Antiproliferative and antimitotic effect, S phase accumulation and induction of apoptosis and necrosis after treatment of extract from *Rhodiola rosea* rhizomes on HL-60 cells

Agnieszka Majewska a,∗, Hoser Grażyna b, Furmanowa Mirosława c, Urbańska Natalia c, Pietrosiuk Agnieszka c, Zobel Alicja d, Mieczysław Kurasiś a

a Department of Plant Morphogenesis, Warsaw University, Miecznikowa Str. 1, 02-096 Warsaw, Poland
b Department of Clinical Cytology, Medical Centre of Postgraduate Education, Marymoncka 99, 01-813 Warsaw, Poland
c Department of Biology and Pharmaceutical Botany, Medical University of Warsaw, Banacha Str. 1, 02-097 Warsaw, Poland
d Department of Chemistry, Trent University, Peterborough, Ont., Canada K9J 7B8

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Abstract

*Rhodiola rosea* L. (Crassulaceae) has been used for a very long time in Russian and Chinese folk medicine (Rege et al., 1999); even orthodox medicine started to use it over 130 years ago. Recently phytochemical studies of the rhizomes and shoots have revealed the presence of flavonoids, proanthocyanidines, tyrosol, and its glycoside salidrose, cinnamyl alcohol and its glycoside rosavine, β-sitosterol, anthraglycosides, as well as other glycosides, organic acids, ethereal oils, sugars, fats, specific alcohols and proteins (Kurkin et al., 1986). In Russia, roots and rhizomes are a pharmacopea herbal standardised for the content of rosavine (qualitative) and salidrose (qualitative and quantitative). The sphere of application of *Rhodiola rosea* extracts is very comprehensive. At present, liquid extracts from *Rhodiola rosea* are used in medical practice as a stimulating and adaptogenic agent (Sokolov et al., 1985). The common

1. Introduction

*Rhodiola rosea* L. (Crassulaceae) has been used for a very long time in Russian and Chinese folk medicine (Rege et al., 1999); even orthodox medicine started to use it over 130 years ago. Recently phytochemical studies of the rhizomes and shoots have revealed the presence of flavonoids, proanthocyanidines, tyrosol, and its glycoside salidrose, cinnamyl alcohol and its glycoside rosavine, β-sitosterol, anthraglycosides, as well as other glycosides, organic acids, ethereal oils, sugars, fats, specific alcohols and proteins (Kurkin et al., 1986). In Russia, roots and rhizomes are a pharmacopea herbal standardised for the content of rosavine (qualitative) and salidrose (qualitative and quantitative). The sphere of application of *Rhodiola rosea* extracts is very comprehensive. At present, liquid extracts from *Rhodiola rosea* are used in medical practice as a stimulating and adaptogenic agent (Sokolov et al., 1985). The common

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usage of Rhodiola rosea deals with its antiarrhythmic effect (Lishmanov et al., 1993). Rhodiola rosea is also a stimulant of the immune system (Kelly, 2001). Rhodiola rosea extract protects the heart against damage caused by stress, most likely by inhibiting the release of catecholamines (Maslova et al., 1994). Many investigations have pointed to Rhodiola rosea’s anticancer role (Kelly, 2001) and shown its reaction on Ehrlich, B16 and Lewis cancers (Razina et al., 2000).

The extract strengthened the activity of cyclophosphamide (Udintsev and Schakhov, 1991) and lowered its hepatotoxicity. Similar activity was shown with adriamycin (Udintsev and Schakhov, 1991) and lowered its hepatotoxicity. The extract strengthened the activity of cyclophosphamide (Duhan et al., 1999).

In spite of these therapeutic effects of Rhodiola rosea extract, little is known about the anticancer mechanism of its action, because no extensive cytological investigations have been conducted on mitotic activity, cell cycle or survival of cancer and normal cells. Our results from tests on the plant Allium cepa showed that extract from Rhodiola rosea rhizomes caused inhibition of the mitotic activity of meristematic cells of Allium cepa root tips and accumulation cells in G2/M phase of cell cycle (Majewska et al., 2003). The aim of this paper is to further investigate the biological action (survival, cell cycle, apoptosis) of the antimitotic activity of Rhodiola rosea on promyelobic leukemia cells on HL-60 line to define the anticancer effect of the extract.

2. Material and methods

2.1. Extract preparation

The rhizomes of ground plants of Rhodiola rosea were grown for 2 years in the experimental field of the Department of Biology and Pharmaceutical Botany of the Medical Academy in Warsaw. The plant material was dried at 80 °C. 10 g of ground dry raw material was dipped in 60 ml of 96% ethanol. Extraction was carried out in flasks on a rotary shaker, at 25 °C, in darkness for 12 h. The extract was filtered, and the filtrate was evaporated at 35 °C, at reduced pressure (20 mbar). The dry residue (0.23 g) was dissolved in 5 ml dimethyl sulphoxide (DMSO) whose final concentration was 2%. This solution was used for dilution with distilled water. During investigation, the following dilutions of the basic solution were used: 4.5, 9, 45, 90, 180, 225, 450 μg/ml, where the maximal concentration of DMSO in the obtained dilutions did not exceed 0.5%. Such a concentration of DMSO was used as a control in all our experiments. 0.5% DMSO does not cause visible structural changes in HL-60 cells according to the results of the experiments of Rowinsky et al. (1988).

2.2. HPLC analysis

Chemical investigation was carried out in the same way as earlier described by Furmanowa et al. (2002). Dried and pulverized rhizomes were extracted with ethyl acetate then with methanol and the extracts were purified. HPLC analysis was performed on a DIONEX HPLC system, equipped with UVD detector. Separation was done in an EC 250/4 Nucleosil® 120-7C18 column (Machery-Negel). The mobile phase consisted of 0.04 M H3 PO4—A, acetonitrile—B, methanol—C, used in the following gradient elution: from 7B/8C in 30 min to 20B/20C. The flow rate was 1 cm3 min−1, detection 220, 250, 275 and 320 nm. The standard substances: salidroside, tyrosol, cinnamyl alcohol, triandine and rosavin fraction were analyzed in the same conditions. Peaks were assigned by spiking the samples with standard substances and comparison of the retention times and UV spectra.

2.3. Cell culture and exposure to the extract from Rhodiola rosea

Promielotic leukemia cells of the HL-60 line were obtained from the Department of Clinical Cytology of the Medical Centre for Postgraduate Education in Warsaw. They were cultured in RPMI 1640 (GIBCO) medium supplemented with 20% inactivated newborn calf serum (Biochrom KG), penicillin and streptomycin (GibcoBRL, Life Technologies) at 37 °C in air supplemented with 5% CO2. For cytological investigation, 105 cells per ml of RPMI medium were used. Cells were treated with different concentrations of the extract for 6, 12, 24, 48 and 72 h. After each time of incubation cells, were collected for further cytological investigations.

2.4. Cytological investigations

2.4.1. Mitotic activity

HL-60 cells were fixed in 3% paraformaldehyde with 0.25 M mannitol (45.54 g/l) for 2 h, rinsed in PBS buffer and stained with DAPI (indolo-4,5,6-dwuamidyno-2-fenylidyny, 480 nm). After rinsing in PBS, the cells were embedded in citifluor. On the basis of microscopic preparations, the mitotic index and phase indices were counted and microphotographs were made with a fluorescent microscope (Optiphot 2. Nikon). For each experiment, the indices were counted per 1000 cells using four replicates.

2.4.2. Survival of cells and detection of percentage of apoptosis and necrosis

Survival of cells was evaluated on the basis of PI (propidium iodide, Sigma P4170) staining. PI (final concentration 5 μg/ml) was added to each examined sample for 10 min
The samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, USA, equipped with an argon laser with an emission spectrum of 488 nm). Ten thousand cells were analyzed using four replicates. The results were analyzed with the CELLQuest program.

2.4.3. Investigations of cell cycle

Cells were fixed in 75% ethanol for 4 h and then washed in PBS and suspended in 0.1% NP40 (Nonidet P40, Biochemica Fluka) and RNase (Ribonuclease A, DNAse-free preparation, 10 μg/ml, Serva) for 20 min at room temperature. Next, PI was added (final concentration 5 μg/ml) for 12 h at 4 °C in darkness. Samples were analyzed with the FACSCalibur flow cytometer (Becton Dickinson, USA). For each sample, 10,000 cells were analyzed using four replicates. The results were analyzed with the CELLQuest program.

2.4.4. Statistical analysis of results

All results were statistically analyzed using ANOVA parameter tests and Student–Newman–Keuls test.

3. Results

3.1. Survivability and induction of apoptosis and necrosis

Rhodiola rosea extract caused a marked decrease in the survival of HL-60 cells (Fig. 1), depending on its concentration and incubation period.

The two lowest concentrations, 4.5 and 9 μg/ml, only slightly increased the survival of HL-60 cells for 6 h of incubation, and reduced it slightly after 12 h. At further incubation times, the survival rate rose, reaching its top value after 72 h (Fig. 1).

A concentration of 45 μg/ml reduced the survival of cells by 30% after 12 h of incubation. During further incubation (24, 48 h), the survival rate gradually rose, finally reaching (72 h) values close to control.

Treatment with a concentration of 90 μg/ml markedly reduced survival, by 50% in proportion to the control, after 12 and 24 h of incubation, then survival started rising after 48 h, not reaching, however, the control value after 72 h (Fig. 1). Treatment with 180 μg/ml reduced the survival to 15% after 24 h, followed by a slight growth after 48 and 72 h (Fig. 1), and the highest concentrations of Rhodiola rosea extract (225 and 450 μg/ml) reduced it to almost 0 after 48 and 72 h of incubation.

During incubation with all concentrations of the Rhodiola rosea extract, the decrease of percentage of live cells was accompanied by proportional growth of the percentage of apoptotic and necrotic cells (Figs. 2–5). The proportion of both types of cells varied throughout the experiment.

During the initial period of incubation (6 and 12 h), all concentrations gave a higher percentage of apoptotic than necrotic cells (with the exception of the concentration of 450 μg/ml, which had an opposite effect after 12 h of incubation) (Figs. 2 and 3).

![Fig. 1. Changes in survival of HL-60 cells after treatment with different concentrations of R. rosea extract during 72 h of incubation (control = 100%).](image)

![Fig. 2. Changes in the percentage of normal, apoptotic and necrotic cells during incubation (6–72 h) in different concentrations of Rhodiola rosea extract. *P < 0.05 in comparison to control value.](image)

<table>
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<th>normal apoptotic necrotic cells (%)</th>
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Fig. 3. Changes in the percentage of normal, apoptotic and necrotic cells during incubation (6, 12, 24 h) in 90 μg/ml (A–C); (A) and (D)—6 h, (B) and (E)—12 h, (C) and (F)—24 h. Cells were incubated in propidium iodide. In R1 gate are alive cells, R2 gate—apoptotic cells, R3—necrotic cells; FSC—forward side scatter, FL-2 fluorescence of propidium iodide.
In the further course of the experiment (24, 48 and 72 h), the percentage of apoptotic cells decreased and the percentage of necrotic cells rose, proportionally to the concentration (Figs. 2 and 3). After 24, 48 and 72 h of treatment with the highest concentration of the extract (225 and 450 µg/ml), the percentage of necrotic cells was much higher than of apoptotic cells (Figs. 2 and 3). Microscopic observation confirmed the death of the cells. Beside normal cells, some cells with distinctly fragmented nuclei or containing apoptotic bodies were observed (Fig. 4).

3.2. Cell cycle analysis

Analysis of the cell cycle after treatment of HL-60 cells with the *Rhodiola rosea* extract showed that, during incubation in the extract, the proportion of G1 and G2/M phase cells was reduced (Figs. 5 and 6), and the percentage of cells at the S phase of the cell cycle grew. This was dependent on the period and concentration of the extract, and was accompanied by the appearance of a large number of apoptotic cells at the sub-G1 phase (Fig. 5).

Treatment with the lowest concentrations of the extract (45 and 90 µg/ml) caused only a slight decrease of G1 and G2/M phase cells after 24 and 48 h (Fig. 6) of incubation, in comparison with the control and higher concentrations of the extract. A significant increase of the percentage of S-phase cells under those concentrations was observed after 24 h of incubation; further incubation caused a reduction of this value, until reaching the control level after 72 h (Fig. 6).

Higher concentrations of the extract (180 and 225 µg/ml) caused an almost two-fold reduction of the percentage of G2/M phase cells as soon as after 12 h of incubation, and approximately about 20% reduction of the percentage of G1 cells. In these concentrations, the percentage of S-phase cells had already risen after 6 h, and it significantly increased during further incubation, reaching the highest values after 24 and 48 h of incubation. After 72 h of incubation, the percentage of cells in the S phase slightly decreased, but it was still much higher than in the control (Fig. 6).

3.3. Mitotic index after treatment with *Rhodiola rosea* extract

The *Rhodiola rosea* extract, in all chosen concentrations, reduced the percentage of cell divisions or led to their total inhibition, which depended on the extract concentration used (Fig. 7).

The lowest one, 45 µg/ml, reduced the mitotic activity of HL-60 cells only slightly after 6 and 12 h of incubation; however, with further incubation, the IM rose to slightly exceed the control value after 48 h (Fig. 7).

The *Rhodiola rosea* extract in a concentration of 90 µg/ml lowered the IM statistically significantly, by about 65%, after 12 h of incubation, which was followed by its increase, to exceed the control value after 48 h of incubation (Fig. 7).

The two highest concentrations of the extract, 180 and 225 µg/ml, led to total inhibition of cell division. The concentration of 180 µg/ml inhibited cell division after 12 h, but they slowly spontaneously reappeared in further incubation (Fig. 7). After treatment with the highest concentration, 225 µg/ml, total inhibition occurred as soon as after 6 h of incubation. This lasted until 48 h of incubation, and small amounts of cell division were seen after 72 h.

3.4. Phase index after treatment with *Rhodiola rosea* extract

Analysis of the phase index after treatment with all chosen concentrations of the extract (45, 90, 180 µg/ml) showed...
a typical significant growth of the prophase index after 6 h of incubation (Fig. 8B–D). In further incubation, this index decreased, which was correlated with a reduction of the mitotic activity of cells. Meanwhile, the telophase index started increasing, and it markedly exceeded the control value, especially after treatment with concentrations of 45 µg/ml and 180 µg/ml. The anaphase index was low, close to 0 (Fig. 8B and D). The highest fluctuation of the index were observed after treatment with the extract at the concentration of 90 µg/ml (Fig. 8C), which caused the highest increase of the prophase index. It remained higher than the control value until the end of incubation, which correlated with a reduction of the metaphase and anaphase indices (Fig. 8C).

After treatment with the *Rhodiola rosea* extract, no morphological changes of chromosomes nor micronuclei were observed; only the highest concentrations of the extract, i.e., 225 and 450 µg/ml, caused thickening and shortening of prophase and metaphase chromosomes (2–3%) (data not shown).
Fig. 6. Percentage of control cells in G1, S, G2/M phases of the cell cycle and after treatment (6–72 h) with different concentrations of *Rhodiola rosea* extract. *P* < 0.05 in comparison to control value.

### 3.5. HPLC analysis

Analysis of the retention times and UV-spectra of compounds present in *Rhodiola rosea* thymone extract, and comparison of standard substances with the same data, showed the presence of phenylpropanoids like rosavin and cinnamyl alcohol (0.13%). The above-mentioned compounds best penetrates into ethyl acetate during extraction. In the UV spectra, the λ_{max} (MeOH) for rosavin and its aglycon cinnamyl alcohol are 206, 251 and 283 nm; thus detection in 250 nm was optimal for these compounds. There were also unidentified compounds (Fig. 9) which together with rosavin and cinnamyl alcohol could caused the observed changes in mitotic activity.

### 4. Discussion

According to recent reports, use of plant extracts is very effective in anticancer therapy, due to the supplementary or synergistic effect of particular compounds of the extract (Li et al., 2000). The cytostatic effect of whole plant extracts on cancer cells is often much better than the effect of their particular biologically active compounds (Yano et al., 1994; Vickers, 2002). From this point of view, it is important to study the mechanism of the anticancer effects of a whole extract and its separate biologically active components. There has been no investigations regarding the effect of *Rhodiola rosea* extracts on the mitotic index, survival, apoptosis induction or cell cycle of healthy and cancerous cells. The results of investigation on in vivo anticancer use of extracts from this plant in mice and rats show their high therapeutic effectiveness (Dement’ev and Iaremenko, 1987; Kelly, 2001). In addition, *Rhodiola rosea* extracts have no side effects, and they act synergistically with some other cytostatics and reduce their toxicity. The anticancer, synergistic and protective effect of *Rhodiola rosea* extracts seems to be very promising for their use in supplementary anticancer therapy.

HPLC analysis of *Rhodiola rosea* root showed the presence of rosavin, which is an adaptogenic agent, and of cinnamyl alcohol (Kucinskaite et al., 2004). However, no salidroside was found, although it is present in roots of *Rhodiola rosea* more than 5 years old, growing in the Botanical Garden of Medicinal Plant Institute in Poznań (Furmanowa et al., 1998). According to reports (Bykov et al., 1999), the content of the above-mentioned compounds in the roots of the plant rises with its age. For therapeutic purposes, roots older than 5 years are used.

The results of our investigations show that extract of *Rhodiola rosea* thymones reduces survival of HL-60 cells by (accelerating them towards) programmed death. However, longer incubation periods result in the appearance of necrotic cells (apart from apoptotic cells), which outnumber the apoptotic cells, especially after treatment with the highest concentrations of the extract. Some reports indicate that low concentrations of some compounds in HL-60 cells induce apoptosis, while high concentrations induce necrosis (Del Bino et al., 1990, 1991). The appearance of necrotic cells may be also a result of incomplete apoptosis (Leist and Nicotera, 1998). Probably, the extract induces apoptosis, and the cells enter necrosis at a later stage; or this result may be connected with a more complex effect of the compounds of the extract, reflected by changes in the percentage of cells at particular stages of the cell cycle after treatment with the extract.
Fig. 8. Changes in the phase index of HL-60 cells after treatment with different concentrations of *Rhodiola rosea* extract.

After treatment, inhibition of mitotic division of HL-60 cells occurred. The inhibition depended on the concentration of the extract and was observed much earlier than the total death of the HL-60 cells. After treatment with the extract at the concentration of 225 ng/ml, total inhibition of cell division occurred as soon as after 6 h of incubation, and the most significant decrease of survival of cells took place between 6 and 48 h of incubation. This could indicate that the cells enter apoptosis and necrosis starting from the interphase of the cell cycle. However, the inhibition of cell division was preceded by an accumulation of cells at the prophase stage. If prophase cells did not proceed to metaphase and entered the interphase instead, the percentage of cells containing double DNA (4C) contents would increase. We did not observe this. Instead, we observed a significant decrease of the prophase index, correlated with the inhibition of cell division, and marked elimination of cells at the G2/M cells. This was accompanied by the appearance of large amounts of apoptotic cells. So it can be speculated that cells enter apoptosis at the border of the G2/M phase.

The results of our earlier work on meristematic cells of *Allium cepa* L. roots showed that, during incubation, a marked accumulation of cells at the G2/M phase of the cell cycle occurs, which was preceded by an accumulation of cells at the prophase (Majewska et al., 2003). The accumulated and changed prophase cells did not enter metaphase, but their division was inhibited, and cells containing 4C passed to interphase and accumulated at the G2/M phase (Majewska et al., 2003).

The present investigation on HL-60 cells has shown that, during incubation with the *Rhodiola rosea* extract, cells at the G2/M phase were eliminated instead of being accumulated. Many reports agree (inter alia, Bhuyan and Groppi, 1989; Gorczyca et al., 1993) that the phase of the cycle in which
the progress is stopped by low concentrations is the same where high concentrations induce apoptosis. This results in a quicker disappearance of cells at those phases of the cell cycle after treatment with higher concentrations.

The results of our investigations showed differences in the effects of low and high concentrations of the extract towards the prophase index, and its specificity towards the cell cycle. Lower concentrations of the extract (45 and 90 μg/ml) caused much weaker elimination of cells at the G2/M phase of the cell cycle, and higher accumulation of cells at the prophase after 6h of incubation. However, after 12h, a marked decrease of the prophase index, connected with reduction of the mitotic index and the simultaneous appearance of a large amount of cells in apoptosis, was observed. This suggests that treatment with lower concentrations results in stopping the cells at the M phase and/or at the border of the G2/M phase, from which they enter apoptosis. The mitotic index and survival of cells rose during further incubation, which was positively correlated with the increase of the prophase index. The proportion of cells in apoptosis was also lower at that moment.

Higher concentrations led to much stronger elimination of cells at the G2/M phase after 12 and 24h of incubation, in comparison with lower concentrations. This was also correlated with a significant increase of the percentage of apoptotic cells at that time. The prophase index rose very slightly after 6h of incubation, in comparison with lower concentrations. This suggests that higher concentrations of the extract force the cells to enter apoptosis from the G2 phase of the cell cycle, rather than from the accumulated prophase.

The percentage of cells at the G1 phase was reduced due to the inhibition of cell divisions, i.e., cells did not enter the G1 phase as they did not begin the division cycle. Cells which were in the G1 phase during incubation passed to the S phase and stopped there. If cells entered apoptosis from the G1 phase, there would be no accumulation of cells at the S phase. Apart from that, especially after treatment with higher concentrations, the share of cells at the G2/M phase was reduced by some 50–60% in comparison to the control after 12h of incubation, whereas the share of G1 cells decreased only by an average of 15–20% at the same time. During prolonged incubation, an accumulation of cells at the S phase occurred, which was, however, not correlated with those cells entering apoptosis. Analysis of the effect of concentrations of 180 and 225 μg/ml shows that the increase of the share of cells at the S phase after 48h, after treatment with both concentrations (58 and 68%), and its further reduction after 72h (42 and 58%) did not correlate with the appearance of a larger amount of apoptotic and necrotic cells after 72h of incubation. On the contrary, the number of both types of cell decreased and their survival rose. Analysis of a lower concentration, 90 μg/ml, leads to a similar conclusion. The percentage of cells at the S phase remained about the same as in the control, but the percentage of apoptotic and necrotic cells rose by about 50% at that time.

The results of our investigations show that the extract of *Rhodiola rosea* leads to inhibition of cell division of HL-60 cells, which is preceded by an accumulation of cells at prophase. This leads to induction of apoptosis and necrosis in HL-60 cells and to a marked reduction of their survival. The cells pass to apoptosis from the G2/M phase of the cell cycle.

The results show that the extract of *Rhodiola rosea* can be used together with other cytostatics for enhancing their effect and reducing of toxicity, or in supplementary cancer therapy.

References


