together with suspicion of some embryo-toxicity, are worrying and eliminate the possibility of use *Rhodiola kirilowii* extracts for long-term treatment in pregnant females.

Key words: mouse, pregnancy, *Rhodiola kirilowii*, polyphenols, spleen, lymphcytes

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Introduction

Everyday diet and most plant-derived pharmaceuticals and diet supplements contain substantial amounts of polyphenolic compounds. The flavonoids, which are the best defined group of polyphenols in human diet, are a large and complex group in themselves. They exert various biological effects, stimulatory or inhibitory, depending on the tested parameter and type of the experiment (Gee et al. 2001). Bioflavonoids are commonly known for their beneficial effects on health. They demonstrate antioxidant, anti-inflammatory, anticancer, cardio-protective, antimicrobial and antiviral properties, and are considered to be safe, both in herbal supplements and in everyday diet. This, however, might not always be the case. Little is known about possible adverse effects of flavonoids. Recently, Dong et al (2014) reported that some flavonoid compounds (daidzein, genistein, hesperidin, naringenin) significantly inhibited influenza virus replication, while others (kaempferol, chrysin, diosmetin, icariin) dramatically promoted virus replication. These opposite effects were due to modulating cell-autonomous immunity (cellular self- defense) by MAPK signaling pathways.

Vanhees et al reported that flavonoids can induce DNA double-strand breaks and prenatal exposure to some of them (genistein and quercetin) resulted in a slight increase in the incidence of malignancies in DNA repair-deficient mice (Vanhees et al. 2011).

There is also insufficient knowledge concerning the effects of flavonoid-rich food supplements and herbal drugs on pregnancy. In humans, polyphenols, through their anti-inflammatory action, when ingested during the third trimester of pregnancy, may influence the dynamics of fetal ductus arteriosus flow (Zielinsky and Busato 2013). On the other hand, the citrus flavone nobiletin, through reduction of pro-inflammatory mediators, decreased the risk of infection- induced preterm birth (Morwood and Lappas 2014).

Polyphenol silibilin in pregnant mice with infection-induced inflammation exerted a protective effect on infection- induced brain injury in this model of preterm birth (Lim et al. 2014).

Curcumin, naringenin and apigenin were shown to exert an anti-inflammatory effect in human gestational tissues by inhibiting the transcriptional activity of NF-kB (Lim et al. 2013). Polyphenols have also been shown to influence fertility and sexual development (Ly et al. 2014). Chu et al. described an uptake in fetal organs, when green tea catechins were orally administered to pregnant rat females (Chu et al. 2007).

Recently, Lesser et al. reported an effect of rutin, (-)-epicatechin and (+)-catechin on mice. It was ingested prior to, and during pregnancy and lactation and had affected reproductive and developmental processes. It also influenced maternal and offspring tissue mineral concentrations (Lesser et al. 2015). Authors observed alterations in maternal and offspring liver mineral concentrations, and no marked developmental effects.

In the past, we have performed studies on the immunotropic (*in vitro*) and angiomodulatory (*in vitro* and *in vivo*) activity of alcoholic and aqueous extracts of roots and rhizomes of these plants in mice, rats and pigs (Siwicki et al. 2007, Skopińska-Różewska et al. 2008, Wójcik et al. 2009). The aim of our present study was to determine the effect of feeding mice (during pregnancy and lactation) *Rhodiola kirilowii* extracts rich in flavonoids, on the splenocytes number, phenotypes, response to mitogens and spleen content of selected polyphenols.

Material and Methods

Plant cultivation

Rhodiola kirilowii (*Crassulaceae*) roots and rhizomes were cultivated, collected and identified in Department of Botany, Breeding and Agriculture of Institute of Natural Fibres and Medicinal Plants, Poznań. Voucher specimen is kept in the herbarium of this department.

Preparation of extracts

Water extract (RKW): finely powdered roots were extracted two times with water (first – 2 hours and second 1 hour long) in the ratio raw material/solvent (1/5), at the temperature 40-45°C. The supernatants were mixed together, spun and lyophilized. Hydro-alcoholic extract (RKW-A): 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 at the temperature 40-45°C. Dry extract ratio (DER) values were: 5.09/1 for RKW and 3.27/1 for RKW-A. Extracts were stored at -70°C until used.

Chemical analysis of phytochemicals from *Rhodiola kirilowii* extracts in spleen tissue

Total extracts' polyphenol/flavonoids concentration was assayed by using the HPLC system (Dionex) equipped with the CoulArray electrochemical detector (ESA Inc). The extraction procedure of poly-

phenols/flavonoids was performed as previously described (Zdanowski et al. 2014).

Concentration of total flavonoids from spleen tissue conjugated with glucuronic acid, sulfate or glycoside groups was analysed according to Erlund et al. (1999). The analysis of flavonoids' separation and contents was done by applying the HPLC system (Dionex) equipped with the CoulArray electrochemical detector (ESA Inc.) The separation was conducted on a Hypersil BDS 150 x 4.6 mm, 5 μm column (Sigma-Aldrich) at mobile phase flow rate of 1.2 ml/min. The mobile phase consisted of sodium phosphate 50 mM: methanol (99:1) pH 3.0. The conditions of electrochemical detection: four electrodes with potentials 400, 500, 600, and 750 mV. The chromatograms were processed by identifying the compounds on the base of standards and areas of chromatographic peaks, taking into account their retention times as well as the ratio of the peak area for dominating electrode to that of neighboring electrodes.

Animals

Experiments were performed on 128 adult inbred females of Balb/c strain, 8-9 weeks old, mated with adult males from the same strain. Females, since copulatory plug was noted, up to the 28-th day after delivery were fed daily with dissolved in distilled water, lyophilized RKW or RKW-A extracts (20 mg/kg b.m.). Dose of extract corresponds to 200 mg given to 70 kg person, applying the coefficient equal 7 for adjusting differences between mouse and human in relation of the surface to body mass. The control group received distilled water. Females were housed separately and to avoid stress connected with gavage the substances were applied on a corn crisp and served to the female in a Petri dish.

For all performed experiments animals were handled according to the Polish regulation concerning the welfare of laboratory animals. All experiments were accepted and conducted according to ethical guidance of Local Bioethical Committee, (permission 73/2011). Mice were maintained under conventional conditions (room temperature 22.5-23.0°C, relative humidity 50-70%, 12h day/night cycle) with free access to breeding rodent feed (Labofeed H, Wytwórnia Pasz „Morawski") and water.

Spleen isolation

Mice were bled in anaesthesia (intraperitoneal injection of ketamine 120 mg/ kg of b.w. and xylazine 12 mg kg of b.w. solution) from retroorbital plexus. After bleeding mice were sacrificed (pentobarbital, 400 mg/kg b.w.) and spleens were isolated in aseptic conditions (laminar flow chamber).

Preparation of splenocytes suspension

Spleens collected from mice were gently pressed through the sterile nylon strainer (40 ,m) to a 50 ml tube (Falcon) with 20 ml of medium (RPMI 1640 with 10 % FBS). Strainers were rinsed twice with medium to remove all remaining cells. Next, the cells were centrifuged (500 x g, 5 min.), pellet was resuspended in medium and cells were counted in the hematological analyzer (Exigo, Boule Medical AB). Cell suspension containing $1x10^6$ cells/ml was used to evaluate response to mitogens. Splenocytes viability was determined by Trypan Blue method and amounted to over 95% of living cells.

Response of spleen cells to mitogens

Spleens cells were seeded on 96 multi-well dish ($1x10^5$ cell/per well) and after 1 hour of incubation the following mitogens were added: LPS (1 μg/ml), ConA (5 μg/ml), or PHA (2 ,g/ml). 24 hours later Alamar-Blue® (1:10, v/v, PAA) was added directly to the wells. Cells were incubated for the next 24h at standard conditions (37°C 5% CO_2, 95% RH). Fluorescence (excitation 544 nm and emission 590 nm) was measured directly in the wells on FLUOstar Omega reader (BMG Labtech) (Zdanowski et al. 2012).

Spleen phenotype determination

Spleen cells suspensions (100 μl, $1x10^6$ cell/ml) were labeled by surface staining with fluorochrome-coniugated anti-mouse antibodies, according to the manufacturer's protocol (BD Biosciences). Three panels were established: panel 1 – Mouse T Lymphocyte Subset Antibody Cocktail (BD Biosciences): CD3e PE-Cy™7, CD4 PE, CD8a APC, panel 2 – Mouse B Lymphocyte Activation Antibody Cocktail: CD 19 APC and panel 3 – CD3 FITC, CD 335 PE. Before all flow cytometry analysis red blood cells were lysed (10 min., cell lysate BD Biosciences). Phenotype analysis was performed by flow cytometry (FACS Calibur, BD). Results are presented as mean % ± SEM.

Statistical analysis

Statistical evaluation of the results obtained in the control and experimental groups was done using Pearson's correlation, unpaired t test, one way ANOVA or two-way ANOVA with Tukey's or Bonferroni multiple comparison post- test (Graph Pad Prism).

Results

Experiments were performed on 128 female inbred mice of Balb/c strain. Out of 45 mice belonging to the control group, 42 females fed water extract and 41 mice fed hydro-alcoholic extract, 32, 18 and 16 became pregnant after mating, accordingly.

Analysis of *R.kirilowii* extracts revealed the presence of phenyloethanoid salidroside, four phenolic acids (chlorogenic, ferulic, ellagic and p-coumaric), and flavonoids (fisetin, naringenin, kaempferol, epicatechin, luteolin, quercetin, epigallocatechin, and (+)-catechin). The RKW-A extract presented higher concentration of identified compounds (except epigallocatechin gallate), the lowest differences were observed for quercetin (18%), ferulic acid (30%) and salidroside (35%) and the highest for (+)-catechin (119%), p-coumaric acid (126%) and naringenin (127%) (Table 1).

From these compounds, the following ones were found in the spleens of mice after feeding them *Rhodiola kirilowii* extracts for 47-51 days (during 19-23 days of pregnancy and 28 days after delivery): kaempferol, epicatechin, quercetin, (+)-catechin, salidroside (Table 1). Kaempferol and salidroside were absent in the spleens of mice belonging to the control group. Epicatechin level in control spleens was also very low. However, spleens from control animals contained significantly higher amounts of quercetin than the spleens of animals from both experimental groups. Concentrations of kaempferol and epicatechin were significantly higher in RKW-A than in RKW spleens.

Pearson's analysis revealed negative correlation between epicatechin and quercetin concentrations in the spleens collected from RKW- fed mice (Fig. 1A), as well as negative correlation between epicatechin and kaempferol concentration in the spleens collected from RKW-A mice (Fig. 1B), but no correlations between content of the remaining compounds. Cellularity of spleens of mothers was significantly lower in both experimental groups in comparison to the controls. There is a similar tendency in groups of non-pregnant females, however no statistical significance had been observed (Fig. 2B). Positive correlation was observed between spleen cellularity and quercetin (Fig. 2C) and negative correlation was present between spleen cellularity and epicatechin content (Fig. 2D). No correlation between spleen cellularity and catechine, kaempferol or salidroside levels was found.

In contrast to spleen cellularity, neither RKW nor RKW-A extracts affected spleen mass and relative spleen mass index (Fig. 2A). Morphology of spleen cells was unchanged after RKW administration,

Table 1. Concentration of polyphenols in *Rhodiola kirilowii* extracts and in spleen tissue (HPLC-ECD). Two extracts were examined: RKW (water extract) and RKW-A (hydro-alcoholic extract). Results are presented as a mean +/- SEM. [a] – significant differences in comparison to control, [b] – significant differences between RKW and RKW-A extracts. *HPLC-DAD, (-) not present.

Polyphenols	Extracts (μg/mg of dry extract)		Spleens (ng/g of tissue)		
	RKW	RKW-A	Control	RKW	RKW-A
kaempferol	0.64 ± 0.02	1.06 ± 0.03[b]	(-)	0.15 ± 0.01	2.13 ± 0.23[ab]
epicatechin	2.66 ± 0.10	4.44 ± 0.06[b]	0.07 ± 0.01	1.54 ± 0.06[a]	2.87 ± 023[a,b]
quercetin	1.88 ± 0.05	2.22 ± 0.05[b]	1.15 ± 0.08	0.24 ± 0.02[a]	0.06 ± 0.01[ab]
(+)-catechin	0.90 ± 0.02	1.96 ± 0.01[b]	1.06 ± 0.33	2.35 ± 0.17[a]	2.97 ± 0.62
asalidroside	2.62 ± 0.12	3.56 ± 0.34[b]	(-)	1.87 ± 0,19[a]	2.81 ± 0.39[a,b]
fisetin	0.80 ± 0.03	1.11 ± 0.03[b]	(-)	(-)	(-)
naringenin	0.04 ± 0.00	0.09 ± 0.01[b]	(-)	(-)	(-)
luteolin	0.04 ± 0.00	0.08 ± 0.01[b]	(-)	(-)	(-)
p-coumaric acid	0.39 ± 0.02	0.87 ± 0.04[b]	(-)	(-)	(-)
ellagic acid	0.68 ± 0.02	1.08 ± 0.03[b]	(-)	(-)	(-)
epigallocatechin	1.34 ± 0.04	1.90 ± 0.06[b]	(-)	(-)	(-)
ferulic acid	0.93 ± 0.04	1.21 ± 0.03[b]	(-)	(-)	(-)
chlorogenic acid	1.02 ± 0.01	1.50 ± 0.01[b]	(-)	(-)	(-)
epicatechin gallate*	1.45 ± 0.01	2.41 ± 0.02[b]	(-)	(-)	(-)
epigallocatechin gallate*	0.77 ± 0.01	0.26 ± 0.01[b]	(-)	(-)	(-)
N	3	3	12	10	7

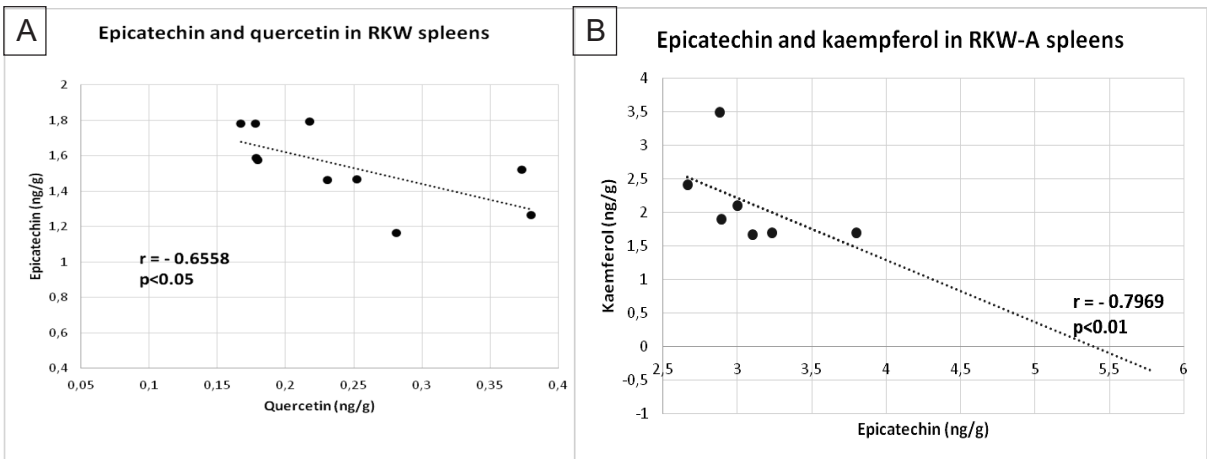
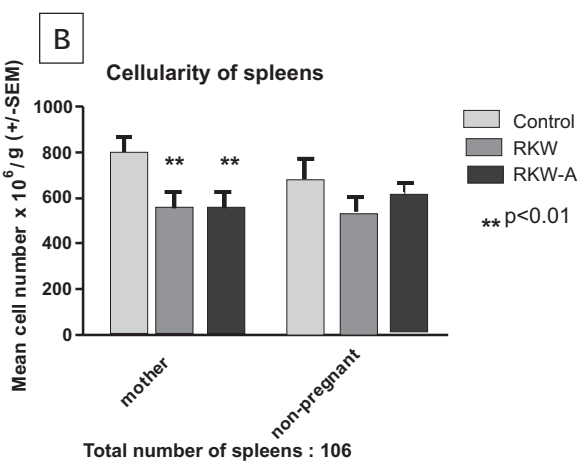
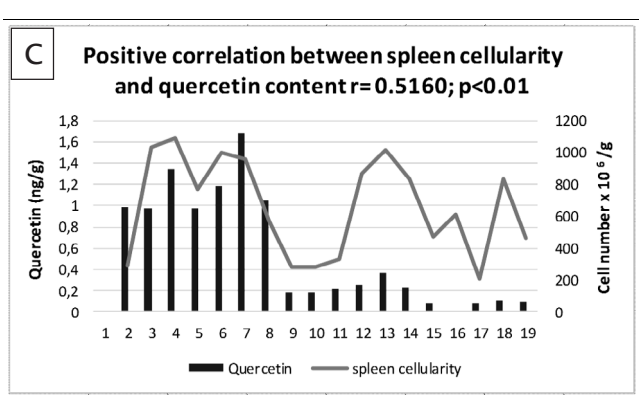


Fig. 1. Negative correlation between epicatechin and quercetin (A) and epicatechin and kaempferol (B) concentration in spleens of mice fed *Rhodiola kirilowii* aqueous (RKW) extract (r = -0.6558; p<0.05) and *Rhodiola kirilowii* hydro-alcoholic (RKW-A) extract (r = -0.7969; p<0.01).

A

	Spleen mass (mg)			Relative spleen mass (index)		
	Control	RKW	RKW-A	Control	RKW	RKW-A
Mean	109,3	111,3	118,0	4,40	4,52	4,79
SEM	2,9	3,5	6,9	0,10	0,13	0,32
N	25	16	16	25	16	16





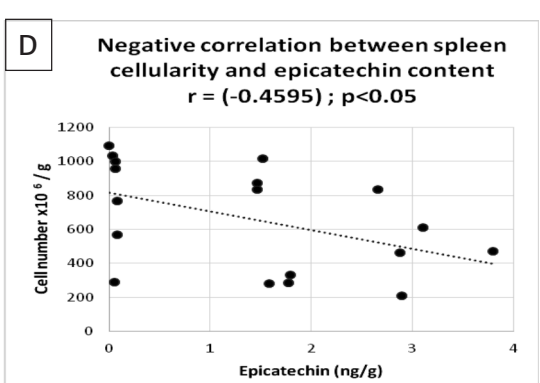


Fig. 2. Spleens analysis: average spleen mass (mg) and relative spleens mass index (mg of spleen/g of animal mass) (**A**), cellularity (**B**) and correlations between spleen cellularity and quercetin (**C**) or epicatechin (**D**).

however some slight, but significant, changes were noted after RKW-A administration (decreased percentage of lymphocytes and increased percentage of monocytes and granulocytes, p<0.05, Table 2). We did not observe percentage differences in spleen lymphocytes phenotype (CD3, CD4, CD8, CD19, CD335 -NK cells and CD4/CD8 ratio, Table 2). Functionality of spleen cells, measured as proliferation potential by AlamarBlue® method, was also not disturbed after RKW and RKW-A administration in both control condition (unstimulated cells) as well as after PHA, ConA or LPS stimulation (Table 3).

Table 2. Morphology and lymphocytes phenotype of spleen cells. Results are presented as mean ± SEM. * – significant differences in comparison to control group ($p<0.05$).

	Lymphocytes (%)			Monocytes (%)			Granulocytes (%)		
	Control	RKW	RKW-A	Control	RKW	RKW-A	Control	RKW	RKW-A
Mean	91,9	91,6	89,4*	2,80	2,92	3,31*	5,29	5,48	7,34*
SEM	0,5	0,7	0,6	0,11	0,17	0,13	0,42	0,51	0,54
N	8	6	7	8	6	7	8	6	7
	CD3			CD19			NK		
	Control	RKW	RKW-A	Control	RKW	RKW-A	Control	RKW	RKW-A
Mean	47,1	45,2	54,6	33,6	34,5	29,7	15,3	13,2	15,6
SEM	2,5	3,5	3,1	4,9	6,3	2,8	2,4	2,6	2,8
N	8	7	10	8	7	10	8	7	10
	CD4			CD8			CD4/CD8 ratio		
	Control	RKW	RKW-A	Control	RKW	RKW-A	Control	RKW	RKW-A
Mean	35,0	35,5	34,5	10,6	12,2	11,3	3,46	3,02	3,32
SEM	2,7	2,3	1,6	0,8	1,0	1,0	0,49	0,32	0,37
N	8	7	11	8	7	11	8	7	11

Table 3. Proliferation index (mean RFU ±SEM) of spleen cells measured by A.Blue® test.

	Control				RKW				RKW-A			
	Unstimulated	PHA	ConA	LPS	Unstimulated	PHA	ConA	LPS	Unstimulated	PHA	ConA	LPS
Mean	69,1	99,8	158,8	111,5	63,1	93,4	157,5	108,0	70,0	97,8	152,8	118,5
SEM	1,9	4,1	4,7	4,1	2,7	5,7	8,7	3,6	3,4	6,1	8,4	6,9
N	19	19	19	19	11	11	11	11	10	10	10	10

Discussion

In both experimental groups the number of mated mice without offspring was significantly higher (almost twice) than in the control one, which confirms our initial observations (Zdanowski et al. 2014). The ability of catechins to cause cell death was also reported. Tu et al. (2010) presented adverse epicatechin gallate influence on the viability and subsequent embryonic development of mouse blastocysts in vitro. The effects of tea catechins on developing embryos were studied in tissue culture and some of them were classified as weakly embryo-toxic. This effect was associated with increased embryo 8- isoprostane (Wang et al. 2007). Accordingly, other authors described inhibitory effects of catechin derivatives on mammalian DNA polymerase and topoisomerase activities and mouse one-cell zygote development (Yoshida et al. 2013). Recently, induction of embryonic toxicity through apoptosis by epigallocatechin gallate in mouse blastocysts was described (Fan and Chan 2014). Therefore, one may suspect a higher rate of embryos mortality in the experimental groups than in the control group.

To our knowledge, this is the first study of spleen levels of polyphenols after repeated treatments of mice with extracts prepared from *Rhodiola kirilowii* rhizoma and roots. As we presented in the Table 1, spleens of control group of mice contained mainly quercetin and (+)-catechin, with trace amounts of epicatechin. Kaempferol and salidroside were not present in control spleens. Concentration of salidroside is high in both experimental groups, higher in RKW-A than in RKW spleens. The same situation is with epicatechin. In the case of catechin, its concentration is significantly higher in spleens of both experimental groups of mice than in the controls.

Therefore, one may argue that, the long-term in vivo exposition to salidroside, epicatechin or catechin, or the sum of them, would be responsible for significantly lower cellularity of spleens from mice belonging to both experimental groups. However, the role of salidroside in this phenomenon is rather doubtful. Salidroside, a phenylpropanoid glycoside, possess anti-stress, anti-hypoxia, anti-inflammatory, anti-viral, anti-cancer (anti-proliferation effect on cancer cell lines, induction of cell-cycle arrest and apoptosis, suppression of tumor angiogenesis) and anti-fatigue effects. Recently, its protecting activity in D-galactose-induced mouse model of aging has been demonstrated (Lu et al. 2013). The immunotropic effects of salidroside are not inhibitory. It was reported that this

compound, in liposome formulation, enhanced the activity of dendritic cells *in vitro* and immune response *in vivo* (Zhao et al. 2013). Salidroside promoted the proliferation of bone marrow mesenchymal stem cells and increased the expression and secretion of stem cell factor (Bai et al. 2014). Moreover, salidroside significantly inhibited the apoptosis of human bone marrow mesenchymal stem cells induced by cytarabine C, through mechanism related to the regulation of BCL-2/BAX expression (Wei et al. 2013). In aged rats, dietary intake of salidroside induced an increase in total T cells and and T-helper cells (Lu et al. 2013). An adjuvant effect of salidroside on the immune responses to ovalbumin in mice was observed (Guan et al. 2011). Therefore, the deleterious role of salidroside on spleens cellularity should rather be excluded.

The role of kaempferol in lowering spleen cells number in both experimental groups is also doubtful because this compound was present in very high concentration in RKW-A spleens, and in very low amounts in the spleens obtained from RKW-fed mice. The role of quercetin could be protective, what is suggested by the existence of positive correlation between the amount of quercetin and spleen cellularity.

Moreover, we observed a negative correlation between quercetin and the main candidate for observed phenomena, epicatechin, which was correlated negatively with spleen cellularity (Fig. 2D).

This is a little surprising, because quercetin and various catechins in some situations act similarly. Their antioxidant capacity is due to their stabilizing effect on the cell membranes. Both quercetin and (-)-epicatechin can augment nitric oxide status and reduce endothelin-1 concentrations (Loke et al. 2008). Catechin and quercetin significantly reduced organophosphorus insecticide chlorpyrifo- induced hepatotoxicity in rats (Uzun and Kalender 2013). Some authors describe their synergistic action. Catechin and quercetin, and to a larger extent the combination of both, attenuated adipose inflammation in fructose-fed rats (Vazquez Prieto et al. 2015). Catechin, quercetin and epigallocatechin gallate, when combined with fluconazole, induce apoptosis in *Candida tropicalis* resistant to fluconazole (DaSilva et al. 2014).

On the other hand, it was recently reported that interaction of (-)-epicatechin with food components, including other polyphenols (among them quercetin, kaempferol, chlorogenic acid), modified its absorption, metabolism and bioactivity (Sanchez-Bridge et al. 2015).

Flavonols and flavanols are anti-cancer agents. They were shown to inhibit tumor angiogenesis and to exert anti-proliferative and pro-apoptotic effects on cancer cells. Quercetin, a natural inhibitor of catechol-O-methyltransferase and multidrug resistance proteins increased bioavailability and decreased methylation of green tea catechins *in vitro* and *in vivo* (Wang et al. 2012) Kaempferol alone, in combination with conventional chemotherapeutic drugs, or in combination with quercetin, inhibits cell proliferation and induces apoptosis in various cancer cells (Rajendran et al. 2014).

Kaempferol and catechins, but not quercetin, were described as inhibitors of mouse and human lymphocyte proliferation. Kaempferol inhibited the activation and proliferation of mouse T lymphocytes in response to ConA, arresting cell cycle at S phase and G2/M *in vitro* (Mu et al. 2009). In humans, green tea catechin EGCG significantly suppressed *in vitro* lymphocyte proliferation in response to stimulation and IFN gamma production (Saleh et al. 2014). Recent studies have shown that green tea catechin, EGCG, affects the differentiation of najve T cells into different effector subsets (Pae and Wu 2013). This might partly explain why in our present study we did not observe percentage differences between various splenocytes subsets.

Conclusions

In the long-term treatment model applied we did not observed immunostimulatory effects in pregnant mice. Extracts of *Rhodiola kirilowii* (RKW and RKW-A) used in the present study did not affect the proportions of various lymphocyte subpopulations in the spleen and their proliferative responses after mitogens stimulation. However, the compounds (probably catechins) present in the extracts had a strong negative impact on the spleen cellularity of the treated animals. The obtained results, together with suspicion of some embryo-toxicity, are worrying and eliminate the possibility of use *Rhodiola kirilowii* extracts for long-term treatment in pregnant females.

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