

The influence of *Rhodiola rosea*, *Rhodiola kirilowii* and *Rhodiola quadrifida* extracts on cutaneous angiogenesis induced in mice after grafting of human kidney cancer tissue

EWA SKOPIŃSKA-RÓŻEWSKA¹, ALEKSANDER WASIUTYŃSKI¹, EWA SOMMER¹, SEBASTIAN MIELCAREK², ALINA MŚCISZ², ANNA KRAJEWSKA-PATAN², MICHAŁ MAZURKIEWICZ¹, KRZYSZTOF PASTEWKA³

¹Department of Pathology, Biostructure Center, Medical University in Warsaw, Poland; ²Research Institute of Medicinal Plants, Poznań, Poland; ³Department of Urology, Postgraduate Medical Center, Warsaw, Poland

Abstract

The aim of the work was to study the effect of aqueous and 50% hydroalcoholic extracts of *Rhodiola rosea*, *Rhodiola kirilowii* and *Rhodiola quadrifida* roots and rhizomes on neovascular reaction induced in the skin of Balb/c mice after grafting of human kidney cancer tissue homogenate or, for comparison, L-1 sarcoma syngeneic cells. Mice were fed 0.4 mg of extracts daily, for 3 days. After 72 hours mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope.

Results: Extracts of underground parts of *Rhodiola rosea* and *Rhodiola kirilowii* significantly decreased cutaneous neovascular reaction induced by human cancer homogenate, extracts prepared from *Rhodiola quadrifida* were ineffective in this model of angiogenesis. In syngeneic tumor cells model all extracts were effective, except aqueous extract of *R. kirilowii*.

Key words: Angiogenesis, mice, human kidney cancer, *Rhodiola rosea*, *Rhodiola kirilowii*, *Rhodiola quadrifida*.

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Introduction

Dr Judah Folkman was the first who recognized that the growth of solid tumor and its metastasis is strongly dependent on the recruitment and ingrowth of new blood vessels from nearby normal tissues. Now, it is general agreement, that inhibition of angiogenesis may become a promising strategy in cancer therapy [1]. We previously reported, that aqueous and hydro-alcoholic extracts of 3 *Rhodiola* species – *R. rosea*, *R. kirilowii* and *R. quadrifida* suppressed angiogenic reaction induced in mice skin by grafting of syngeneic L-1 sarcoma cells [2-4]. The question arise whether this suppression was dependent on inhibition of angiogenic factors production by transplanted cells, or was the result of attenuation of angiogenic signaling for endothelial cells. For

this purpose we decided to repeat our previous experiments using sonicated homogenate of human kidney cancer tissue instead of living murine sarcoma cells, and comparing the results to the results of simultaneously performed experiments with L-1 sarcoma living cells. In both type of experiments we used 0.4 mg daily dose of *Rhodiola* extracts. From our earlier studies we have known that tissue homogenates and cell suspensions prepared from various types of human cancers (kidney, ovary, lung, urinary bladder), as well as human sera and human recombinant angiogenic factors, when injected intradermally to recipient mice induce strong neovascular reaction, which may be suppressed or attenuated by various substances of synthetic and natural origin [5-13].

Correspondence: Ewa Skopińska-Różewska, Department of Pathology, Biostructure Center, Medical University, Chałubińskiego 5, 02-004 Warsaw, Poland, Email: ewaskop@hotmail.com

Materials and Methods

R. rosea and *R. kirilowii* plants originated from many years' cultivation of the Research Institute of Medicinal Plants (RIMP) in Poznań, thanks to prof. P. M. Mrozikiewicz and dr W. Buchwald. Rhizomes and roots of *R. quadrifida* were collected in Altai mountain in Mongolia, thanks to dr H. Wiedenfeld and prof. M. Furmanowa. Samples extractions were prepared by the methods as described [14, 15]. Briefly, aqueous extracts: finely powdered roots were extracted with water two times in the temperature 40–45°C. The supernatants were combined and after centrifugation at 3000 rpm for 15 min, were lyophilized. Hydro alcoholic extracts: finely powdered roots were extracted with ethanol/water solution (1/1, V/V) by the percolation method. The percolates were lyophilized which was preceded by the distilling off the ethanol in the temperature 40–45°C.

Biological studies

The study was performed on 7–8 weeks old inbred Balb/ mice, weighing about 20 g, females, delivered from the Polish Academy of Sciences breeding colony. *Rhodiola* extracts were administered to mice *per os* in daily doses 0.4 mg. These doses corresponded to 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 Eppendorff pipette, in 40 µl of 10 % ethyl alcohol, for 3 days after tumor homogenate or cells grafting. Control animals received 10% alcohol.

Tumour tissue was obtained surgically from patient with kidney clear cell carcinoma. 2.5 g of tissue were suspended in 5 ml of phosphate-buffered saline (PBS), homogenized with an ultrasonic disrupter VirSonic (Virtis) for 2 minutes, at frequency 22.5 KHz and stored at -70°C, in 1 ml aliquots.

Sarcoma cells were delivered from Warsaw's Cancer Centre collection, thanks to dr Henryk Skurzak, and then passaged through several generations of Balb/c mice, according to the method described [16]. Briefly, sarcoma cells were grafted (10⁶/0.1 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 concentration of 4 × 10⁶/ml.

Cutaneous angiogenesis assay was performed according to [17] with own modifications [18]. Briefly, multiple 0.05 ml samples of homogenate or cell suspension were injected intradermally into partly shaved, narcotised Balb/c mice (3–4 mice per group, 4–6 injections per mouse). In order to facilitate the localisation of injection sites later on, the suspension was coloured with 0.1% of trypan blue. On the day

of grafting and on the following two days mice were fed 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 (3.6% chloral hydrate, 0.1 ml per 10 g of body mass).

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.

Statistical evaluation of the results was performed by one-way ANOVA and the significance of differences between the groups was verified with a Dunnett's Multiple Comparison Test (GraphPadPrism software package).

Results

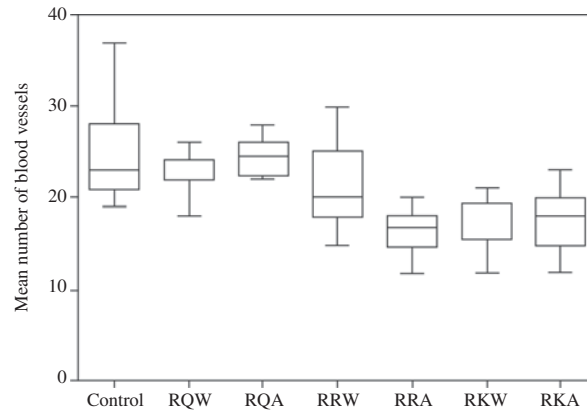
The results of *Rhodiola* extracts administration to mice injected with human kidney tumor homogenates are presented on the Fig. 1. *R. rosea* and *R. kirilowii* extracts significantly diminished neovascular reaction, *R. quadrifida* extracts, however, were ineffective. Water extract of *R. rosea* (RRW) presented lower activity than aqueous (RKW) and hydro-alcoholic (RKA) *Rhodiola kirilowii* extracts, as well as *Rhodiola rosea* hydro-alcoholic (RRA) one.

The main differences between the results obtained for human kidney cancer homogenate and living L-1 sarcoma cells (Fig. 2) was: firstly, lack of the effect of both *R. quadrifida* extracts in the former and significant inhibition in the latter type of experiment, secondly, lack of the effect of *R. kirilowii* water extract on neovascular response induced by L-1 sarcoma cells, and its full activity in the experiment with human cancer homogenate.

R. kirilowii hydro-alcoholic and *R. rosea* extracts were effective in both types of experiment.

Discussion

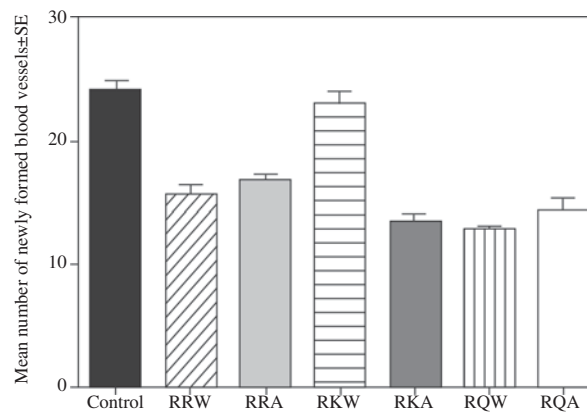
Present experiments show, that attenuation of neovascular reaction, observed by us in mice grafted with L-1 sarcoma living cells and fed *Rhodiola quadrifida* extracts, is probably dependent on the suppressing effect of these herbal preparations on the production, (or/and on the release), of pro-angiogenic factors by transplanted cells. In situation when cocktail of various angiogenic cytokines present in the homogenate of human kidney cancer was introduced to mice skin, *R. quadrifida* extracts were ineffective, not influencing the efferent arc of cutaneous angiogenic response. Other extracts (except aqueous extract of *R. kirilowii*) probably influenced both arcs of neovascular reaction.



Total number of TIA tests: 124

Dunnet's Multiple Comparison Test	Mean Diff.	q	Significant? p<0.05?	Summary	95% CI of diff
Control vs RQW/W	2.609	1.927	no	ns	-0.9188 to 6.138
Control vs RQA/A	0.9352	0.7119	no	ns	-2.487 to 4.357
Control vs RRW/W	4.376	4.284	yes	***	1.715 to 7.036
Control vs RRA/A	8.930	7.618	yes	***	5.876 to 11.98
Control vs RKW/W	8.733	7.003	yes	***	5.484 to 11.98
Control vs RKA/A	7.785	6.385	yes	***	4.609 to 10.96

Fig. 1. Mean number of newly-formed blood vessels in mice grafted with human kidney cancer homogenate and fed *R. quadrifida* (RQ), *R. rosea* (RR), or *R. kirilowii* (RK) water (W) or hydro-alcoholic (A) extracts (0.4 mg daily) for 3 days



Total number of TIA tests: 175

Dunnet's Multiple Comparison Test	Mean Diff.	q	Significant? p<0.05?	Summary
Control vs RRW/W	8.329	5.795	yes	***
Control vs RRA/A	7.204	4.691	yes	***
Control vs RKW/W	1.022	0.9788	no	ns
Control vs RKA/A	10.51	10.07	yes	***
Control vs RQW/W	11.14	9.800	yes	***
Control vs RQA/A	9.665	6.920	yes	***

Fig. 2. The effect of feeding mice *Rhodiola* extracts (3 × 0.4 mg) on neovascular response induced by intradermal grafting of L-1 Sarcoma syngeneic cells

The differences may be connected with different chemical composition of our various *Rhodiola* extracts [14, 19, 20]. We have found angioinhibitory effect of rosavin and salidroside in L-1 sarcoma model [2, 3]. Rosavin is characteristic for *R. rosea* extracts, salidroside is a common compound for all of them. We also observed in other model (serum-induced angiogenesis) inhibitory activity of epigallocatechin gallate (EGCG), one of the compounds present in *R. kirilowii* extracts [10, 21]. Many questions remain unanswered and a lot of experimental studies should yet be done before recommending selected *Rhodiola* extracts or some of their active compounds as safe and effective antiangiogenic agents.

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