The effect of *Rhodiola kirilowii* extracts on tumor-induced angiogenesis in mice

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Abstract

Rhodiola kirilowii is an adaptogenic plant indigenous to the high altitude of Qinghai-Tibet Plateau in China. The plant is used in the Chinese traditional medicine for various purposes, mainly as general adaptogen for enhancement of the ability of anti-anoxia, protecting people against cardiopulmonary function problems when moving to high altitude, mild anti-stress and anti-inflammatory action. In our previous studies we obtained for the first time evidence of in vitro and in vivo immunotropic activity of Rhodiola kirilowii aqueous (RKW) and hydro-alcoholic (RKA) extracts in mice, rats and pigs. The first aim of the present work was to compare the in vivo influence of these two extracts on cutaneous angiogenesis induced in Balb/c mice by grafting sarcoma L-1 syngeneic tumor cells. The second aim was to compare the in vitro influence of RKW and RKA extracts on the migration as well as on the proliferation of murine endothelial (HECa10) cells and on the proliferation of murine tumor (L-1 sarcoma) cells in tissue culture.

Results: *In vivo, RKA administered orally significantly suppressed neovascular reaction to L-1 sarcoma cells. Rhodiola kirilowii aqueous had no influence on cutaneous angiogenesis reaction. In vitro, experiments showed that both extracts have stimulated the proliferation of HECa10 cells, and both of them suppressed proliferation of L-1 sarcoma cells. Inhibitory effect of RKA extract and stimulatory influence of RKW extract on HECa10 cells migration in the presence of L-1 sarcoma cells were observed. These findings might partly explain the differences in their in vivo effect.*

Key words: *angiogenesis, mice, HECa10, migration, proliferation, L-1 sarcoma, Rhodiola kirilowii.*

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Introduction

Genus *Rhodiola* (*Crassulaceae*) consists of a number of species that are popular in traditional Chinese Tibetan medicinal herbs. *Rhodiola* plants are native to high mountains and arctic regions of Asia, Europe and North America, and are known as general adaptogens, anti-depressant, stimulating and anti-stress remedies. In Europe the best known is *Rhodiola rosea*. Its anti-stress properties appear to depend on its ability to modulate the activation of several components of stress-response systems [1-3]. In our earlier papers we reported for the first time immunostimulatory, anti-oxidant and anti-angiogenic effects of *Rhodiola rosea* roots aqueous and hydro-alcoholic extracts [4-9]. To the less known, used in traditional medicine of Russia and China belong species: *Rhodiola quadrifida* and *Rhodiola kirilowii.* We reported for the first time some data on the immunotropic and anti-angiogenic effects of *Rhodiola quadrifida* [10-16]. Previously, Yoshikawa *et al.* described chemical structures and antiallergic activity of some com-

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pounds present in underground parts of *Rhodiola quadrifida* [17].

Rhodiola kirilowii was described by Chinese authors, in the earliest available publication from 1989 year, as an adaptogen preventing high altitude sickness [18]. *Rhodiola kirilowii* extract protected highland villagers from abnormalities in their cardiopulmonary function as they move from 2500 m altitude to 4475 m altitude areas. Recently, *Rhodiola kirilowii* has been used in divers, astronauts, pilots and mountaineers, and also for resisting anoxia, microwave radiation and fatigue. Some phenolic compounds from this plant expressed *in vitro* activity against HCV NS3 serine protease [19] what may be of value in future perspectives of hepatitis C treatment [20]. Antituberculous activity of *Rhodiola kirilowii* roots was also described [21]. Rhizoma and roots of *Rhodiola kirilowii*, similarly to other *Rhodiola* species, contain a lot of various phenolic compounds. The main difference from *Rhodiola rosea* is higher content of catechins (epigallocatechin, epigallocatechin gallate, epicatechin, epicatechin gallate) and lack of phenylpropanoids (rosavin etc.) [22-26].

In the last years, we described for the first time the stimulatory effect of *Rhodiola kirilowii* extracts on some parameters of cellular immunity in mice, rats and pigs. We also obtained some preliminary evidence of different effects of aqueous and hydro-alcoholic extracts on tumor-induced angiogenesis [6, 13, 27-29].

The first aim of the present work was to compare the *in vivo* influence of these two extracts on cutaneous angiogenesis induced in Balb/c mice by grafting sarcoma L-1 syngeneic tumor cells. The second aim was to compare the *in vitro* influence of *Rhodiola kirilowii* aqueous (RKW) and hydro-alcoholic (RKA) extracts on the migration and proliferation of murine endothelial (HECa10) cells and on the proliferation of murine tumor (L-1 sarcoma) cells in tissue culture.

Material and methods

Preparation and chemical analysis of extracts

Rhodiola kirilowii (*Crassulaceae*) roots and rhizomes were cultivated, collected and identified in the Research Institute of Medicinal Plants (RIMP), presently Institute of Natural Fibres and Medicinal Plants, Poznań. The cultivation was established by vegetative propagation. 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. Voucher specimen is kept in the herbarium of Department of Botany, Breeding and Agriculture in Plewiska near Poznań.

Sample extractions were prepared by the methods as described below. Aqueous extract: finely powdered roots were extracted two times with water (extraction was performed: first – 2 hours 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: 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. Extracts were stored at –70˚C until used.

The content of phenolic compounds was determined by means of two methods: ultra performance liquid chromatography (UPLC) and high-performance liquid chromatography (HPLC).

Ultra performance liquid chromatography. An UPLC connected to a tandem mass spectrometer (UPLC-MS/MS; Waters) was used for chemical analyses. The separation of analytes was performed on an Acquity UPLC BEH C18 column, 1.7 µm 2.1 × 50 mm (Waters). Mobile phase: phase A: 0.1% (v/v) HCOOH solution in water, phase B: 0.1% (v/v) HCOOH solution in acetonitrile. Mobile phase flow rate was: 0.20 ml/min. The assay was performed in gradient elution: 0.0 min – 97% of phase A, 7.5 min – 68% of phase A, 9.0 min – 97% of phase A. Column temperature was 25˚C; ion source temperature: 120˚C; desolvation temperature: 350˚C. Gas flow rate: desolvation gas: 700 L/h; cone gas: 10 L/h. Peaks were identified by spiking the samples with standard compounds.

High-performance liquid chromatography. High-performance liquid chromatography analysis was performed (with the samples diluted with methanol) on Agilent 1100 HPLC system, equipped with photodiode array detector. For all separations a LiChrospher 100 RP18 column (250.0 × 4.0 mm, 5 µm) 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 95 A/5 B to 80 A/20 B for 30 min then from 80 A/20 B to 20 A/80 B for 5 min and an isocratic elution for 15 min. Each run was followed by an equilibration period of 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 were injected. All separations were performed at a temperature of 25˚C. Peaks were identified by spiking the samples with standard compounds and comparison of the UV-spectra and retention times.

Spectrofotometric analysis of tannins was performed according to the methods of European Pharmacopoeia on UV-Visible Spectrometer Cintra 20 GBC 9 (Determination of tannins in herbal drugs. European Pharmacopoeia, 6th ed. 2008; 1: 255).

Mice

The study was performed on 7-8 weeks old inbred Balb/c mice, weighing about 20 g, females, delivered from

the Polish Academy of Sciences breeding colony. *Rhodiola kirilowii* extracts were administered to mice *per os* in daily doses 50, 100, 200 or 400 μg. These doses corresponded to 25, 50, 100 or 200 mg given to 70 kg person (applying the counter 7 for differences between mouse and human in relation of the surface to body mass). Mice received drugs by Eppendorf pipette, in 40 μl of 10% ethyl alcohol, for 3 days after tumor cells grafting. Control animals received 10% alcohol.

Tumor cells

Sarcoma cells were delivered from Warsaw's Cancer Center collection and then passaged through several generations of Balb/c mice, according to the method described [30]. For experiments cells from the 7^{th} and 8^{th} passage were used. Briefly, sarcoma cells were grafted (10^6/0.1 ml) subcutaneously into subscapular region. After 14 days the tumours were excised, cut to smaller pieces, rubbed through sieve and suspended in 5 ml of PBS. The suspension was left for 10 min at room temperature. After sedimentation the supernatant was collected and centrifuged for 10 min at 300 × g. Obtained sarcoma cells were washed once with PBS for 10 min, then centrifuged at 300 × g, and resuspended in Parker medium in a concentration of 4×10^6/ml.

Angiogenesis induced in the skin of Balb/c mice after grafting of L-1 sarcoma cells (TIA test)

Tumor-induced cutaneous angiogenesis assay was performed according to Sidky and Auerbach [31] with own modifications [32-34]. Briefly, multiple 0.05 ml samples of 200 thousand of cells were injected intradermally into partly shaved, narcotised Balb/c mice (at least 3 mice per group). In order to facilitate the localisation of cell injection sites later on, the suspension was coloured with 0.1% of trypan blue. On the day of cells grafting and on the following two days mice were fed with tested substances or 10% ethyl alcohol as a control. After 72 hours mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at magnification of 6×, in 1/3 central area of microscopic field. Identification was based on the fact that new blood vessels, directed to the point of cells injection, differ from the background vasculature in their tortuosity and divarications. All experiments were performed in anaesthesia.

For all experiments animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted by the local Ethical Committee.

HECa10 cells

A cell line HECa10 (endothelial cells from mouse peripheral lymph nodes, immortalized by cationic liposome-mediated transfection and isolated on the basis of its capacity to specifically bind fucoside carrying glycoconjugates) was kindly provided by Laboratory of Glycobiology and Cell Interactions Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wrocław. These cells present the main characteristics of endothelial cells: production of angiotensin converting enzyme and of factor VIII-related antigen. Upon stimulation, they express E-selectin and the MECA 79 addressin which is characteristic for the peripheral lymph node high endothelium and is a L-selectin ligand. HECa10 cells also express a second fucoside binding protein which differs from E-selectin, constitutively expressed on unstimulated cells [35].

Cell culture procedures

HECa10 and L-1 cells were maintained in 75 cm^2 (BD Bioscience) culture flasks under standard culture conditions of 5% CO_2 in air at 37°C with medium renewal every 2-3 days. The culture media used was DMEM (1 g/l glucose, PAA) or RPMI-1640 containing 200 mM glutamine (PAA), 1/100 penicillin – streptomycin solution and 1/1000 gentamicin solution supplemented with 10% FBS (PAA). When subconfluent (~80%), cells were split 1 : 3. Cells passaged for 20 times, were used for the experiments.

Rhodiola kirilowii extracts incubation assay

HECa10 or L-1 cells in log phase growth stage were harvested (Accutase, PAA) and cell count was determined. Afterward, cells were suspended in culture medium [DMEM, 4.5 g/ml glucose (PAA) or RPMI-1640 (PAA) with L-glutamine and antibiotics], and then seeded into the wells. Next, lyophilized aqueous or hydro-alcoholic extracts of *Rhodiola kirilowii* dissolved in PBS were added to the cultures, to the final concentration 1, 10, 20, 50 or 100 μg per ml. The plate was returned to the incubator for the next 24 hours.

HECa10 cell migration assay (DilC12(3) pre-labeling test)

L-1 cells in log phase growth stage were harvested (Accutase, PAA) and cell count was determined. Afterward cells were suspended (~4×10^4 cells/ml) in culture medium [DMEM, 1 g/l glucose (PAA) with L-glutamine, antibiotics and 1% FBS], and then seeded (750 μl) into wells of 24-well plate.

HECa10 cell suspensions (obtained as described in cell culture procedure) in DMEM with 10% FBS and 10 μg/ml DilC12(3) were incubated for 1 h in standard conditions. After staining, the cells were washed with DMEM without FBS by centrifuging. Next, they were suspended in culture medium without FBS and adjusted to $4 \times 10^4 – 5 \times 10^4$ cells/ml. The cells were placed into the inserts (250 μl, 24-well plate with inserts, 3 μm, BD) where lower chambers were filled with the L-1 sarcoma cells and RK extracts in complete medium with 1% of FBS. After 24 h incuba-

tion in standard conditions (37°C, 5% CO_2), the inserts were relocated into new 24-well plate containing 1 ml of PBS and the fluorescence was measured directly in the plate with inserts (Ex 549/Em 565) using the reader with bottom optic option. The results were presented in the form of relative fluorescence units (RFU).

Alamar Blue Assay for L-1 cell proliferation

After a 24-h incubation with *Rhodiola kirilowii* extracts, media were discarded and 0.2 ml of fresh RPMI-1640 medium (supplemented with 1% FBS, without Fenol Red) with Alamar Blue (1 : 10 Alamar Blue in medium) was added directly to the wells. Cells were incubated for 3 h at 37°C and 5% CO_2. After this time, fluorescence was measured (FLUOstar Omega, BMG Labtech) at excitation 544 nm and emission 590 nm.

The results are shown as the mean inhibition indices calculated by dividing each experimental result by the mean of the respective control values.

^{3}H thymidine assay for cell proliferation

Cells were incubated at standard culture conditions of 5% CO_2 in air at 37°C for 24 hours with *Rhodiola kirilowii* extracts. Following this, ^{3}H thymidine (^{3}HTdR, Amersham, UK, spec. act. 2 Ci/mM) was added into the cultures in a dose of 0.4 µCi/culture. After 3 h the cultures were harvested and incorporation of ^{3}HTdR was measured in Packard Tri-Carb 2100 TR scintillation counter. The results were calculated as a mean value of dpm ±SEM.

Statistical evaluation of the results

Statistical analysis was done by 2-way ANOVA test and the significance of differences between the groups was verified by Bonferroni post-test (Graph Pad Prism software).

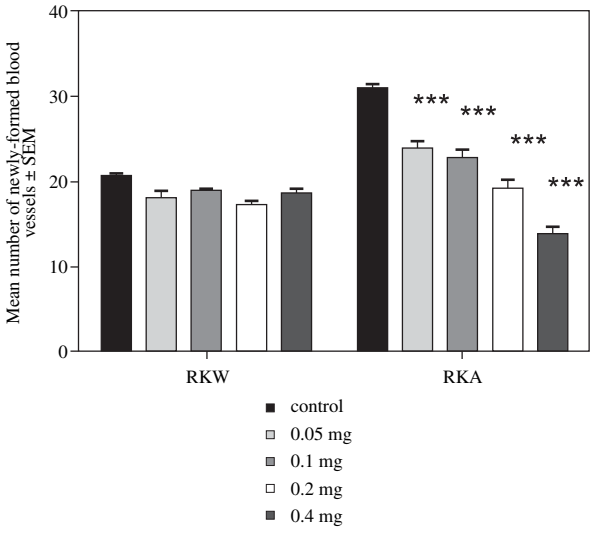

Fig. 1. The effect of *Rhodiola kirilowii* extracts on cutaneous angiogenesis induced in Balb/c mice by L-1 sarcoma syngeneic cells. Total number of TIA tests: 159; *** $p < 0.001$

Results

In vivo angiogenic activity of *Rhodiola kirilowii*

As shown in Fig. 1, in the TIA test, the administrations of *Rhodiola kirilowii* hydro-alcoholic extract (RKA) resulted in marked and statistically significant ($p < 0.001$) reduction in the total number of newly formed blood vessels, at each dose tested. The observed effect was dose dependent and was strongest for a dose of 0.4 mg/kg. At the same time, there was no effect of administration of aqueous extract of *Rhodiola kirilowii* (RKW) on the number of newly formed blood vessels in the study.

In vitro migration and proliferation tests

The results of the study on the effect of two extracts of *Rhodiola kirilowii* on endothelial cell migration and proliferation of endothelial and tumor cells are shown in Figs. 2-5.

As our studies have shown, migration of HECa10 endothelial cells in the presence of tumor L-1 cells was significantly changed by the addition of *Rhodiola kirilowii* extracts (Fig. 2). The aqueous extract significantly increased the number of migrating cells compared to the control (without *Rhodiola kirilowii*), while the use of hydro-alcoholic extract had the opposite effect (decrease of migration).

The study of cell proliferation was carried out by two independed methods: Alamar Blue and ^{3}H thymidine incorporation. Alamar Blue is an indicator dye which incorporates an oxidation-reduction (REDOX) indicator that changes color in response to the chemical reduction of growth medium, resulting from cell growth. This assay is designed to quantitatively measure the proliferation of various cell lines. The assay is simple to perform since the indicator is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays.

Figure 3 shows the mean inhibition index for four concentrations of RKW and RKA (1, 10, 20 and 50 µg per milliliter), designated by Alamar Blue assay. There were no differences between the aqueous and hydro-alcoholic extracts. At the same time the index was significantly lower for all used doses, except the lowest one (1 µg/ml), compared to corresponding control groups. The observed effect was, similar to the *in vivo* experiments, dose dependent and strongest for the highest dose – 50 µg/ml.

Comparison of the effect of both extracts on HECa10 and L-1 cell proliferation (measured by DNA synthesis) are shown in Figs. 4 and 5, respectively. In case of endothelial cells (HECa10), we have found that hydro-alcoholic extract more strongly stimulated cells to proliferate in comparison to the aqueous extracts (Fig. 4). Moreover, hydro-alcoholic

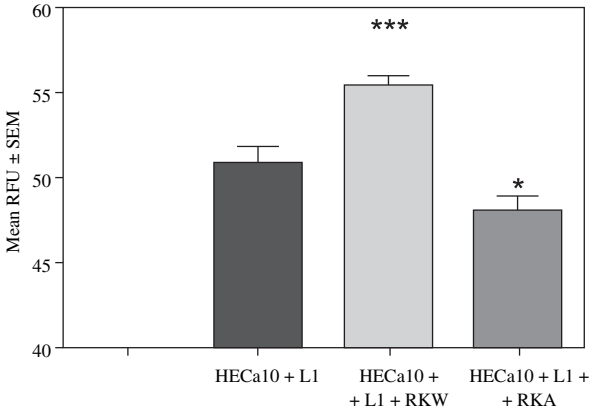

Fig. 2. The *in vitro* effect of *Rhodiola kirilowii* extracts (20 µg/ml) on HECa10 cells migration in the presence of L-1 sarcoma cells. Averaged results of two independent experiments (total number of cultures 55); * $p < 0.05$, *** $p < 0.001$

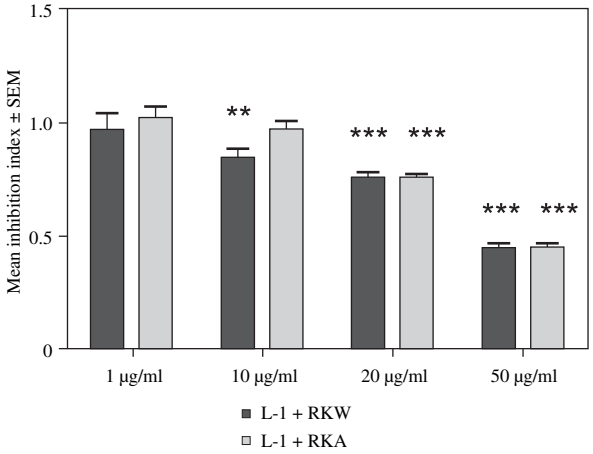

Fig. 3. The *in vitro* effect of *Rhodiola kirilowii* extracts on L-1 sarcoma cells proliferation presented as inhibition indices in comparison to the control. Averaged results of five independent experiments performed with Alamar Blue test (total number of cultures 296); ** $p < 0.01$, *** $p < 0.001$

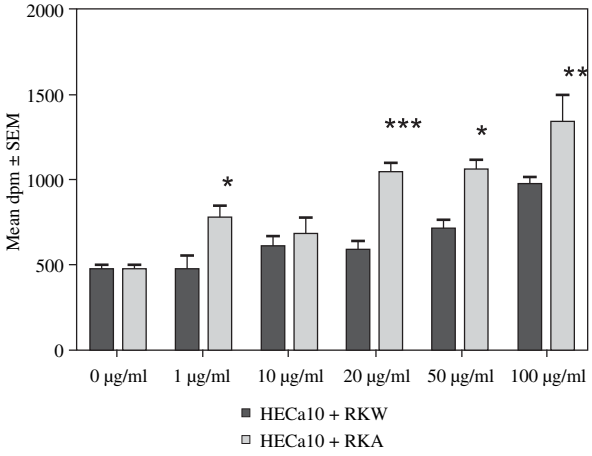

Fig. 4. Comparison of the *in vitro* effect of RKW and RKA extracts on DNA synthesis in cultures of HECa10 cells (^{3}H thymidine incorporation test). Total number of cultures 72; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

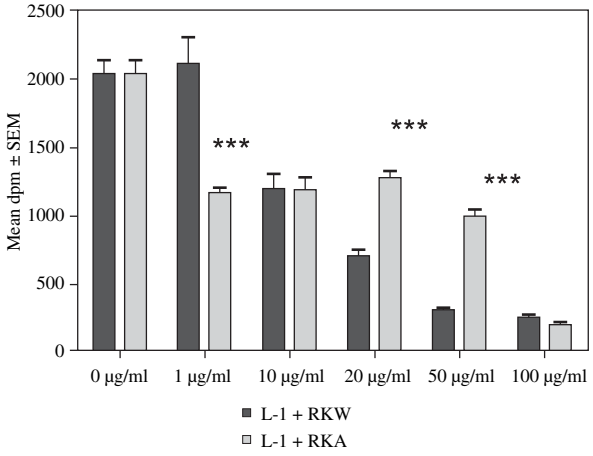

Fig. 5. Comparison of the *in vitro* effect of RKW and RKA extracts on DNA synthesis in cultures of L-1 sarcoma cells (^{3}H thymidine incorporation test). Total number of cultures 72;*** $p < 0.001$

extract significantly increased HECa10 cell proliferation in all concentrations, while aqueous extract – only at the doses: 50 µg/ml ($p < 0.01$) and 100 µg/ml ($p < 0.001$). In cultures with lower RKW concentrations (10 and 20 µg/ml), stimulation was on the border of statistical significance ($0.05 < p < 0.1$).

Rhodiola kirilowii extracts supplementation caused an opposite DNA synthesis effect, depending on the cell line used in the experiment, measured by ^{3}H thymidine incorporation test. Generally both aqueous and hydro-alcoholic extracts increase DNA synthesis in cultures of HECa10 and decrease DNA synthesis in cultures of L-1 sarcoma cells in comparison to corresponding control groups. Interestingly, the various observed effects (stimulation/inhibition) were dose dependent and the strongest for the highest dose – 100 µg/ml (Figs. 4 and 5).

As we described above, supplementation with the extract from *Rhodiola kirilowii* inhibit DNA synthesis measured by ^{3}H thymidine incorporation test in culture of L-1 sarcoma cells (Fig. 5). This effect was observed in concentrations ranging from 10 µg/ml for RKW and 1 µg/ml for RKA. In both the aqueous and hydro-alcoholic extracts the strongest reduction of DNA synthesis at 100 µg/ml concentration was noticed. *Rhodiola kirilowii* aqueous sup-

plementation caused significant decrease in a dose dependent manner, while we didn't noticed differences between 1-20 μg/ml concentrations for RKA supplementation.

Discussion

L-1 sarcoma tumor employed in this study arose spontaneously in the lung of Balb/c mouse and was described by Przemysław Janik from Warsaw Oncology Center/Institute [36]. Since then, tumor has been maintained by subcutaneous passages in syngeneic mice, frozen and stored in Oncology Center Cells Collection. Cells isolated from L-1 tumors were adapted to the growth *in vitro*, retaining their ability to form tumors in *in vivo* conditions. Previously, we often used sarcoma L-1 cells as a model for the study of pro- and anti-angiogenic activity of various substances of synthetic and natural origin [37-44].

Mean number of newly-formed blood vessels 3 days after intradermal grafting of 0.2×10^6 L-1 tumor cells oscillate on average from 20 to 30 between various passages [30]. In the present experiments, cells originated from the 7^{th} and 8^{th} *in vivo* passage (performed after defrosting and *in vitro* culture) were used, and their angiogenic activity was comprised in this range. Intradermal injection of medium alone induced 3-5 newly formed blood vessels. In our present experiments only hydro-alcoholic (RKA) but not water (RKW) extract of *Rhodiola kirilowii* reduced the cutaneous angiogenesis induced by L-1 sarcoma cells. The results of presently performed *in vivo* tests, in mice fed with increasing doses of *Rhodiola kirilowii* extracts, confirmed previously obtained results for the highest, 0.4 mg daily dose [6]. In those previous experiments *Rhodiola kirilowii* hydro-alcoholic extract diminished neovascular response induced by L-1 sarcoma living cells, as well as the reaction to inoculation of the homogenate of human kidney cancer tissue. *Rhodiola kirilowii* aqueous extract behaved differently-suppressing reaction to homogenate and not influencing response to L-1 living cells. This finding has suggested that in the situation when the mixture of various angiogenic stimulators, present in kidney cancer homogenate, are introduced into mice skin, both *Rhodiola kirilowii* extracts may influence efferent arc of angiogenic reaction, blocking activity of these factors, or their receptors on endothelial cells. However, in the situation, where living tumor cells were grafted, hydro-alcoholic extract probably suppressed production or release of some tumor-derived proangiogenic substances. Surprisingly, aqueous *Rhodiola kirilowii* extract was ineffective in this situation.

To explain the background of these phenomena, we compared the influence of both *Rhodiola kirilowii* extracts on migration abilities of endothelial HECa10 cells in the presence of tumor cells, an *in vitro* model of cancer metastasis neovascularization.

The results of these *in vitro* experiments suggest, that lack of the influence of RKW extract on *in vivo* angiogenesis may be connected with its inability to suppress the production (release) of factors stimulating endothelial cells migration. Moreover, this extract presented direct stimulatory influence on endothelial cells migration and proliferation. This is in agreement with the results of studies performed in rats with myocardial infarction, where promoting effect of *Rhodiola kirilowii* aqueous extract on myocardial angiogenesis was observed [45]. We suppose, that two chemokines may be involved – CXCL8/IL8, and CC chemokine, monocyte chemotactic protein 1 (MCP-1) [46, 47]. Interleukin 8 induces the endothelial cell migration through the activation of phosphoinositide 3-kinase – Rac1/RhoA pathway [48]. Angiogenic effects of IL-8 are mediated by CXCR2 receptors. Acting on this receptor, IL-8 upregulates VEGF mRNA and protein what results in the autocrine activation of VEGFR2 [49]. Reduced migration of endothelial cells treated with anti-IL-8 and anti-CXCR2 antibody, but not anti-CXCR1 antibody was observed [50, 51]. Angiogenic effect of MCP-1 is consistent with the expression of CCR2, the receptor for MCP-1 on endothelial cells. This chemokine that is abundantly produced by some tumors can directly contribute to tumor progression, and antagonists of MCP-1 may be of value in combined anti-angiogenic therapy. There are some drugs which inhibit proliferation of endothelial cells *in vitro*, not affecting angiogenesis *in vivo* (cisplatin), and drugs which inhibit migration and invasiveness of endothelial cells in very low concentration, which did not affect endothelial cell proliferation, *in vitro* and *in vivo* (Paclitaxel). Some *in vivo* data suggested that docetaxel (Taxotere) had selectivity for endothelial cell migration and microvessel formation in not-cytotoxic and non-antiproliferative concentrations [52-55].

Table 1. Chemical analysis of aqueous and hydro-alcoholic extracts of *Rhodiola kirilowii* (values in [%])

	Compound						
Extract	**Epicatechin**	**Epigallocatechin**	**Epicatechin gallate**	**Epigallocatechin gallate**	**Tyrosol**	**Salidroside**	**Tannins**
Aqueous (RKW)	0.00086	0.031	0.0051	0.311	0.023	0.00013	2.90
50% hydro-alcoholic (RKA)	0.0019	0.109	0.0042	0.273	0.019	0.00005	7.47

From the results obtained it seems reasonable to conclude that the neo-vascularization stimulatory potential of L-1 sarcoma is stronger than neovascularization inhibitory effect of RKW, while, at the same dose, RKA inhibits endothelial cells migration more effectively than L-1 stimulates it (Fig. 2). Both extracts show the stimulation potential to endothelial cell proliferation (described as the increase in DNA synthesis detected by ^{3}H thymidine incorporation in Fig. 4) although RKA influence is statistically higher. One might conclude that RKA in the absence of tumor cells is more effective in promoting vascularization maturation processes like cell proliferation instead of cell migration.

Our *in vitro* experiments also showed for the first time anti-proliferative effect of *Rhodiola kirilowii* extracts on L-1 sarcoma cells in culture. This was independently tested by two methods: Alamar Blue and ^{3}H thymidine incorporation. It remains to be established, whether this effect was direct, or may result from inhibition of substances stimulating tumor cells proliferation. For example, IL-8 has been found to be mitogenic for some cancer cells [56]. As we have shown in the case of both *Rhodiola kirilowii* extracts that they decrease the proliferative potential of L-1 sarcoma cells, it seems that brake in tumor angiogenesis *in vivo*, exerted by RKA, is not dependent simply on brake of proliferation of tumor cells.

It should be remembered that various pro- and anti-inhibitory cytokines interplay during the process of angiogenesis. Recent study provided evidence for the existence of negative feed-back regulation between PAI-1 and its potent activator TGF-β in endothelial cells. No such regulation was demonstrated for cancer cells which might implicate different regulatory mechanism in neoplastic cells [57]. There are some papers on the effect of other *Rhodiola* species on tumors and tumor cells. *Rhodiola rosea* extracts and its active compound salidroside decreased the bladder cancer cell line and induced apoptosis in human breast cancer cells [58]. *Rhodiola sachalinensis* suppressed T241 fibrosarcoma tumor cells proliferation *in vitro* and growth *in vivo* [59], *Rhodiola crenulata* and salidroside induced death and inhibited growth of various cancer cell lines [60, 61], *Rhodiola imbricate* inhibited proliferation of an erythroleukemic cell line K-562 by inducing apoptosis and cell cycle arrest at G2/M phase [62]. There are also older papers from Russian authors, briefly reviewed elsewhere [9].

The question arise, which compounds present in *Rhodiola kirilowii* extracts may be responsible for the difference in their action. As presented on the Table 1, hydro-alcoholic extract contained 2 times more epicatechin, three times more epigallocatechin and almost three times more tannins than aqueous one. In our previous studies in mice fed during pregnancy with chocolate or chocolate-derived catechins, we observed negative correlation between epigallocatechin content and angiogenic activity of fetal tissues [63]. Anti-tumor activity of tannins and catechins is known, as well as their anti-angiogenic properties [64-66]. Then, we may suppose that, may be, these compounds played essential role in the angio-inhibitory effect of *Rhodiola kirilowii* hydro-alcoholic extract presented in this paper.

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