RHODIOLA ROSEA IN VITRO CULTURE – PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTION

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(Received: October 28, 1997. Accepted: January 15, 1998)

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 adaptogenic and stimulating agent (Ssaratikov 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 biosynthesize compounds which belong to different chemical groups such as: flavolignans, flavonoids, phenylpropanoides, 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 Kiryanov et al. (1991) the main constituents of the extracts from *R. rosea* rhizomes include cinnamoyl-glycosides 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.

Barnaulov 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 the all 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 simultaneous 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 Kiryanov 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 (Hellium) DGU-10A, detector ultraviolet-visible spectrophotometric SPD-10A module, integrator Chromatopac C-R6A, injector manual – 20 μ l. Column: MN LiChrosorb 100 RP-18 5 μ 250 mm $\times$ 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), rosavin, salidroside and triandrin (supplied by Prof. Dr. G.G. Zapesochayna 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 μ g/ml.

Isolation of neutrophils (polymorphonuclear leukocytes – PMNs). Neutrophils were obtained using the method of Zeman and Tchórzewski (1989). Heparinised peripheral blood

of healthy donors was centrifugated ($400\times g$ for 25 min) on Gradisol G (Polfa Kutno). The lower band containing PMNs was removed and the neutrophils were washed twice in PBS ($400\times g$ 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 $4\times 10^6/\text{ml}$, purity 95%, viability $> 97\%$ by exclusion of trypan blue.

Chemiluminescence assay (CI)

PMNs chemiluminescence activity was measured in LKB-Wallac 1251 luminometer according to the method of Easmon 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.

CI response (mV) was measured for 15 min at 8 sec intervals. Mean activity of 30 control (without plant extract) samples was $107,91\pm 12,22$ mV.

The results were analyzed by Student's *t* test and expressed as inhibition index – the ratio of peak value CI 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 CI-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

Extract	Salidroside	Triandrin	Rosavin	Caffeic acid
Rr 1	+	+	+	+
Rr 2	–	+	+	+/-
Rr 3	+	+	+	+
Rr 4	–	+	–	+
Rr 5	+	+	+	+
Rr 6	–	+	+/-	+
Rr 7	–	+	+/-	+

+ detected, – not found, +/- traces

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

Dose	Inhibition index $\pm$ SE						
	Rr 1	Rr 2	Rr 3	Rr 4	Rr 5	Rr 6	Rr 7
2 $\mu\text{g}/\text{ml}$	$x = 1,00\pm 0,03$ $n = 17$	$x = 1,02\pm 0,06$ $n = 15$	$x = 1,00\pm 0,04$ $n = 9$	$x = 0,94\pm 0,03$ $n = 12$	$x = 1,04\pm 0,04$ $n = 9$	$x = 0,67\pm 0,07$ $n = 12$ $p < 0,01$	$x = 0,77\pm 0,04$ $n = 9$ $p < 0,01$
20 $\mu\text{g}/\text{ml}$	$x = 0,94\pm 0,08$ $n = 12$	$x = 1,16\pm 0,07$ $n = 12$	$x = 0,91\pm 0,06$ $n = 9$	$x = 0,71\pm 0,06$ $n = 12$ $p < 0,01$	$x = 0,71\pm 0,04$ $n = 9$ $p < 0,01$	$x = 0,47\pm 0,06$ $n = 10$ $p < 0,001$	$x = 0,43\pm 0,03$ $n = 9$ $p < 0,001$

FMLP-stimulated PMNs without *Rhodiola rosea* extract (mean mV $\pm$ SE): $107,91\pm 12,22$ $n = 30$.

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.

ACKNOWLEDGMENTS

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

- ALEKSANDROVA I. V., DANILINA A. N., GALKINA L. V., ANISIMOV O. L., 1981. Sposob poluchen'ya biologicheskikh aktivnykh veshchestv. Otkryt'ya, izobreten'ya, promyshlennyye obrazcy, tovarnye znaki 44, A.s. 885252: 102.
BARNAULOV O. D., LIMARENKO A., KURKIN V. A., ZAPESCHONAYA G. G., SHCHAVLINSKII A. N., 1986. Sravnitel'naya ocenka biologicheskoy aktivnosti soedineniy vydelenykh iz vidov *Rhodiola* L. Khim.-Farm. Zh. 20: 1107-1112.

- DEMENT'EVA L. A., IAREMENKO K. V., 1987. Effect of a *Rhodiola* extract on the tumor process in an experiment. Vopr. Onkol. 33: 57-60.
EASMON C. S. F., COLE P. J., WILLIAMS A. J., HASTINGS M., 1980. The measurement the opsonic and phagocytic function by Luminol-dependent chemiluminescence. Immunology, 41: 67-74.
ENGLER A., 1964. Syllabus der Pflanzenfamilien. Vol. 2., Borntraeger, Berlin, pp. 199-200.
FURMANOWA M., MICHALSKA M.*, ALFERMANN A. W., SKOPINSKA-ROZEWSKA E., MALKOWSKA G., ROGALA E., 1995a. *Rhodiola rosea* tissue and cell cultures-phytochemical and immunological aspects of studies. In: 16th Sci. Meet. of Polish Pharm. Soc. "Science and pharmaceutical practice in health prevention". Warsaw, 20-21.10.1995. Abstr. A 50, pp. 103-104.
FURMANOWA M., OLEDZKA H., MICHALSKA M.*, SOKOLNICKA I., RADOMSKA D., 1995b. *Rhodiola rosea* L. (Roseroor). In vitro regeneration and the biological activity of roots. In: Biotechnology in Agriculture and Forestry, Vol. 33.; Medicinal and

- Aromatic Plants VIII. Bajaj, Y.P.S. (ed.). Springer-Verlag, Berlin, Heidelberg, New York, pp. 412-426.
- GALAKTYONOV S. G., NIKOLAYCHIK V. V., YURIN V. M., BIRGER P. S., KOZLOVSKAYA E. V., MIHNEVA L. M., MAZUR L. I., 1991. Issledovan'ye protektorov modiliruyushchikh povrezdayushcheye deystv'e peptidov grupy "srednikh molekul" na kletki krovi. Khim.-Farm. Zh. 11: 8-10.
- GOSSUDARSTVENNAYA FARMAKOPEYA SSSR, 1984. Extractum Rhodiolae Fluidum 10th Ed., Medicina. Moskva.
- HUSHBAKTOVA Z. A., SYROV V. N., KULIEV Z., 1989. Issledovan'ye gipolipidemicheskikh svoystv polimernykh proantocyanidinov iz rasteniy ispolizuyemykh v narodnoy medicinie. Khim.-Farm. Zh. 9: 1111-1115.
- KIRYANOV A. A., BONDARENKO L. T., KURKIN V. A., ZAPESOCHNAYA G. G., 1988. Opredelen'ye rozavidina v kornevishchakh Rodioly rozovoy. Khim.-Farm. Zh. 22: 451-455.
- KIRYANOV A. A., BONDARENKO L. T., KURKIN V. A., ZAPESOCHNAYA G. G., DUBICHEV A. A., VORONTSOV E. D., 1991. Opredelen'ye biologicheskikh aktivnykh komponentov kornevishch *Rhodiola rosea*. Khim. Prir. Soedin. 3: 320-323.
- KURKIN V. A., ZAPESOCHNAYA G. G., 1986. Khimicheskii sostav i farmakologicheskiye svoystva rasteniy roda *Rhodiola* (Obzor). Khim.-Farm. Zh. 20: 1231-1244.
- KURKIN V. A., ZAPESOCHNAYA G. G., DUBICHEV A. G., VORONTSOV E. D., ALEKSANDROVA I. V., PANOVA R. V., 1991. Fenilpropanoidy kallusnoy kultury *Rhodiola rosea*. Khim. Prir. Soedin. 4: 481-490.
- LARSON R., 1988. The antioxidants of higher plants. Phytochemistry, 27: 969-978.
- MOLOKOVSKII D. S., DAVYDOV V. V., T'ULENEV V. V., 1989. The action of adaptogenic plant preparations in experimental alloxan diabetes. Probl. Endokrynol. Moskva. 35: 82-87.
- MURASHIGE T., SKOOG F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
- SOKOLOV S. J., IVASHIN V. M., ZAPESOCHNAYA G. G., KURKIN V. A., SHCHAVLINSKII A. N., 1985. Issledovan'ye neurotropnoy aktivnosti novykh veshchestv vydelenykh iz *Rhodioly rozovoy*. Khim.-Farm. Zh., 19: 1367-1371.
- SSARATIKOV A. S., KRASNOV E. A., CHNIKINA L. A., DUVIDSON L. M., SOTOVA M. I., MARINA T. F., NECHODA M. F., AXENOVA R. A., TSCHERDINTSEV S. G., 1969. Rhodiolsid, a new glycoside *Rhodiola rosea* and its pharmacological properties. Pharmazie. 23: 392-395.
- UDINTSEV S. N., SHAKHOV V. P., 1991. Decrease of cyclophosphamide haematotoxicity by *Rhodiola rosea* root extract in mice with Ehrlich and Lewis transplantable tumors. European Journal of Cancer. 27: 1182.
- WAGNER H., NÖRR H., WINTERHOFF H., 1994. Plant adaptogens. Phytomedicine. Vol. 1/1: 63-76.
- ZEMAN K., TCHÓRZEWSKI H., 1989. Prosta i szybka metoda równoczesnej izolacji z krwi obwodowej wysoko oczyszczonych limfocytów i granulocytów obojętnochnych. Immunol. Polska. XIII: 217-224.
- ZONG Y., LOWELL K., PING J. A., CHE C. T., PEZUTTO J. M., FONG H. H., 1991. Phenolic constituents of *Rhodiola coccinea*, a Tibetan folk medicine. Planta Medica. 57:589.

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

STRESZCZENIE

Przeprowadzono badania różnorodnego materiału roślinnego *Rhodiola rosea* otrzymanego in vivo oraz in vitro. Obserwacje dotyczyły: poszukiwania wybranych biologicznie czynnych związków fenolowych – salidrozydu, rozawiny, triandryny i kwasu kawowego przy użyciu wysokosprawnej chromatografii cieczowej (HPLC) oraz określenia właściwości przeciwutleniających suchych pozostałości wyciągów etanolowych metodą chemiluminescencyjną.

Analiza fitochemiczna wykazała obecność salidrozydu w organach roślin gruntowych *Rhodiola rosea*, rozawiny – w kłączach i korzeniach pochodzenia in vivo i in vitro oraz w pędach roślin gruntowych. Triandrynę charakterystyczną dla kultur tkankowych 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 próbkach.

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

Dalsze badania są w toku.

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