RHODIOLA ROSEA IN VITRO CULTURE – PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTION

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ABSTRACT

Various Rhodiola rosea organs and tissues from in vitro culture were studied in two areas: searching for the biological active four phenolic compounds and measurement of antioxidant activity of dry residues of EtOH-extracts soluble in PBS using chemiluminescence method. For phytochemical investigation HPLC method was used. Salidroside was typical for organs of intact plant, rosavin for roots of different origin and shoots of intact plants, triandrin was more typical for tissue cultured in vitro, but it was found in all studied samples. Caffeic acid was detected mainly in green and yellow lines of callus, less in other Rhodiola rosea plant material.

The highest antioxidant activity showed the extract from 1.5-years-old callus of green and yellow line, which contained caffeic acid and triandrin. Further observations are in progress.

KEY WORDS: Rhodiola rosea, Crassulaceae, tissue culture, phenolics, antioxidant activity, Luminol-dependent chemiluminescence.

INTRODUCTION

Rhodiola rosea L. Sp. Pl. 1035 (1753) known also as Sedum roseum (L.) Scop. and Sedum rhodiola DC. belongs to the family Crassulaceae, subfamily Sedoideae (Engler 1964). This species is distributed mainly in North Asia and in mountains of central Europe.

Rhodiola rosea plants can be propagated by seeds or vegetatively by rhizome and root cuttings. The plants grow wild and are cultivated in several places but mainly in Russia.

The process of organ regeneration (Aleksandrova et al. 1981) and plant micropropagation was elaborated in different laboratories, also by Furmanowa et al. (1995b).

The medicinal value of the plants comes from its compounds, which may be used to treat several illnesses. The extract from roots known as “golden roots” was used in traditional medicine as an adaptogetic and stimulating agent (Saratikov et al. 1969, Sokolov et al. 1985, Zong et al. 1991 and Wagner et al. 1994). Antistress, endocrine, CNS activities in animals after oral administration of Rhodiola rosea extracts containing salidroside and rosavin (syn. rosavin) and other biological properties of R. rosea extracts have also been described (Dement’eva and Iaremenko 1987, Hushbaktova et al. 1989, Molokovskii et al. 1989, Galaktyonov et al. 1991, Udintsev and Shakhov 1991). Kurkin and Zapesochnaya (1986) gave a general review of the chemical compounds and pharmacological activity of R. rosea plants. They biosynthesized compounds which belong to different chemical groups such as: flavolignans, flavonoids, phenylpropanoic acids, coumarins, sterols, monoterpenes, lactones and organic acids. The adaptogenic activity was demonstrated by phenylethanoides: salidroside, tyrosol and phenylpropanoides: cinnamic alcohol and its glycosides: rosin, rosavin, rosarin, all of them being the main components of the raw material, roots.

According to Kryyanov et al. (1991) the main constituents of the extracts from R. rosea rhizomes include cinnamoylglycosides and salidroside. It seems, that all of them should be used for standardization of the preparation. On the base on the HPLC method the content of these compounds oscillated between 7,8 and 11,8% of DW.

Barnaful et al. (1986) compared some biological effect of chemical compounds isolated from Rhodiola species with the activity of extracts from Eleutherococcus senticosus (Rupr. et Maxim.) Maxim. and Aralia mandshurica Rupr. et Mey. Wagner et al. (1994) stressed the chemical relationship between R. rosea phenylpropanoides and syringin (= eleutheroside B) isolated from Eleutherococcus senticosus, one of its bioactive compounds.
Now, the "Liquid Extract of Rhodiola", prepared from the roots is used in Russia as a tonic drug (Goss. Farm. SSSR 1984).

Other compounds were determined in callus and suspension culture of *R. rosea*. Triandrin (0.19% of DW) is the main component of suspension culture and one of the most biologically active agents (Kurkin et al. 1991). No cinnamoylglycosides and salidroside typical for roots were detected by them using HPLC method. The authors (Kurkin et al. 1991) suggested that determination of triandrin should be used for standardization of extracts obtained from *R. rosea* cell suspension. Apart of triandrin in suspension culture p-coumaric alcohol, p-coumaric acid, glucosides of coumaric and caffeic acids were found. In callus, from all the mentioned above compounds, caffeic acid and lignans were also observed.

Suspected caffeic acid was detected by Furmanowa et al. (1995a) in roots, shoots, green and yellow callus of *R. rosea* cultured in vitro.

Furmanowa et al. (1995b) described the effect of *Rhodiola rosea* extract obtained from roots growing in vivo or in vitro on the formation of new blood vessels (angiogenesis) in mice and the effect of extracts on the release of angiogenic cytokines by human blood mononuclear cells. The extract of rhizomes and roots of intact plants (5-years-old) inhibited the second phenomenon, but the extract prepared from roots excised from plantlet (2-months old), regenerated in vitro, stimulated it.

Free radicals, e.g. hydrogen peroxide, superoxide anion radical, hydroxyl radical and singlet oxygen, have been implicated as mediators of cellular injury in a variety of clinical conditions. Caffeic acid known as antioxidant (Larson 1988) found in different organs and tissue of *Rhodiola rosea* (Furmanowa et al. 1995a) stimulated us to begin present investigation.

In this research work we studied extracts of various *Rhodiola rosea* plant material obtained from intact plants as well as from organ and tissue cultured in vitro.

Preliminary qualitative HPLC analysis and measurement of antioxidant activity of the extracts using chemiluminescence method are presented.

**MATERIALS AND METHODS**

**Plant materials**

For phytochemical and immunological experiments the following *Rhodiola rosea* plant materials were used:

**Rr 1** - rhizomes and roots of intact plants (5-years-old) collected in 08., 1991,

**Rr 2** - roots excised from plantlets regenerated in vitro (3-months-old),

**Rr 3** - shoots of intact plants grown in garden (5-years-old),

**Rr 4** - shoot of plantlets regenerated in vitro (3-months-old),

**Rr 5** - green callus (10th-14th passages i.e. about 1-year-old),

**Rr 6** - green line of callus (17th-21st passages i.e. 1,5-years-old),

**Rr 7** - yellow line of callus (17th-21st passages i.e. 1,5-years-old)

Female intact plants of *Rhodiola rosea* were collected from the garden of Department of Biology and Pharmaceutical Botany, Medical University of Warsaw (Poland). Plantlets were regenerated in vitro using the method described by Furmanowa et al. (1995b). For callus induction and maintenance the best of various methods (Furmanowa et al. 1995b) was selected and modified. Callus was induced from leaves of young intact plants before florescence in April, 1990. Green callus tissue was formed from the sterile leaf segments on Murashige and Skoog's (MS) solid medium (Murashige and Skoog 1962) in own modification containing 2.0 mg/l 6-benzylaminopurin (BAP) (Lachema, Czechoslovakia), 2.0 mg/l α-naphthylacetic acid (NAA) (Loba-Chemie, Austria), 1 g/l casamino acids (c.a.) (Difco, USA), 6% sucrose and 8 g/l Purified-agar (Difco, USA); pH 5.6 with 0.1M NaOH. After four passages, callus was subcultured on a new media with various combinations of auxins and cytokinins to find the best growth conditions, in case of MS solid medium (Murashige and Skoog 1962) with 2.0 mg/l BAP, 2.0 mg/l indole-3-butyric acid (IBA) (Gibco, USA), 1 g/l c.a., 3% sucrose and 8 g/l Purified-agar; (pH 5.75). From the 17th passage, two lines: a dense, deep green and a friable yellow-green heterogeneous line were cultured simultaneously under regime of 12h light (2000 Lux). Callus cultures were subcultured every four weeks. Growth rate of fresh weight of 30-days-old cultures in 17th passage for green callus line was 887.8, and for yellow one - 713.

**Extraction procedure**

Air-dried and powdered plant material was extracted three times with 96% EtOH p.a. at ratio 1 : 20 using the method of Kyrnanov et al. (1988): 1 g : 20 ml for phytochemical analysis and 3 g : 60 ml for immunological research at the same temperature 25°C for 1,5 h on a rotary shaker (80 rpm). Combined extracts were filtered and evaporated under vacuum. Each dry residue of the extracts used for HPLC determination of selected biological active compounds was dissolved in water for immunological study - in the phosphate buffered saline (PBS).

HPLC samples were centrifuged for 10 min at 10000 rpm and analysed using the modified method described by Kurkin et al. (1991).

**HPLC analysis**

Equipment: Shimadzu High Performance Liquid Chromatograph consisted of: solvent delivery module LC-10AD, degasser (Helium) DGU-10A, detector ultraviolet-visible spectrophotometric SPD-10A module, integrator Chromatopac C-R6A, injector manual - 20 μl. Column: MN LiChrosorb 100 RP-18 5μ, 250 mm x 4 mm. Mobile phase: acetonitrile (Sigma) - 0.2% acetic acid (in water), 125 : 875. Flow rate: 1,0 ml/min. Range: 0.1 AUFS. Detection: 264 nm, 280 nm. Time of analysis using isocratic system: 45 min. Control using external standards in water solutions: caffeic acid (Sigma), rosinavin, salidroside and triandrin (supplied by Prof. Dr. G.G. Zapesochyn from the Russian Research Institute of Medicinal and Aromatic Plants in Moscow, Russia). All reagents used were analytical or HPLC grade.

Studies of antioxidant activity were performed using formyl-methionyl-leucyl-phenyl-alanine (FMLP)-stimulated human neutrophil granulocyte chemiluminescence as a model.

Preparation of samples was described in the part 'Extraction procedure'. Dry residues were dissolved in the phosphate buffered saline (PBS). Dosages of *Rhodiola rosea* extract were: 2 and 20 μg/ml.

Isolation of neutrophils (polymorphonuclear leukocytes - PMNs). Neutrophils were obtained using the method of Zeman and Tchorzewski (1989). Heparinised peripheral blood
of healthy donors was centrifuged (400×g for 25 min) on Gradisol G (Polfa Kutno). The lower band containing PMNs was removed and the neutrophils were washed twice in PBS (400×g for 10 min). Next they were counted and resuspended in PBS supplemented with 0.1% glucose and 0.1% bovine serum albumine (PBS-GA), at a density of 4×10^6/ml, purity 95%, viability >97% by exclusion of trypan blue.

**Chemiluminescence assay (Cl)**

PMNs chemiluminescence activity was measured in LKB-Wallac 1251 luminometer according to the method of Eason and Cole (1980). Each test-tube contained 50 μl of neutrophil suspension, 850 μl of luminol dissolved in PBS-GA (10^-3 M final concentration). One of the seven of *Rhodiola rosea* extract (50 μl) was added to cells. At time '0', 100 μl of stimulating agent — FMLP, or PBS-GA (chemiluminescence background) was added.

Cl response (mV) was measured for 15 min at 8 sec intervals. Mean activity of 30 control (without plant extract) samples was 107.9±12.22 mV.

The results were analyzed by Student's t test and expressed as inhibition index — the ratio of peak value Cl in the stimulated samples to the nonstimulated (Table 2).

**RESULTS AND DISCUSSION**

The qualitative determination of the four studied chemical compounds: salidroside, rosavin, triandrin and caffeic acid in seven samples of *Rhodiola rosea* organs and tissue showed some differences in their localization. Triandrin was found in all seven samples, caffeic acid was in six samples except the extracts of roots excised from plantlets regenerated in vitro, when only traces were detected. Salidroside was typical for organs (roots and shoots) of intact plant. Rosavin was in roots of different origin, shoots of intact plant and green callus (one-year-old) (Table 1, Fig. 1, 2, 3).

Our results are in some accordance with the observation of Kurkin et al. (1991) who presented the opinion that phenylpropanoids as rosin typical for the intact plant can be in vitro transformed to p-hydroxyrosin (= triandrin) by phenylhydroxylases. In our experiments suspected triandrin was always observed in callus.

Antioxidant activity of various *Rhodiola rosea* extracts (sample Rr 1 to Rr 7) (Table 2) are in some relationship with the phytochemical analysis. The highest activity showed the extracts from older (1,5-years-old) callus of green (Rr 6) and yellow line (Rr 7), which contained caffeic acid and triandrin. The sample Rr 2 (extract from roots excised from plantlets regenerated in vitro), which contained only traces of caffeic acid does not show antioxidant activity.

Possibly, this effect is caused by caffeic acid and other polyphenols. According to Larson (1988), acidic compounds incorporating phenolic groups have been repeatedly implicated as active antioxidants; to this group belong caffeic acid. Qualitative and quantitative phytochemical differences in various generations of studied *Rhodiola rosea* plant material could influence their Cl-suppressing activity. Quite new in our investigation was also to find suspected triandrin in the roots and shoots of intact plants. To confirm our observation further studies will be done.

**TABLE 1.** HPLC qualitative analysis of selected phenolic compounds in organ and tissue cultures of *Rhodiola rosea* developed in vivo and in vitro

<table>
<thead>
<tr>
<th>Extract</th>
<th>Salidroside</th>
<th>Triandrin</th>
<th>Rosavin</th>
<th>Caffeic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rr 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rr 2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Rr 3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rr 4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rr 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rr 6</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Rr 7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ detected, — not found, +/− traces

**TABLE 2.** Antioxidant effects of extracts prepared from *Rhodiola rosea* organs and tissues

<table>
<thead>
<tr>
<th>Dose</th>
<th>Rr 1</th>
<th>Rr 2</th>
<th>Rr 3</th>
<th>Rr 4</th>
<th>Rr 5</th>
<th>Rr 6</th>
<th>Rr 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 μg/ml</td>
<td>x = 1,00±0,03 n = 17</td>
<td>x = 1,02±0,06 n = 15</td>
<td>x = 1,00±0,04 n = 9</td>
<td>x = 0,94±0,03 n = 12</td>
<td>x = 1,04±0,04 n = 9</td>
<td>x = 0,67±0,07 n = 12</td>
<td>x = 0,77±0,04 n = 9</td>
</tr>
<tr>
<td>20 μg/ml</td>
<td>x = 0,94±0,08 n = 12</td>
<td>x = 1,16±0,07 n = 12</td>
<td>x = 0,91±0,06 n = 9</td>
<td>x = 0,71±0,06 n = 12</td>
<td>x = 0,71±0,04 n = 9</td>
<td>x = 0,47±0,06 n = 10</td>
<td>x = 0,43±0,03 n = 9</td>
</tr>
</tbody>
</table>

FMLP-stimulated PMNs without *Rhodiola rosea* extract (mean mV±SE): 107.91±12.22 n = 30.
Fig. 1. HPLC chromatograms of standard substances: 1 — salidroside, 2 — triandrin, 3 — caffeic acid, 4 — rosavin (—— UV, 264 nm; ———— UV, 280 nm).

Fig. 2. HPLC chromatogram of the extract from roots of *Rhodiola rosea* excised from plantlets regenerated in vitro, 2 — triandrin, 3 — caffeic acid, 4 — rosavin.

Fig. 3. HPLC Chromatogram of the extract from leaves derived yellow callus line of *Rhodiola rosea* cultured on MS medium with BAP and IBA, 2 — triandrin, 3 — caffeic acid, 4 — rosavin.

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We are grateful to Prof. Dr. A. W. Alfermann (University of Düsseldorf, Germany) for valuable suggestions in the area of phytochemical analysis during the scholarship of M. Michalska-Hartwich (Pharm. M.) in Institute of Developmental and Molecular Biology of Plants in Düsseldorf.

LITERATURE CITED


RÓŻENIEC GÓRSKI, RHODIOLA ROSEA W HODOWLI W IN VITRO.
BADANIA FITOCHIMICZNE I DZIAŁANIE PRZECIWUTLENIAJĄCE

STRESZCZENIE


Analiza fitochemiczna wykazała obecność salidrozydu w organach roślin gruntowych Rhodiola rosea, rozawiny – w kłączach i korzeniach pochodenia in vivo i in vitro oraz w pędach roślin gruntowych. Triandryn charakteryzuje dla kultur tkankowych w in vitro tego gatunku, wykryto w śladowych ilościach w całym badanym materiale Rhodiola rosea. Podobnie, kwas kawowy znaleziono głównie w zielonej i żółtej linii tkanki kalusowej, w mniejszych ilościach w pozostałych próbках.

Najniższe wartości supresji chemiluminescencji świadczące o najsilniejszym działaniu przeciwtleniającym wykazały wyciągi otrzymane z 1,5 rocznej kultury tkanki kalusowej obu linii zielonej i żółtej, zawierających w swoim składzie chemicznym kwas kawowy i triandryn.

Dalsze badania są w toku.

SŁOWA KLUCZOWE: Rhodiola rosea, Crassulaceae, kultura tkankowa, fenole, działanie przeciwtleniające, chemiluminescencja.