The influence of *Rhodiola rosea* extracts and rosavin on cutaneous angiogenesis induced in mice after grafting of syngeneic tumor cells

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Abstract

Anticancer research on the plant species Rhodiola rosea L. (Crassulaceae) has begun in animal models in 1987. In combination with cyclophosphamide, R. rosea extract enhanced the anti-tumor and anti-metastatic effects of drug treatment and reduced drug-induced toxicity. Combined with Adriamycin®, it improved inhibition of tumor dissemination and prevented liver toxicity. The aim of the present work was to study the effect of rosavin and aqueous and 50% hydroalcoholic extracts of R. rosea roots and rhizomes on neovascular reaction induced in the skin of Balb/c mice after grafting of L-1 sarcoma cells. Mice were treated per os with 50, 100, 200 and 400 μg of extracts, or were fed rosavin in daily doses 2, 4 and 8 μg. After 72 hours mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope. Both extracts in 100-400 μg daily doses and rosavin in the highest dose highly significantly decreased neovascular reaction.

Key words: *angiogenesis, tumor cells, L-1 sarcoma, mice, Rhodiola rosea, rosavin.*

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Introduction

Roseroot, *Rhodiola rosea* L. (syn. *Sedum roseum*/L./Scop.) is an arctic-alpine species from the family *Crassulaceae* distributed in Asia, Europe, North America. It has been described as a "future anti-aging plant'' or a "phytoadaptogen'' with CNS stimulating, antidepressant, tonic, hepatoprotective and hepatoregenerative, antidiabetic, antiviral (a potential inhibitor of HIV-1 protease), antibacterial, anti-inflammatory, antistress, antioxidative, antiradiative and phytoestrogenic properties [1-9]. Rosavin (Figure 1) is a diagnostic compound only for the rhizomes and roots of two plant species i.e. *R. rosea* and *Rhodiola sachalinensis*. The content of rosavin in the underground organs is evaluated on approx. 3% D.W. Higher amounts of rosavin are more abundant in female plants [10]. The improvement in the yield of rosavin production was studied in biotransformation processes of trans-cinnamyl alcohol by *R. rosea* cell cultures [11-14]. The isolation method of rosavin was patented by Kurkin et al. [15]. The method of the synthesis of rosavin was elaborated by Kishida et Akita in 2005 [16] and Patov et al.

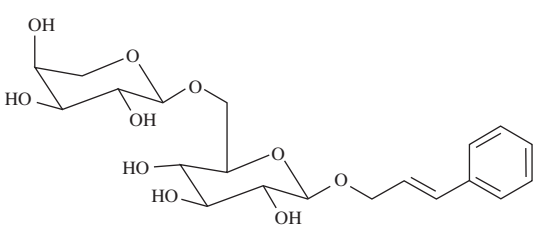

Fig. 1. Chemical structure of rosavin

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in 2006 [17]. Nowadays the commercial sale of rosavin has a broad scale e.g. via Chemical Abstracts database.

There are some reports on anti-cancer effect of *R. rosea*, but often published in Russian language. Dement'eva and Iaremenko [18] have begun this kind of studies on *R. rosea.* The antitumor and antimetastatic effects of an official extract of *R. rosea* rhizomes "Extractum Rhodiolae fluidum" (Pharmacopoeia of Soviet Union, 1984) were described. Experiments on mice and rats with transplantable NK/Ly tumor, Ehrlich's adenocarcinoma, melanoma B16 and Lewis lung carcinoma after application of the preparation to sarcolysin-treated animals followed by an increase in survival. Since 1989, the other Russian scientists, Udintsev with co-authors, documented the next anticancer bioassays. In experiments with transplantable tumors after partial hepatectomy, *R. rosea* extracts or their combination with anticancer drugs inhibited the growth of Ehrlich's tumor and Pliss' lymphosarcoma as well as dissemination of the latter. These effects were to a certain extent attributed to the production of humoral factors by the liver inhibiting clonogenic activity of tumor cells *in vivo* and *in vitro* [19]. The clonogenic activity of tumors has been further studied on CBA, BALB/C and C57B1/6 mice with the Ehrlich adenocarcinoma and Lewis lung carcinoma treated with adaptogenic drugs of *R. rosea* extract, a synthetic analog of *Rhodiola* phenolic derivative, methyluracil and their combinations with cyclophosphamide. The extract and the derivative protected the myelopoietic tissue from the toxic action of cyclophosphamide, retaining or increasing the suppressive effect of the latter towards clonogenic tumors cells. The authors suggested, that the extract and derivative might be used during the antitumor chemotherapy as biological response modifiers [8]. It was shown also in rats with Pliss lymphosarcoma (PLS) that partial hepatectomy (PHE), a course application of *R. rosea* extract (RRE) or combined effects inhibited the growth of tumors by 37, 39 and 59%, respectively, and that of metastases by 42, 50 and 75%. In combined treatment the process of hepatic regeneration was completed in earlier terms versus the animals which underwent PHE, and proliferative activity of the tumor and metastases decreased by 15 and 59%, respectively, judging by the degree of 3H-thymidine incorporation into DNA of these tissues. The assessment of clonogenic activity of PLS cells taken in the animals of this group, using the method of diffusion chambers, revealed a significant decrease in this index versus the rats which underwent PHE or which were given RRE. The assumption that these effects were determined by factors originating from the regenerating liver was confirmed in experiments with double-layer agar systems. Inhibition of colony-forming activity of PLS cells was the maximum in application of the hepatocytes of the rats which underwent a complex of effects, as a feeder, versus the hepatocytes taken in intact or hepatectomized animals, or the rats which were given RRE. In experiments on mice with Ehrlich adenocarcinoma, the factors isolated from the liver of animals subjected to PHE against a background of RRE administration and from the liver of mice which were given RRE only, as well as operated or intact ones, inhibited the tumor growth to 63, 38, 35 and 21%, respectively [20].

Bocharova et al. [21] studied different schemes of *R. rosea* usage per os for prophylaxis of mouse hereditary hepatomas. A long-term effect on stimulation of tissue integration, T-immune activity and sufficient decrease of tumour frequency has been shown for *R. rosea* usage in early ontogenesis. A short-term effect on tissue and immune parameters has been demonstrated for *R. rosea* usage in middle ontogenesis. High-tumour frequency (as in the control), but tumour size decrease (comparing to the control) has been noticed in this case.

As was shown by Baltina and Serdyuk [22], 40% ethanolic extract from *R. Rosea* completely suppressed cell growth of cultured human lymphoblastoid cells in concentrations of 50-200 μg/ml. Ming et al [5] in studies on cytotoxicity of gossypetin-7-O-L-rhamnopyranoside and rhodioflavonoside isolated from *R. rosea* extracts displayed activity against the prostate cancer cell line with IC50 values of 50 μg/ml and 80 μg/ml, respectively. The plant preparation AdMax (Nulab Inc., Clearwater, Fl, USA), containing dried ethanolic extracts from roots of *R. rosea* and of other three plants increased the mean numbers of the T cell subclasses CD3, CD4, CD5, CD8 and IgG, IgM in patients with stage III-IV epithelial ovarian cancer after 4 weeks usage following the chemotherapy [23]. Recently, Hoser et al. reported anti-proliferative effect and induction of apoptosis and necrosis of HL-60 cells treated with an extract from *R. rosea* rhizomes [24].

Rosavin is known as a stimulator of the spontaneous motor activity [25], antistress and adaptogenic agent [2, 26]. Its potential antidepressant [4, 27], nootropic [28], UV-protective [3] properties were reported. No anticancer data on rosavin were found. In the available literature there were no reports concerning the possible effect of *R. rosea* extracts and rosavin on angiogenesis induced by tumor cells, except our preliminary conference communication [29]. Then, we wanted to check, whether this mechanism may contribute to antitumor activity of *R. rosea* extracts. It is known, that forming of new blood vessels in tumors is necessary for the process of their growth and for metastasis formation. The aim of the present work was to study the *in vivo* effect of aqueous and 50% hydroalcoholic extracts of *R. rosea*, and its compound, rosavin, on the cutaneous angiogenesis induced in Balb/c mice by L-1 Sarcoma syngeneic cells.

Materials and Methods

R. rosea plants originated from many years' cultivation of the Research Institute of Medicinal Plants (RIMP) in Poznań. Extracts of *R. rosea* roots and rhizomes were prepared and their chemical analysis performed by Wiedenfeld et al.

[30] and by the scientists from RIMP (Mrozikiewicz PM, Mścisz A, Krajewska-Patan A, Mielcarek S, Buchwald W), and Furmanowa M from Warsaw Medical University, as was described in details previously [31, 32]. Briefly, aqueous extracts: finely powdered roots were extracted two times with water (extraction was performed: first – 2 hour 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 lyophylized. Hydroalcoholic extracts: 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 lyophylized which was preceded by the distilling off the ethanol in the temperature 40-45°C. Dry extracts were stored under silica gel in the exsiccator in the room temperature. Rosavin was supported by Chromadex (USA, Santa Ana).

Animals

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. *R. Rosea* extracts was 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). Rosavin was administered in daily doses 2, 4 and 8 μg. Mice received drugs by Eppendorff pipette, in 40 μl of 10% ethyl alcohol, or water, for 3 days after tumor cells grafting. Control animals received 10% alcohol or water.

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 [33]. 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 concentration of 4 × 10^6/ml. Cutaneous angiogenesis assay was performed according to Sidky & Auerbach [34, 35] with own modifications [36-39]. Briefly, multiple 0.05 ml samples of 200 thousand 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, 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 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, are thin and/or 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 results was performed by Student's *t* and Mann-Whitney tests.

Results

The results of *R. rosea* extracts administration to mice for 3 days after transplantation of L-1 sarcoma cells are presented on the Table 1 (aqueous extract) and Table 2 (50% hydroalcoholic extract). Aqueous extract in tested doses significantly (the lowest dose, $P<0.05$) and highly significantly (higher doses, $P<0.01$) decreased neovascular reaction induced in syngeneic mice skin and evaluated 3 days after cells grafting.

Table 1. Inhibitory effect of *R. rosea* (Rr) aqueous extract on neovascular reaction induced in Balb/c mice skin by syngeneic L-1 Sarcoma cells

Daily dose of Rr aqueous extract	Number of tests	Mean number of newly-formed blood vessels ±SE	Statistical significance of difference from the control
control (0 μg)	17	20.5±0.48	
Rr 50 μg	17	18.9±0.43	P<0.05
Rr 100 μg	17	14.5±0.51	P<0.01
Rr 200 μg	17	15.9±0.52	P<0.01
Rr 400 μg	18	14.7±0.45	P<0.01

Table 2. Inhibitory effect of *R. rosea* (Rr) 50% hydroalcoholic extract on neovascular reaction induced in Balb/c mice skin by syngeneic L-1 Sarcoma cells

Daily dose of Rr extract	Number of tests	Mean number of newly-formed blood vessels ±SE	Statistical significance of difference from the control
control (0 μg)	16	20.1±0.72	
Rr 50 μg	17	19.6±0.75	NS
Rr 100 μg	16	16.2±0.47	P<0.01
Rr 200 μg	18	16.2±0.65	P<0.01
Rr 400 μg	17	14.7±0.67	P<0.01

Similar inhibitory effect was observed for higher concentrations of 50% hydro-alcoholic extract, however, the dose of 50 μg was ineffective (Table 2). Rosavin inhibited neovascular response in the highest dose (8 μg) only.

Discussion

We reported previously that theobromine (present in Theobroma cacao seeds), adenosine receptor antagonist, suppressed cutaneous neovascular reaction induced in mice by human lung and ovary cancer cells, and inhibited induction of angiogenesis and VEGF-mRNA expression in v-raf transfectants of human urothelial cells [40-42]. We also described inhibitory effect of low-molecular weight heparin enoxaparine and various medicinal plant extracts as well as some isolated compounds, among them catechins, shikonine, ursolic acid, convallamaroside [43-53] on L-1 Sarcoma growth and tumor-induced angiogenesis in mice. In this paper we present evidence, that *R. rosea* aqueous and hydro-alcoholic extracts and rosavin also posses anti-angiogenic activity in the model of tumor-induced angiogenesis. The antiangiogenic and, possibly, antitumor activities of rosavin might be explained in the studies of structure-activity relationship to observe effects both of cinnamyl alcohol derivatives, as sugar units. Cinnamaldehydes have been shown to have inhibitory effects on farnesyl protein transferase, angiogenesis, cell-cell adhesion, tumor cell growth and to be immunomodulators [14]. The results of studies of Jeong et al [54] support the hypothesis that the cinnamaldehyde derivatives exert cytostatic properties by inducing mitotic arrest in cancer cells.

The mechanisms responsible for angioinhibitory effect of our *R. rosea* extracts are unknown. Firstly, it may be connected with the direct inhibitory action of some compounds present in *R. rosea* extracts on mouse vessels endothelial cells proliferation. According to this, Sui et al. reported recently [55] inhibitory effect of *R. Rosea* on the growth of human umbilical vein endothelial cell line EVC-304.

Secondly, some substances present in *R. rosea* extracts may indirectly influence angiogenesis, suppressing pro-angiogenic factors release from transplanted tumor cells. It was presented by Bałan et al [46] that some phenolic acids (caffeic and chlorogenic acids) suppressed angiogenic activity and angiogenic factors release (VEGF and bFGF) *in vitro* in cultures of human ovarian cancer cells. The presence of chlorogenic and gallic acids in *R. rosea* extracts was documented [31, 32] as well as the presence of epigallocatechin gallate (EGCG) [30]. This last compound is also known as angiogenesis inhibitor. Its action is partly connected with inhibition of binding of VEGF to its thyrosine kinase receptors on endothelial cells, inhibition of matrix-degrading proteases, and inhibition of urokinase activity [44]. In the other study, we present evidence of anti-angiogenic effect of another *Rhodiola* extract compound, common for *R. rosea* and *R. quadrifida* species, salidroside [56]. We suppose, that anti-angiogenic action of rosavin present in our *R. rosea* extracts is of not great importance in cutaneous angiogenesis inhibition produced by these extracts. In fact, the concentration of rosavin [32] in aquaeous extract (which is more anti-angiogenic), was several times lower than in hydroalcoholic one (less antiangiogenic).

Table 3. The effect of rosavin on neovascular reaction induced in Balb/c mice skin by syngeneic L-1 Sarcoma cells

Daily dose of rosavin	Number of tests	Mean number of newly-formed blood vessels ±SE	Statistical significance of difference from the control
control (0 μg)	21	21.7±0.56	
2 μg	16	21.2±0.81	NS
4 μg	18	20.6±0.33	NS
8 μg	15	15.2±0.88	P<0.01

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