Activity-guided extraction optimization of highly efficient antioxidant plant species: study of *Rhodiola rosea* L. (Golden root)

D. I. Ivanova[1]*, S. S. Boyadzieva[1], G. Angelov[1], P. T. Nedialkov[2], D. Nedeltcheva-Antonova[3], F. V. Tsvetanova[1]

[1] Institute of Chemical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[2] Faculty of Pharmacy, Medical University, 1000 Sofia, Bulgaria
[3] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

The species of genus *Rhodiola* L. (Crassulaceae) are mainly distributed in different cold regions of the Northern Hemisphere of the world. The Golden root (*Rhodiola rosea* L.) extract is known in the traditional medicine as adaptogen to treat fatigue, depression and stress-associated diseases. The Golden root extract was selected for a comprehensive study because it exhibited superior antioxidant properties in a group of other efficient antioxidant plant species studied here. Consequently, we aimed our study at an antioxidant activity – guided optimization of *Rh. rosea* extraction by selection of experimental conditions leading to maximum total polyphenolic content and antioxidant activity of the extract. Using a set of variable parameters (solvent composition, temperature, ratio solvent-to-raw material, process duration), it was suggested that extraction in 25% ethanol, at 50ºC, using solvent-to-solid ratio of 15 (v/w) and process duration of 3-5 min are optimal extraction conditions for obtaining of Golden root extracts with maximum total phenolic content (TPC) and antioxidant activity. Metabolite identification in the extract with the best antioxidant activity was performed by both HPLC and UHPLC/HRMS methods. It was found that *Rh. rosea* extract, obtained at optimal conditions, contained 2.29±0.05% of rosavin and 0.80±0.02% of salidroside. The Golden root extract, obtained in this study at optimal conditions regarding its antioxidant activity, has potential application in the production of high-quality plant extracts for prevention of cancer and oxidative-stress associated diseases, including cardio-, neuro- and hepato-degenerative disorders.

Keywords: Antioxidant activity, Extraction optimization, *Rhodiola rosea* L., Rosavin, Salidroside, Total phenolic content

INTRODUCTION

Rhodiola rosea L. (Crassulaceae) is a perennial plant, known as Golden root, Roseroot, Arctic root. This plant species is found at high altitudes in cold regions of Europe, Asia and Nord America. The Golden root is known from centuries in the traditional medicine [1-4]. The plant is used as adaptogen, to treat depression, fatigue, psychological disorders, for improvement of memory and cognitive functions of the central nervous system [5]. *Rhodiola rosea* has very low toxicity [6, 7] and has demonstrated induction of no mutations in humans [8]. The antioxidant activity of *Rhodiola rosea* points to its potential application in the prevention of cancer, neuro-, cardio-, and hepato-degenerative diseases by protection of the cells from the harmful action of radicals obtained in different metabolic pathways.

The extraction of biologically active compounds from *Rhodiola rosea* is an object of increasing scientific and industrial interest [9]. Classical and ultrasonic extractions have been used for determination of appropriate conditions for isolation of bioactive compounds from different species of the genus *Rhodiola* L.: phenylethanoids (salidroside, *p*-tyrosol), phenylpropanoids (rosavin, rosarin, rosin), monoterpenes (rosiridin) [10], flavonoids [11] etc. Microwave-assisted [12] and supercritical fluid [13, 14] extractions have been also employed for recovery of bioactive compounds content in *Rhodiola* species. The effects of the origin, plant part, harvest season and processing of *Rhodiola rosea* cultivars have been studied in order to obtain high-quality extracts for medicinal use [15, 16]. HPLC with mass-spectrometry and rapid resolution liquid chromatography were applied for analyses of salidroside, rosavins and other bioactive compounds from the Golden root [17, 18]. However, the conclusions about the optimal extraction conditions in terms of solvent composition, solvent-to-solid ratio (v/w) and extraction duration differ considerably in the literature, depending on the method, experimental parameters or target compounds used for extraction optimization.

Considering the scientific and industrial interest in obtaining of high quality plant extracts, we studied the antioxidant properties of *Rh. rosea* rhizomes in a group of other efficient antioxidant

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* To whom all correspondence should be sent:
E-mail: dianadoc@abv.bg

species: *Smilax excelsa* L., *Sideritis scardica* Griseb., *Achillea collina* (Becker ex Rchb.f.) Heimerl, *Achillea thracica* Velen., *Inula helenium* L., *Clinopodium vulgare* L. In this comparative study we determined a superior antioxidant activity of the Golden root, which was the reason to initiate an activity-guided extraction optimization of this species complemented with antioxidant metabolite quantification of the extract obtained at optimal experimental conditions.

EXPERIMENTAL

Materials and methods

Rhodiola rosea rhizomes (Russian origin) were delivered from a pharmaceutical supplier '*Bilki*' Ltd., Sofia; *Sideritis scardica* and *Achillea thracica* were collected from the Botanical garden of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (IBER-BAS); *Inula helenium* was from experimental botanical field 'Beglica' (the Rhodope mountains); *Achillea collina* - from village Bistritsa (Vitosha mountain); *Clinopodium vulgare* – from village Zelenigrad (Province Pernik); *Smilax excelsa* – from village Belopolyane (Province Haskovo). The plant species were authenticated by a botanist Dr. Dessislava Sopotlieva (IBER, BAS).

Chemicals and reagents

Salidroside (98%), rosavin (98%), DPPH, Folin-Ciocalteu's (FC) reagent (2N), gallic acid were supplied from Sigma-Aldrich Co. (Saint Louis, MO, USA). LC-MS grade solvents were purchased from Fischer Scientific (Waltham, USA).

Extraction procedure

Dried ground *Rh. rosea* rhizomes (1.5 g) were mixed with the solvent (at different solvent/solid ratios). The extraction was carried out in a shaker water bath at various experimental conditions (Table 1). The mixture was filtered and antioxidant properties of the extracts were analyzed by Folin-Ciocalteu- and DPPH-assays.

Folin-Ciocalteu method for determination of total polyphenol content in different plant extracts

The total polyphenol content (TPC) of the extracts of different plant species was determined by the Folin-Ciocalteu (FC) method [19-21]. In brief, stock solutions of freeze-dried plant extracts, dissolved in the corresponding solvent at a concentration of 10 mg/ml were prepared by ultrasonication (2×5 min, 55°C). The stock solution of *Rhodiola rosea* extract was 5-fold diluted before starting the spectrometric measurements

because of its strong antioxidant activity. The stock solutions of the other plant extracts were 3-fold diluted. Then, 20 µl of the corresponding diluted extract were mixed with 1.58 ml of distilled water and 100 µl of FC-reagent were added. The control sample contained the same reagents without plant extract. After 3-5 min, 300 µl of sodium carbonate (20% w/v) were added and the samples were kept at room temperature for 2 h. The sample absorbance at 765 nm was registered on a spectrophotometer.

The calibration curve was generated using a gallic acid standard. The TPC was given in gallic acid equivalents (GAE), according to the formula $C = c.V/m$, where C is concentration of the TPC in mg GAE/g dry extract; c – gallic acid concentration [mg/ml], determined from the calibration curve; m – weight [g] of the plant extract; V – volume [ml] of the extract.

DPPH method for determination of antioxidant activity of different plant extract

IC_{50} values of the radical scavenging activity of extracts were determined by DPPH-method [22, 23]. In brief, 1 ml of the corresponding plant extract (10 mg/ml) was mixed with 4 ml of DPPH solution (0.004% w/v) in a test tube. The control sample was prepared with the same reagents excluding the plant extract. The blank sample contained only solvent. The solutions were kept at room temperature for 1 h in the dark and then decrease of the absorption was measured on a spectrophotometer at 517 nm. DPPH inhibition was calculated according to the formula:

$$\% \ inhibition = [(Ac - As)/Ac]x100,$$

where A_c is the absorbance of the DPPH in the control sample without extract and A_s is the absorbance of the DPPH in the sample with plant extract. The half-maximum inhibitory concentrations (IC_{50}) were determined as the concentration of the extract in the test sample that decreased the initial DPPH concentration by 50%.

An *UV-1600PC* spectrophotometer (*VWR int.*) was used for FC- and DPPH-assays.

Statistical analysis was done by single-factor analysis of variance (ANOVA) using Microsoft Excel software and *p*-value≤0.05 was set as the lowest level of statistical significance.

Antioxidant metabolite quantification of the extract with optimal antioxidant activity

UHPLC/HRMS analyses for the antioxidant metabolite identification in the Golden root extract were performed on a Thermo Scientific Dionex Ultimate 3000 RSLC (Germany), consisting of 6-

channel degasser SRD-3600, high pressure binary gradient pump HPG-3400RS, autosampler WPS-3000TRS and column compartment TCC-3000RS. The LC system was coupled to Thermo Scientific Q Exactive Plus mass-spectrometer (Germany) with heated electrospray probe HESI-II. Data acquisition and processing were done using Thermo Scientific Xcalibur 3.0 software. An Akzo Nobel Kromasil Externity XT-1.8-C18 (Bohus, Sweden) narrow-bore column (2.1×100 mm, 1.8 μm) with Phenomenex Security Guard ULTRA UHPLC EVO C18 (Torrance, USA) column was used and maintained at 40°C. The mobile phase consisted of systems A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The following gradient was employed: the mobile phase was held at 5% B for 1 min, gradually turned to 95% B over 27 min, kept at 95% B for 2 min and the system was turned to the initial condition of 5% B in 1 min. The system was conditioned at 5% B for 4.5 min before injection. The flow rate and the injection volume were set to 300 μL/min and 2 μL, respectively. The instrument was set at spray voltage 3.5 kV, ion transfer tube and HESI-II vaporizer temperatures at 320 °C.

HPLC analyses for quantitative determination of antioxidant metabolites in the Golden root extract were performed on a HP1100 system with a manual injector (Rheodyne, model 7725), fitted with a 20 μL sample loop and a diode–array detector (G1365B), controlled by ChemStation software (Rev. 04.03, Agilent Technologies). Analytical column ChromSep SS, Inertsil 5 ODS-2 (250 × 4.6 mm i.d., 5 μm particle size) with a ChromSep guard column (Varian, Palo Alto, CA) was used. The mobile phase was acetonitrile/water (5:95, v/v) containing 0.1% formic acid (pH 3) at a flow rate of 1.0 ml/min. The column compartment was kept at 30ºC. The detector signal was monitored at 205, 254 and 280 nm. The extract was filtered (PTFE, 0.22 μm) prior to the analysis.

Calibration curves: The absolute calibration method (external standard method) was used to establish the calibration curve and to quantify the analytes. The standard compounds (0.005 g) were diluted with acetonitrile in volumetric flasks. Five standard solutions with concentrations in the range of 0.2-1 mg/ml were prepared and analyzed in triplicate; the results were presented graphically (peak area versus concentration). The equations of the linear calibration curves are given below:

for salidroside: $Y = 1436.5 X - 11.8$; regression coefficient 0.992;

for rosavin: $Y = 38016.0 X + 36.1$; regression coefficient 0.999;

where Y is the DAD peak area and X is the compound concentration [mg/ml].

RESULTS AND DISCUSSION

Selection of plant species with superior antioxidant activity

The variety of experimental methods and target compounds (salidroside, rosavins), used in the literature for extraction optimization, led to different conclusions about the optimal extraction conditions of the Golden root. Due to the interest in identification of efficient antioxidants for prevention of oxidative stress-associated disorders, we performed an antioxidant activity-guided optimization of *Rh. rosea* rhizome extraction. We established the best antioxidant properties of *Rhodiola rosea* rhizomes extract (1) in a group of extracts of other efficient antioxidant plant species, such as: *Smilax excelsa*, aerial parts (2); *Sideritis scardica*, aerial parts (3); *Achillea thracica*, flower heads (4); *Achillea collina*, flower heads (5); *Inula helenium*, leaves (6); *Clinopodium vulgare*, aerial parts (7). The antioxidant activity of the extracts was analyzed by their DPPH-radical scavenging activity. The IC_{50} values of DPPH-inhibitory activity of the extracts were calculated as the concentration of the extract, required to decrease the initial amount of DPPH by 50%. A lower IC_{50} revealed higher antioxidant activity. The comparison of TPC (Fig. 1) with the DPPH-radical scavenging activity (Fig. 2) of the total extracts revealed superior TPC value (374±17 GAE mg/g dry extract) of the Golden root extract, which corresponded to its best DPPH-radical scavenging activity (IC_{50} 29±1 μg/ml). Hence, *Rhodiola rosea* rhizome extract was selected for extraction optimization experiments because it demonstrated the best antioxidant properties in the group of the studied efficient antioxidant plant species.
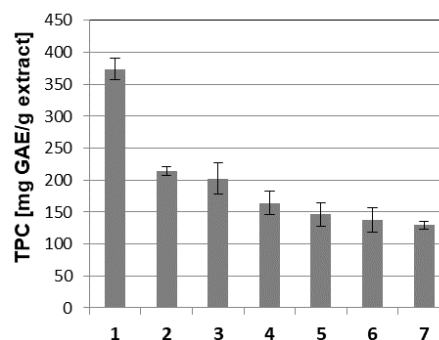

Fig. 1. Comparison of total polyphenolic content of different plant extracts. *Rhodiola rosea* rhizomes (1), *Smilax excelsa*, aerial parts (2), *Sideritis scardica*, aerial parts (3), *Achillea thracica*, flower heads (4), *Achillea collina*, flower heads (5), *Inula helenium*, leaves (6), *Clinopodium vulgare*, aerial parts (7). The extraction

was performed in 80% methanol, at solvent-to-solid ratio 10 (v/w) and 65°C for 1 h (3-fold).
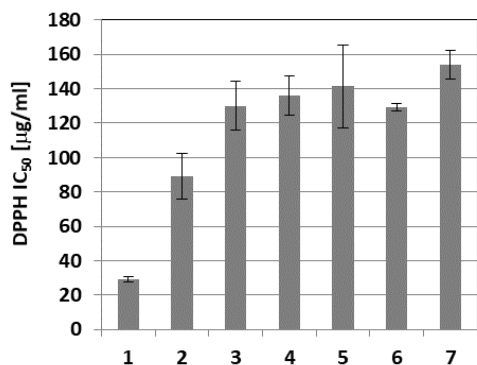

Fig. 2. Comparison of half-maximum DPPH-radical scavenging concentrations (IC_{50}) of plant extracts. The extracts numbers are the same as in Fig. 1. Lower IC_{50} values reveal higher antioxidant activity.

Antioxidant activity-guided optimization of Rhodiola rosea rhizome extraction

Effect of solvent, temperature, hydromodule and extraction duration on the antioxidant activity of Rhodiola rosea rhizome extract. Different conditions for *Rhodiola rosea* rhizome extraction were applied in order to determine their effect on the TPC, resp. on the DPPH-radical scavenging activity of the extracts. We started with determination of the influence of various concentrations of aqueous ethanol (0%, 25%, 50%, 80% and 96%), chosen as an ecological solvent, on the antioxidant activity of the Golden root extract. The TPC, DPPH-radical scavenging activity and the yields of the extracts increased using concentrations from 0% to 25% EtOH (Fig. 3), however further increase of the ethanol concentration did not improve the values of the analyzed experimental parameters. ANOVA test confirmed that the experimental parameter values in the range of 25-96% ethanol were statistically equal. Hence, 25% ethanol was selected as optimal solvent for *Rh. rosea* extraction.

Variation of the extraction temperature from 30°C to 70°C revealed that the half-maximum DPPH-inhibitory concentrations and the extraction yields were similar at all tested temperatures, while some higher TPC values were registered at 50°C

(Table 1). Therefore, operational temperature of 50°C for *Rh. rosea* rhizome extraction in 25% ethanol was preferred for further process optimization.

The effect of hydromodule (HM, solvent-to-solid ratio v/w) on the antioxidant activity of the *Rh. rosea* extract was also examined (Table 1). The extraction yields significantly decreased at solvent-to-solid ratios less than 10 (v/w) due to insufficient quantity of the solvent needed for complete recovery of the extracted matter. The extractions at HM15 and HM20 showed the best and statistically similar values (confirmed by ANOVA) of the analyzed parameters (TPC, DPPH-IC_{50}, extraction yields); However, HM15 was chosen as economically more advantageous parameter over HM20 because of the lower solvent consumption at HM15. Consequently, 25% ethanol, temperature 50°C and solvent-to-solid ratio 15 (v/w) were selected as beneficial conditions for *Rh rosea* rhizome extraction.

Variation of the process duration (3, 5, 15, 30, 60 min) at the above selected conditions revealed that the antioxidant metabolite extraction of the Golden root proceeds very rapidly and only 3-5 min were sufficient to obtain *Rh. rosea* rhizome extract with optimal TPC, DPPH-radical scavenging activity and yields (Table 1).

In conclusion, analysis of the impact of experimental variables (solvent, temperature, solvent-to-solid ratio, process duration) on the antioxidant activity parameters, such as half-maximum DPPH-inhibitory concentrations in correspondence with total phenol content and process yields, revealed that 25% ethanol at temperature 50°C, solvent-to-solid ratio 15 (v/w) and process duration of 3-5 min were assumed as optimal conditions for Golden root extraction.

Antioxidant metabolite quantification in the extract, obtained at optimal conditions

Using UHPLC/HRMS we identified and quantified both rosavin and salidroside as efficient antioxidant metabolites in the Golden root extract, obtained at optimal extraction conditions in view of exhibiting optimal antioxidant activity.
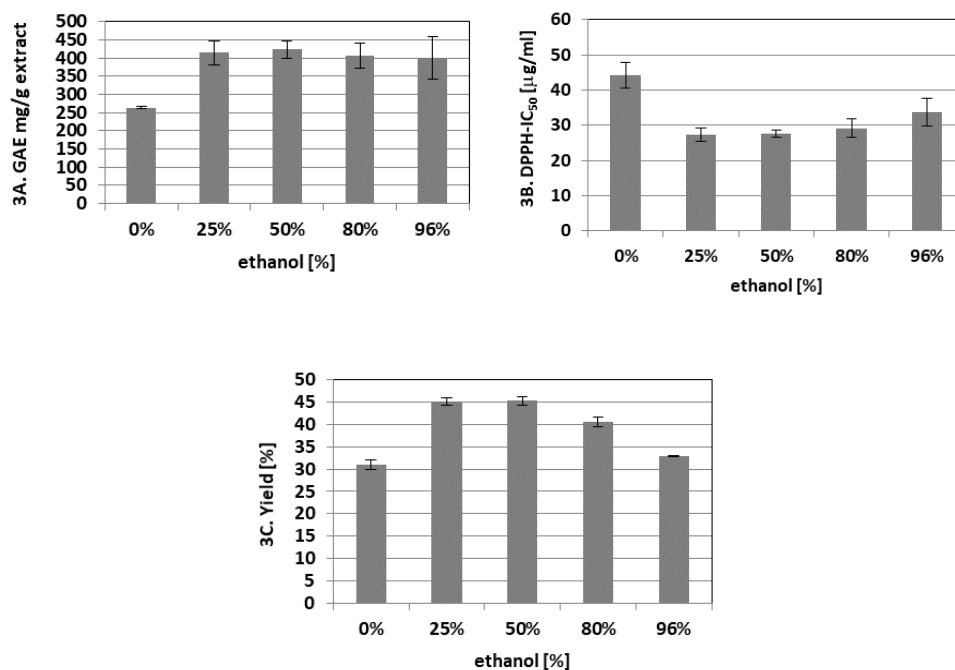
Fig. 3. Results for TPC (3A), DPPH-IC$_{50}$ (3B) and yields (3C) of the Golden root extract depending on the solvent composition. **3A.** Total polyphenol content (TPC) is given in GAE mg/g dry extract, obtained at initial conditions – duration 2 h, temperature 70ºC, using HM 20 (v/w) and different solvent composition; 3**B.** IC$_{50}$ [µg/ml] of DPPH-radical scavenging activity of the extracts, obtained at the same conditions, as in Fig. 3A. A lower IC$_{50}$ revealed higher antioxidant activity; 3**C.** Total extracted matter [% of raw material], obtained at the same conditions, as in Fig. 3A.

Table 1. Effects of variation of the experimental conditions on total polyphenol content (TPC), DPPH-antioxidant activity and experimental yields of the Golden root extraction after selection of 25% ethanol as optimal solvent.

Exp. №	*Variable experimental parameter*	*Constant experimental parameters*	TPC [GAE mg/g]	DPPH-IC$_{50}$ [µg/ml]	Extraction yield [%]
1a	70ºC	HM20, 1 h	384.7 ± 30.0	27 ± 5	43 ± 4
1b	50ºC	HM20, 1 h	420.6 ± 33.5	27 ± 3	42 ± 3
1c	30ºC	HM20, 1 h	396.0 ± 30.0	28 ± 1	40 ± 2
2a	HM 20	50ºC, 1 h	420.3 ± 23.5	27 ± 0	42 ± 3
2b	HM 15	50ºC, 1 h	406.7 ± 17.7	26 ± 3	37 ± 1
2c	HM 10	50ºC, 1 h	388.3 ± 13.5	29 ± 3	30 ± 1
2d	HM 7	50ºC, 1 h	362.0 ± 11.6	31 ± 1	21 ± 1
3a	3 min	50ºC, HM 15	370.4 ± 11.4	29 ± 1	38 ± 2
3b	5 min	50ºC, HM 15	380.0 ± 2.1	34 ± 1	37 ± 2
3c	15 min	50ºC, HM 15	389.6 ± 15.5	33 ± 0.4	37 ± 2
3d	30 min	50ºC, HM 15	377.7 ± 3.4	33 ± 1	38 ± 2
3e	60 min	50ºC, HM 15	387.3 ± 15.0	34 ± 2	35 ± 5

The values are given as an average of two independent experiments ± SD; TPC - total phenolic compounds, given in milligrams GAE (gallic acid equivalents) per gram dry extract; IC$_{50}$ - concentration of the plant extract (in µg/ml), which decreases the initial DPPH concentration by 50%; HM – hydromodule [solvent-to-solid ratio (v/w)]. All experiments, described in the table, were performed in 25% ethanol, selected as optimal solvent for *Rh. rosea* extraction; *Experiment 1 (a-c)* set the parameters for optimization of the extraction temperature at a constant solvent-to-solid ratio; *Experiment 2 (a-d)* set the parameters for optimization of the solvent-to-solid ratio at a constant temperature, selected in the previous experiment 1; *Experiment 3 (a-e)* set the parameters for optimization of the extraction duration at constant temperature and solvent-to-solid ratio, selected in the previous experiments 1 and 2.

Exact mass of the protonated molecular ion of rosavin was found at 429.1751 *m/z* (mass-to-charge ratio, calculated for C$_{20}$H$_{29}$O$_{10}$ 429.1755 *m/z*). Exact mass of the protonated molecular ion of salidroside

was found at 301.1286 *m/z* (calculated for $C_{14}H_{21}O_7$ 301.1282 *m/z*). Quantitative HPLC-analysis determined that the chemical composition of the Golden root extract obtained at optimal experimental conditions (given above) corresponded to a content of 2.29±0.05% of rosavin and 0.80±0.02% of salidroside.

CONCLUSION

In this study we performed an activity-guided extraction optimization and antioxidant metabolite quantification of *Rhodiola rosea* rhizome extract, obtained at optimized extraction conditions. We found that extraction of the Golden root in 25% ethanol, at 50°C, solvent-to-solid ratio 15 (v/w) and extraction duration for 3-5 min creates the most beneficial conditions for obtaining of extracts with optimal TPC and DPPH-radical scavenging activity. The chemical composition of the Golden root extract, obtained at optimal extraction conditions for its antioxidant properties, corresponded to a content of 2.29±0.05% of rosavin and 0.80±0.02% of salidroside. Being a powerful antioxidant with no major toxicity, the Golden root extract with optimized antioxidant properties is a feasible pharmaceutical agent in the prevention of cancer and oxidative stress-associated neuro-, cardio-, hepato-degenerative disorders.

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ОПТИМИЗИРАНЕ НА ЕКСТРАКЦИЯТА НА РАСТЕНИЯ С ВИСОКА АНТИОКСИДАНТНА АКТИВНОСТ: ИЗСЛЕДВАНЕ НА Rhodiola rosea L. (ЗЛАТЕН КОРЕН)

Д.И. Иванова[1]*, С.С. Бояджиева[1], Г. Ангелов[1], П.Т. Недялков[2], Д. Неделчева-Антонова[3], Ф.В. Цветанова[1]

[1] *Институт по инженерна химия, Българска академия на науките, 1113 София, България*
[2] *Фармацевтичен факултет, Медицински университет, 1000 София, България*
[3] *Институт по органична химия с Център по фитохимия, Българска академия на науките, 1113 София, България*

(Резюме)

Растенията от род *Rhodiola* L. (Crassulaceae) са разпространени главно в различни студени райони на северното полукълбо. Екстрактът от златен корен (*Rhodiola rosea* L.) се използва в традиционната медицина като адаптоген за лечение на умора, депресия и болести, предизвикани от стрес. Екстрактът от Златен корен беше избран за задълбочено изследване поради превъзходните му антиоксидантни свойства сред групата от други ефективни антиоксидантни растителни видове, изследвани в настоящата работа. Екстракцията на *Rh. rosea* беше оптимизирана въз основа на активността на екстракта чрез подбор на експериментални условия, водещи до максимално тотално фенолно съдържание и максимална антиоксидантна активност. Чрез оптимизиране на променливи експериментални параметри бяха намерени следните оптимални условия на екстракция за получаване на екстракт от Златен корен с максимално съдържание на полифеноли и максимална антиоксидантна активност: разтворител 25% етанол, температура 50ºС, съотношение разтворител/суровина 15 (v/w) и продължителност на процеса 3-5 минути. Идентифицирането на метаболити в екстракта с най-добра антиоксидантна активност беше проведено чрез HPLC и UHPLC/HRMS методи. Установено беше, че екстрактът от *Rh. rosea*, получен при оптималните условия, съдържа 2.29±0.05% розавин и 0.80±0.02% салидрозид. Екстрактът от златен корен, получен в настоящето изследване при оптимални условия относно антиоксидантната му активност, има потенциално приложение при производство на висококачествени растителни екстракти за превенция на онкологични заболявания и болести, свързани с оксидативен стрес, вкл. кардио-, невро- и хепато-дегенеративни заболявания.