Chemical composition of golden root (*Rhodiola rosea* L.) rhizomes of Carpathian origin

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S u m m a r y

Golden root (*Rhodiola rosea* L.) is a valuable medicinal plant that naturally grows in mountains of many parts of the world, including Carpathian Mountains. In the rhizome it accumulates the secondary metabolites that have been shown to possess different medical activities and valuable adaptogens effects. The interest in *Rhodiola* is focused on the phenylpropanoids (cinnamic alcohol and its glycosides – rosavin, rosarin and rosin), phenolic compounds (salidroside, p-tyrosol), flavolignans (rhodiolin) and terpenes. Also, practical interest is paid to other groups of chemical compounds (volatiles, terpenes etc.). Sensitive RP-HPLC methods were developed for quantitative and qualitative analysis of secondary metabolites in *Rhodiola rosea* plants. Chromatographic data was acquired using photodiode array (PDA) detection. While analyzing the extracts from the Carpathian rhizomes of *Rhodiola rosea* L. we were able to reveal the complex spectrum of secondary metabolites including phenylpropanoids, monoterpenes, flavonoids, coumarins and organic acids. The structure of some compounds was confirmed by nuclear

magnetic resonance spectroscopy (NMR) and infrared analysis (IR). It is also important to point that essential oils from these rhizomes also contained compounds characteristic for *Rhodiola rosea*. Therefore, this quantitative and qualitative applicability of the methods offers efficient and reliable means for the evaluation of *Rhodiola rosea* L. and products thereof.

Key words: *Rhodiola rosea, terpenes, glycosides, phenylpropanoids, salidroside, rosavin, aroma volatiles, RP-HPLC, NMR, IR*

Abbreviations: s – singlet, d – doublet, t – triplet, dd– doublet of doublets, dt – doublet of triplets, m – multiplet. Melting points (mp) were determined on a Boetius hot stage.

INTRODUCTION

Rhodiola rosea L. is a valuable medicinal plant belonging to the *Crassulaceae* family. This species is historically endemic to many parts of the world, including North Asia, Alaska, northern parts of Europe and the mountains of Central Europe, including the Carpathian Mountains. The adaptogen effects of *R. rosea* are manifested in the prevention of tiredness, increase of learning and memory capacity, prevention of age related disorders and diseases as well as avoidance and elimination of depression and sexual disorders [6]. *R. rosea* can be seen as a unique, balancing, anti-stress and immunoprotective herb which improves or restores resistance against diseases and enhances immune system tolerance, with no known side effects or detectable level of toxicity. The major curative capacity of extracts from *R. rosea* is due to the anti-stress and anti-oxidative activities of its chemical compounds. The following curative effects of *R. rosea* have been documented: anti-arrhythmic properties, cardioprotection against ischemic damage, protective effects for the liver, strong general antioxidative protection at the cellular level, prevention of age related disorders and diseases, antitumor effects and attenuation of adverse effects of chemotherapy [6-8]. By limiting the adverse effects of free radical damage *R. rosea* extracts are able to combat the diseases associated with aging and cell mutagenesis, an immediate cause of cancer.

The active substances found in the extracts from *Rhodiola rosea* belong to several chemical groups, namely: phenylpropanoids, phenyletanoids, monoterpenes, flavonoids, coumarins and organic acids [1-5, 8]. The most unique active chemical constituents are the phenylpropanoids, rosavin, rhodiolin, rosarin, rosin, salidroside, p-tyrosol. Out of about 200 species of *Rhodiola* genus only *Rhodiola rosea* contains rosavin. The quality of *Rhodiola rosea* extract is determined by the content of rosavin and salidroside. Salidroside is contained in other similar *Rhodiola* species and should not be used as the only marker compound [8].

Golden root is highly valued in many herbal remedy treatments and traditional medicines. Due to its intensive collection from the nature, indeed, its rapidly growing acceptance in the herbal supplements industry suggests that demand for this plant will continue to grow at a rapid rate in the future. Based on these

factors, as a part of INTAS program we have initiated a multidisciplinary research project to enable the rapid introduction of *R. rosea* in agriculture. The first step in this project was to determine the content of secondary metabolites in rhizomes of *Rhodiola rosea* L. growing in the Romanian Carpathian Mountains that serve as a source of genotypes for introduction in agricultural practice. The Carpathian *Rhodiola rosea* was not analyzed yet regarding the content of secondary metabolites. The obtained experimental results are included in present paper.

MATERIAL AND METHODS

Collection and drying of rhizomes

Rhizomes of *Rhodiola rosea* were collected at the beginning of September at the altitude of 1700–1800 m on the Ineu Mountain, Eastern Carpathian massive, Romania. The typical *Rhodiola rosea* plants grown on mountain Ineu are presented in photo 1. Rhizomes were cut on sites 5–10 cm long, splinted and dried in drying case at 40–45°C. The mean content of water in rhizomes was 59.2±3.28%.

Photo 1. Typical *Rhodiola rosea* plants, grown on the Mountain Ineu (Romanian Eastern Carpathian Mountains)

Extraction of secondary metabolites

Rhodiola rosea L. rhizomes at the amount of 2 g were macerated into small pieces (2–3 mm) and then extracted with MeOH (60%) at room temperature and at constant strong stirring within 1.5 h. The methanolic extract was filtered and chromatography was performed on 1 ml of extract on a silica gel column (1.5 cm x 25 cm, SiO_2, 5 g, L 40/100), then eluted with a mixture of $CHCl_3$: MeOH (1:1, 100 ml). The four last fractions

were combined, the solvent was removed at the reduced pressure. The residue (20 mg) was dissolved one more time in MeOH (1 ml) and subjected to HPLC analysis.

HPLC analysis of secondary metabolites

Analyses were carried out using an Agilent 1100 series HPLC. The analytic column was a Zorbax RX 300 C-18 with guard column 300 SB C-18. Mobile phase was a mixture of MeCN: H_2O (in gradient from 2 to 100%, after that to 0%) with preliminary washing with phosphate buffer (0.025 M). Flow rate – from 0.2 up to 0.8 ml/min. Maximal pressure – 300 bar, analyze time was 65 min. Detection was performed by DAD on 222, 254 and 280 nm [2].

Steam distillation (SD) of volatiles from rhizomes of *Rhodiola*

The distillation apparatus consisted of a heating cap, 0.1 L extraction flask, a 5 ml graduated receiver (Dean and Stark) and condenser.

2 g of *Rhodiola rosea* L. rhizomes were macerated into small pieces (2–3 mm) and 50 ml of water was used. The distillation was carried out for 6 h after reaching the boiling point. The distillate obtained was extracted supplementary with diethyl ether. After filtration it was dried up and used for sample preparation by diluting in acetonitrile.

HPLC analysis of secondary volatiles from *Rhodiola* rhizomes

Analyses were carried out using an Agilent 1100 series HPLC. The analytic column was Zorbax XDB C-18 with guard column Extend C-18. Mobile phase was a mixture of MeCN: H_2O (in gradient), flow rate from 0.4 up to 1.2 ml/min. Maximal pressure was 300 bar, temperature 40^0C, time of analysis was 43 min. Detection was performed by DAD on 195, 200 and 210 nm.

Extraction, isolation and characterization of p-tyrosol

Dried up rhizomes of *Rhodiola rosea* (200 g) were macerated into small pieces (2–3 mm) and then were extracted with MeOH in Soxhlet, within 8 h. The extract was filtered and the removal of the solvent under reduced pressure yielded a dark brown extract (82 g), which was dissolved in water and extracted one more time with buthanol. The received extract was dried up, and the residue (24.2 g) was purified by column chromatography on polyamide, eluted with water. The collected fractions were monitored by TLC, using the system: toluene – EtOAc – EtOH (2:2:1). The fractions containing some products were combined and extracted with BuOH, then concentrated and the rest (0.16 g) was purified by column chromatography on silica gel eluted with mixture of chloroform – MeOH (1:1). One from isolated compound was tyrosol (21 mg, 0.01%) as white crystals identified by m.p., NMR and IR analysis.

Tyrosol, m.p.=87^{0}C, (92-93^{0}C)$^{lit.}$ IR γ_{max} (nujol) cm^{-1}: 710, 805, 1045, 1215, 1385, 1460, 1595, 2920, 3395

^{1}H NMR (400 MHz, DMSO, ppm) δ 2.61 (t, 2H, C_2-CH_2, J= 7.2 Hz), 3.51 (d, 1H, C_1-H, J=7.2 Hz), 3.54 (d, 1H, C_1-H, J=6.8 Hz), 4.57 (t, 1H, J=5.2 Hz, OH), 6.66 (d, 2H, J=8.2 Hz), 6.99 (d, 2H, J=8.2 Hz), 9.12 (s, 1H, OH)

^{13}C NMR (100 MHz, DMSO, ppm) δ 38.17 (C_1), 62.45 (C_4^1), 62.57 (C_2), 114.81 (C_3^1), 114.91 (C_5^1), 129.43 (C_2^1), 129.64 (C_6^1), 155.41 (C_1^1)

Acetylation of tyrosol

In order to confirm the structure a small sample of tyrosol **1** was acetylated acetic anhydride in Py for 3 hours at the room temperature. Afterwards, the reactionary mixture was diluted with water and extracted with diethyl ether for three times. The combined ether extract was washed with a solution of HCl (15%) to remove pyridine from system, then by water and next was dried on Na_2SO_4 (anh.). After a filtration and removal of the solvent the product was purified by chromatography column on SiO_2, eluted with 15% EtOAc in petroleum ether, to give tyrosol diacetate as white crystals. Its spectral data confirm structure of tyrosol [12].

^{1}H NMR (400 MHz, DMSO, ppm) δ 2.04 (s, 3H), 2.29 (s, 3H), 2.93 (t, 2H, C_2-CH_2, J=7.0 Hz), 4.27 (t, 2H, J=7.0 Hz, C_1-CH_2), 7.02 (d, 2H, J=8.4 Hz), 7.22 (d, 2H, J=8.4 Hz)

^{13}C NMR (100 MHz, DMSO, ppm) δ 20.97 ($\underline{CH_3}$CO), 21.13 ($\underline{CH_3}$CO), 34.47 (C_1), 64.75 (C_2), 121.58 (C_3^1, C_5^1), 129.85 (C_2^1, C_6^1), 135.44 (C_6^1), 149.36 (C_1^1), 169.57 ($CH_3\underline{CO}$), 171.01 ($CH_3\underline{CO}$)

General

IR spectra were obtained on Specord 70 in Nujol. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 400 in DMSO and $CDCl_3$ solution (400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C, respectively). Chemical shifts are given in ppm values and coupling constants in Hertz.

For analytical TLC, Merck silica gel 60G in 0.25 mm think layers was used. Chromatographic separations were carried out on Merck silica gel 60 using petroleum ether-ethyl acetate mixture in increasing polarity. All solvents were purified and dried by standard techniques just before use. Usual work-up means that water was added to the reaction mixture, which was afterwards extracted with diethyl ether, the combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated.

RESULTS AND DISCUSSIONS

There is a large information on the composition of secondary metabolites in rhizomes of the genotypes grown in the mountains of Siberia and Northern Russia [1-5, 8, 9]. To our knowledge, our analysis of rhizomes collected in Romanian Carpathian Mountains was conducted for the first time. Extracted and purified secon-

dary metabolites were separated into components in column filled with the SiO_2, with use of the RP-HPLC method. Monitoring of samples for of content of secondary metabolites was carried out at in 222 nm and 280 nm (the maximum adsorption of salidroside and cinnamic alcohol) and at 254 nm, at which the specific adsorption of phenylpropanoids (rosavin, rosin and rosarin) is known [2]. The results of the HPLC separation extract from *Rhodiola rosea* are presented in figure 1.

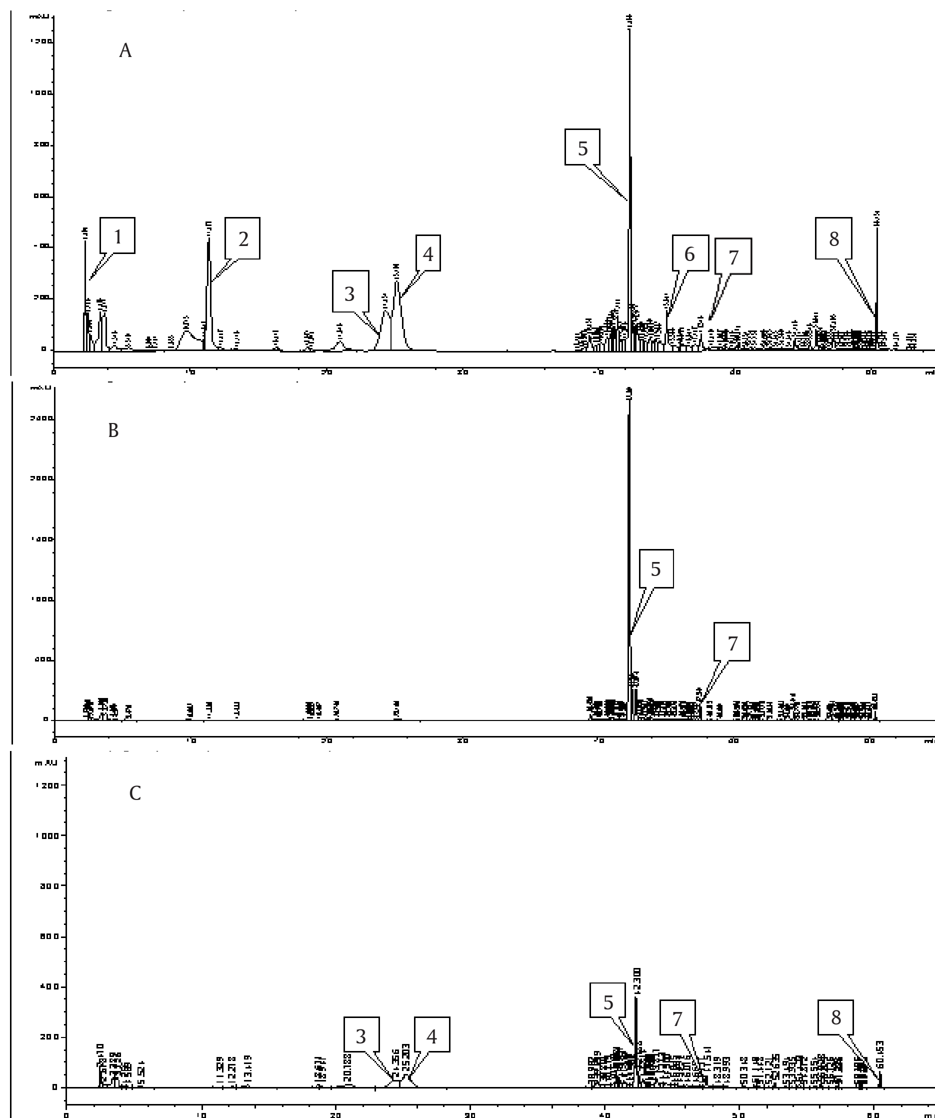


Figure 1. The chromatogram of HPLC analysis of secondary metabolites extracted from rhizomes of *Rhodiola rosea* and detected due adsorption at: A - 222 nm; B – 254 nm; C – 280 nm. Separation was carried out on Zorbax XDB C-18 with guard column Extend C-18. 1 – methylgallate; 2 – tyrosol; 3 – gallic acid; 4 – salidroside; 5 – rosavin; 6 – rosin; 7 – rosarin; 8 – cinnamic acid.

The results presented in fig. 1 show that all identified secondary metabolites of Rhodiola rosea rhizomes have a maximal absorption at 222 nm (see fig. 1a). In contrast with that at 254 nm only compounds 5 and 7 demonstrated a detectable absorption (see fig. 1b). This fact in connection with retention times values coinciding with markers retention times and data from the literature [2] prove that compounds 5 and 7 are phenylpropanoids rosavin (pic. 5) and rosarin (pic. 7, see fig. 1b). All numbered compounds were identified by comparison with authentic markers. The retention time and relative area (peak area related to integral areas of all peaks) of these compounds are presented in table 1.

Table 1.

The retention time, peak area and intensity of adsorption of major compounds of secondary metabolites of *Rhodiola rosea* rhizomes separated by HPLC and detected at 222 nm (see fig. 1a).

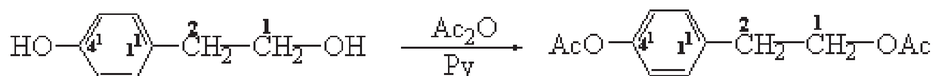
compound	retention time [min.]	peak area [%]	intensity [mAU]
methylgallate	2.29	6.61	450
tyrosol	11.38	10.05	475
gallic acid	24.35	8.34	150
salidroside	25.20	13.45	200
rosavin	42.30	11.01	2500
rosin	45.03	0.90	250
rosarin	47.54	1.40	100
cinnamic alcohol	60.45	2.40	600

Analyzing the chromatogram presented in figure 1 and quantitative characteristics of different components presented in table 1 it could be concluded that the main components extracted from *Rhodiola rosea* rhizomes are salidroside, rosavin and tyrosol. The peak areas and the intensity of signals that characterize the maximum adsorption of these compounds are the most significant ones. The sum of the peak area of gallic acid and product of its transformation, methylgallat, is exceeding the value of peak area for other components. The peak area and the intensity of adsorption of rosin, one of the biologically active compounds of *Rhodiola rosea*, were at rather low levels.

The identity of components was confirmed by the comparison of retention time and intensity of adsorption characteristics of different compounds with those described in scientific literature [1-5, 8]. Some confirmation has been obtained by utilizing markers and also by ^{1}H, ^{13}C NMR and IR analyses. Among compounds separated preparative we could mention tyrosol, the structure of which was confirmed by ^{1}H, ^{13}C NMR and IR analyses.

Tyrosol showed IR absorption bands for benzoic double bonds at γ_{max} 805, 1595 cm^{-1} and hydroxilic groups at γ_{max} 1045, 1215 and 3395 cm^{-1}.

The ^{1}H NMR spectra of tyrosol 1 showed three signals for metilenic groups at 2.61 (t, 2H), 3.51 (d, 1H) and 3.54 (d, 1H), two signals for no substitution protons from benzoic ring at 6.66 (d, 2H), 6.99 (d, 2H), two signal at 4.57 (t, 1H) and at 9.12 (s, 1H) ppm indicate the presence of two hydroxilic groups. The structure of tyrosol was confirmed also by ^{13}C NMR spectra and especially by DEPT (see Materials and methods). Finally, after acetylation with acetic anhydride in pyridine (1) and analysis the spectral data of acetylate confirmed the structure depicted in scheme 1.



Scheme 1

The ^{1}H NMR spectra of diacetate 2 showed the presence of two methyl groups at 2.04 (s, 3H) and 2.29 (s, 3H) which means than both hydroxilic groups in tyrosol were acetylated. The ^{13}C NMR spectra confirms the presence of two acetates by signals of two carbon atoms at 20.97 and 21.13 ppm and those of two carbonyls at 169.57 and 171.01 ppm.

As a result of the steam distillation of 2 g of dry rhizomes of *Rhodiola rosea* we have obtained yellow aromatic oils (0.05% of d.w.). That is in accordance with the oils content of in rhizomes of Norwegian origin [10] and less than that in rhizomes of Russian origin [8]. A chromatogram of HPLC analysis of these oils is presented in figure 2. Among main compounds determining aroma and spirits of *Rhodiola* roots are linalool, geraniol, thymol, caryophyllene, p-cymene, limonene, phenylethyl alcohol, carvacrol and carvona.

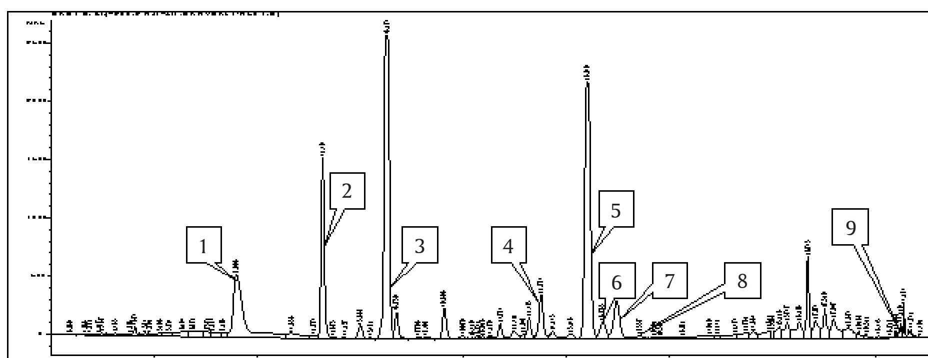


Figure 2. The chromatogram of HPLC analysis of volatile compounds of steam distillate from rhizomes of *Rhodiola rosea*. Separation was carried out on Zorbax XDB C-18 with guard column Extend C-18. 1 – limonene; 2 – phenylethyl alcohol; 3 – p-cymene; 4 – carvona; 5 – geraniol; 6 – linalool; 7 – carvacrol; 8 – thymol; 9 – caryophyllene

The quantitative characteristics of *Rhodiola rosea* aromatic compounds separated by HPLC are presented in table 2. Our results confirm the data on composition of essential oils in *Rhodiola rosea* rhizome published by other authors [10, 11]. The most representative compounds are p-cymene, geraniol and limonene. The peak areas and intensity of signals of these compounds after HPLC separation dominated in comparison to other components (tab. 2). What is interesting, geraniol was identified as the most important rose-like odor compound of *Rhodiola rosea* rhizomes [10].

Table 2.

The retention time, peak area and intensity of adsorption of major components of aromatic components of *Rhodiola rosea* rhizomes separated by HPLC and detected at 195nm (see fig. 2)

compound	retention time [min.]	peak area [%]	intensity [mAU]
limonene	13.18	7.73	1600
phenylethyl alcohol	13.67	0.11	25
p-cymene	16.27	21.24	2500
carvona	23.78	2.64	300
geraniol	26.02	19.97	2200
linalool	26.76	1.38	150
carvacrol	27.43	3.19	300
thymol	28.55	0.38	50
caryophyllene	41.38	0.56	250

By means of HPLC analysis, in *R. rosea* rhizomes of Carpathian origin we have detected the same main compounds (salidroside, rosarin, rosavin, rosin, methylgallate, tyrosol, gallic acid, and cinnamic alcool) that were described for rhizomes of Russian origine [1-5, 8]. It is also important to point that essential oils from these rhizomes also have contained compounds characteristic for *Rhodiola rosea* described in literature [10]. The results of secondary metabolites analysis in callus cells and artificial cultivated plants will be presented in further paper.

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SKŁAD CHEMICZNY KŁĄCZY RÓŻEŃCA GÓRSKIEGO (*RHODIOLA ROSEA* L.) POCHODZĄCEGO Z KARPAT

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S t r e s z c z e n i e

Różeniec górski (*Rhodiola rosea* L.) jest wartościową rośliną zielarską rosnącą dziko w górach w różnych częściach świata, w tym również w Karpatach. W jej kłączu gromadzą się wtórne produkty przemiany materii, których właściwości lecznicze, w tym adaptogenne zostały odkryte i opisane. Szczególne zainteresowanie wzbudzają fenylopropanoidy (alkohol cynamonowy i jego glikozydy: rozawina, rozaryna i rozyna), związki fenolowe (salidrozyd, p-tyrozol), flawonolignany (rodiolina) i terpeny. Znaczenie mogą mieć także inne grupy składników (olejki eteryczne i inne).

Do wykonania analizy jakościowej i ilościowej wtórnych metabolitów roślin *Rhodiola rosea* L. zastosowano czułą metodę RP-HPLC. Dane chromatograficzne uzyskano za pomocą szeregowego czujnika fotodiodowego (PDA). Podczas analizy wyciągów z kłączy *Rhodiola rosea* L. pochodzących z Karpat znaleziono pełne spektrum wtórnych metabolitów, w tym fenylopropanoidy, monoterpeny, flawonoidy, kumaryny i kwasy organiczne. Cechy niektórych składników oznaczano przy użyciu spektroskopii i magnetycznego rezonansu jądrowego (NMR) oraz analizy w podczerwieni (IR). Trzeba także zaznaczyć, że olejki eteryczne uzyskane z tych kłączy również zawierały składniki charakterystyczne dla *Rhodiola rosea* L. Można więc stwierdzić, że metody stosowane do analizy jakościowej i ilościowej składników pochodzących z kłączy *Rhodiola rosea* L. i produktów z nich uzyskiwanych są skuteczne i godne zaufania.

Słowa kluczowe: *Rhodiola rosea L., terpeny, glikozydy, fenylopropanoidy, salidrozyd, rozawiny, olejki eteryczne, RP-HPLC, NMR, IR*