Antioxidant Activities of Bulgarian Golden Root – Endangered Medicinal Species

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Abstract

***Rhodiola rosea* (Golden Root, Roseroot or Arctic Root) is a yellow-flowered herbaceous cool climate species distributed in the sub-arctic areas of the northern hemisphere (North America, Greenland, Iceland, Scandinavia), in high mountains of Asia (the Altai, the Tien-Shan, and the Himalayas) and in Europe (Central Europe and the Balkan peninsula) above 1800 m. In Bulgaria, this species is endangered and is included in the Red Book. It is protected by law in several other countries (e.g. UK, Finland, Russia, Mongolia). *Rhodiola* is a multipurpose medicinal herb with adaptogenic properties. Its radix and rhizome extracts increase the body's nonspecific resistance and normalize body functions, having antidepressant, anticancer, cardio protective and central nervous system stimulating effects. The present study aims at establishing the optimal in vitro conditions for in vitro cultivation to stimulate bioactive substances production. Calli were cultivated on various phytoregulators and concentrations media. Total phenolic/flavonoid content and radical scavenging activity was determined in these calli. Antioxidant properties were influenced by the composition of the cultivation media. A good correlation was obtained between the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radical with both the total phenolic content and the total flavonoid content. No strict relation between the callus colour/structure/texture and the secondary metabolite amounts was found. Despite the low accumulation of phenolic and flavonoid compounds in the calli of Bulgarian *R. rosea*, the presence of these metabolites indicates the possibility for the production of biologically active substances by non differentiated cells. This could be a basis for in vitro metabolic engineering and biotransformation for alternative production of valuable substances in Roseroot.**

INTRODUCTION

Rhodiola rosea L. (Roseroot, Golden root or Arctic root) is an endangered medicinal species that is included in the Red Book of Bulgaria and is protected by law in other countries. *Rhodiola* radix and rhizome is a multipurpose medicinal herb with adaptogenic properties: it increases the body's nonspecific resistance and normalizes body functions. It has antidepressant, anticancer, cardio protective and central nervous system stimulating effects (Kelly, 2001; Brown et al., 2002).

Rhizomes and roots of *Rhodiola rosea* contain 6 different groups of chemical components with pharmaceutical value (Satsiperova et al., 1993; Ganzera et al., 2000; Linh et al., 2002). Beneficial health effects are mainly due to the antioxidant content in Golden Root tissues. Phenolic compounds like flavonoids have a very important antioxidant activity by inactivating free radicals and preventing decomposition of hydroperoxides into free radicals. Phenolic compounds have received considerable attention as potentially protective factors against cancer and heart diseases because of their antioxidant potential and their ubiquity in a wide range of commonly consumed foods of plant origin (Burda and Oleszek, 2001; Edreva et al., 2008). The antioxidant activity of *Rhodiola imbricata* rhizome extracts was proved by examining total phenolic

content, total flavonoid content, DPPH scavenging activity and other parameters (Gupta et al., 2009). Intensive harvesting has brought to exhaustion of Golden Root in natural habitats with limited possibilities for restoration of this resource, since plant development of this specie needs four to six years. In these circumstances, production of pharmacologically important compounds by in vitro cultures could be a good alternative. György (2006) obtained and maintained callus growth of *R. rosea* in liquid culture media supplemented with various precursors of biological active substances (BAS) examining glycoside production. Different reports indicated the multifactor dependence of successful establishment of callus cultures and manipulation of their biosynthetic properties (Furmanowa et al., 1995, György et al., 2004, Krajewska-Patan et al., 2007).

The present study is the first one about evaluation of antioxidant and free radical scavenging properties of calli from the Bulgarian ecotype of Rose Root. The aim of the investigations is to determine the total phenolics and flavonoids content in calli and evaluate the callus antioxidant activity for possible practical use.

MATERIALS AND METHODS

Leaves excised from in vitro micropropagated *Rhodiola rosea* plants were used for callus induction as in Tasheva and Kosturkova (2010). Different variants of nutritious media containing Murashige and Skoog (1962) basic medium (MS) supplement with phytoregulators in various combinations and concentrations were used for efficient induction and maintaining of callus (Table 1).

Plant Material and Extract Preparation

Extracts were obtained from calli cultured for 60 days (3rd passage) on their relevant callus induction media. Extracts from roots of *R. rosea* in vitro regenerants were used as controls (K1 – first generation regenerants growing in green house and K2 – second generation regenerants growing in mountains). Air-dried and ground plant material (1 g of roots, 0.5 g of callus from samples 4 and 7, 0.25 g of callus from samples 5, 6 and 18) was treated with methanol (3x3 ml, 3x3 ml and 3x1 ml, resp.) in an ultrasonic bath for 10 min each. The extracts were centrifuged, combined and diluted with methanol to final volume of 10 ml for roots and samples 4 and 7 and to 5 ml for samples 5, 6 and 18.

Total Phenolic Content

The total phenolic content was measured using the Folin–Ciocalteu method (Yoo et al., 2008). One ml of appropriately diluted extract was mixed with 9 ml of distilled water, 1 ml of Folin-Ciocalteu phenol reagent in 25 ml volumetric flask. After 5 min, 10 ml of 7% (w/v) Na_2CO_3 were added and diluted with distilled water to a final volume of 25 ml. After incubation for 90 min at room temperature, the absorbance was read at 750 nm by a spectrophotometer. The concentration was calculated using gallic acid as standard and the results were expressed as milligram (mg) gallic acid equivalents (GAE) per 1 g of plant material ($y = 3.908x - 0.02280$, $R^2 = 0.9990$).

Total Flavonoid Content

The flavonoid content was measured using a colorimetric assay developed previously (Zhishen et al., 1999). The absorbance was measured at 510 nm against the blank (methanol) and flavonoid content was expressed as mg quercetin equivalents (QE) per 1g of plant material ($y = 1.404x + 0.0075$, $R^2 = 0.9982$).

Free Radical Scavenging Activity on DPPH Radical

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was determined as described by Molyneux (2004) following procedures of Gupta et al. (2009).

The IC_{50} value was defined as the extract concentration (mg/ml) inhibiting formation of DPPH radicals by 50% and was calculated from the plotted graph of radical scavenging activity against the concentration of the extracts.

Statistical Analysis

Experimental results were mean of three parallel measurements. Correlation coefficient (R) for determination of the relationship between the radical scavenging activity and the total phenolic content was calculated using MS Excel software.

RESULTS AND DISCUSSION

The long-term callus cultures of Bulgarian Golden root were established previously (Tasheva and Kosturkova, 2012).

Total Phenolic Content

Total phenolic contents are presented in Table 1. Root extracts from regenerants grown, both in greenhouse and in the mountains showed considerably higher amounts (49.15 and 56.11 mg/g, respectively) compared to callus cultures. Among the calli samples, the highest amount of total phenolic compounds (5.18 and 4.98 mg/g) was detected in samples 18 and 6, respectively. Samples 4 and 7 had similar phenolic content (4.51, and 4.62 mg/g, respectively), while the smallest amount of total phenols was found in sample 5 (3.39 mg/g). These differences could be due to differences in callus structure and callus growth media. The highest phenolic content was found in callus developing on media 18 containing only auxins (2,4-D and IAA) at low concentrations (0.1 and 0.2 mg/L, respectively).

Total Flavonoid Content

Total flavonoid contents are presented in Table 1. Root extracts from regenerants grown in greenhouse and in the mountains were 9.50 mg/g and 12.48 mg/g, respectively. Among the calli samples, the highest amount of flavonoids (1.40 mg/g) was detected in sample 18, while the smallest amount of total flavonoids was found in sample 5 (0.21 mg/g).

Free Radical Scavenging Activity on DPPH Radical

The DPPH (1,1-diphenyl-2-picrylhydrazyl) molecule that contains stable free radical has been widely used to evaluate the radical scavenging ability of antioxidants. IC_{50} values varied between 1.91 to 2.94 mg/ml (Table 1), with the highest IC_{50} value being obtained for sample 5 and the lowest for sample 18, showing thus the highest antioxidant power. A good correlation was obtained between scavenging activity of DPPH radical with both total phenolic content and total flavonoid contents (Figs. 1 and 2). These results indicated that the radical scavenging capacity of each extract might be mostly related to their concentration of phenolic compounds and flavonoids. The IC_{50} of studied extracts was also compared with that of the standard antioxidants caffeic acid (0.002 mg/ml) and quercetin (0.005 mg/ml), as well as, with that of root extracts (0.030 mg/ml and 0.015 mg/ml, respectively). It was found that their radical scavenging potential was less than that of the standard antioxidants and of the corresponding root extract.

Searching for in vitro cultivation conditions stimulating bioactive substances production, callus tissues cultivated on media varying in phytoregulators' nature and concentrations were chosen. Plant hormones and other cell development regulators could interfere with metabolic pathways for induction or increasing synthesis of certain substances. The highest phenolic and flavonoid content and antioxidant activity were found in callus developing on media 18 which contained low concentrations (0.1 and 0.2 mg/L, respectively) of auxins (2,4-D and IAA), and the lowest was observed in callus from medium variant 5 having the highest auxin content (1 mg/L 2,4-D). This shows a negative correlation between the level of auxins in the media and the level of phenolic and flavonoid compounds and their scavenging properties, as well as a positive correlation between the content of cytokinines in the media with the phenolic and flavonoid contents and the antioxidant activity (var. 5 vs. 4; var. 5 vs. 6). Enrichment of the nutrition medium with casein hydrolysate had no significant effect on the levels of phenolic and flavonoid compounds in the calli but slightly influenced their antioxidant

activity (var. 7 vs. 4). No correlation between callus structure/texture and secondary metabolites amounts was observed. However, extraction of biologically active substances from callus tissue (especially loosy one) was easier compared to roots. Despite the low accumulation of phenolic compounds and flavonoids in *R. rosea* calli, their presence indicates the possibility of producing of biologically active substances by undifferentiated cells. This could be a basis for in vitro metabolic engineering and biotransformation (György, 2006) as alternative production of valuable substances.

CONCLUSIONS

For the first time in Bulgaria, phenolic content and DPPH scavenging activity were determined in callus cultures of *Rhodiola rosea*. Linear correlation was noted between total phenolic content and scavenging activity, as well as between total flavonoids and scavenging activity. Antioxidant properties depended to a certain extent on the cultivation media composition.

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Tables

Table 1. Total phenolic compounds content, total flavonoid content and free radical scavenging activity on DPPH radical of *Rhodiola rosea* extracts.

Sample	MS media with phytoregulators (mg/L)	Weight of the extract[A]	Total phenolic content[B,*]	Total flavonoid content[C,*]	DPPH IC_{50}[D,*]
CA			-		0.002
Q			-		0.005
K1 root		0.200	49.15 ± 0.14	9.50	0.030
K2 root		0.150	56.11 ± 0.16	12.48	0.015
Callus No 4	BAP – 1.0 2,4-D – 0.5	0.357	4.51 ± 0.01	0.61	2.37 ± 0.07
Callus No 5	BAP – 0.5 2,4-D – 1.0	0.274	3.39 ± 0.18	0.21	2.94 ± 0.04
Callus No 6	BAP – 1.0 2,4-D – 1.0 CAS - 1000	0.350	4.98 ± 0.18	1.20	1.93 ± 0.03
Callus No 7	BAP – 1.0 2,4-D – 0.5 CAS - 1000	0.374	4.62 ± 0.18	1.09	1.97 ± 0.03
Callus No 18	2,4-D – 0.1 IAA – 0.2 CAS – 1000	0.392	5.18 ± 0.20	1.40	1.91± 0.03

*Values are expressed as mean ± SD of three determinations.
[A]Weight of extracts was expressed as a gram per 1 g of plant material.
[B]Total phenolic content was expressed as milligrams gallic acid equivalent in 1 g of plant material.
[C]Total flavonoid content was expressed as mg quercetin equivalent in 1 g of plant material.
[D]IC_{50} value was defined as extract concentration (mg/ml) inhibiting formation of DPPH radicals by 50%.
MS: Murashige and Skoog medium, 1962; BAP: N^6-benzylaminopurine; 2,4-D: 2,4- dichlorophenoxyacetic acid; IAA: indolyl-3-acetic acid; CAS: Casein Hydrolysate; CA: caffeic acid; Q: quercetin; K1: root from *Rh. rosea* regenerants grown in greenhouse for one year; K2: root from *Rh. rosea* regenerants grown in the mountain for 2 years.

Figures

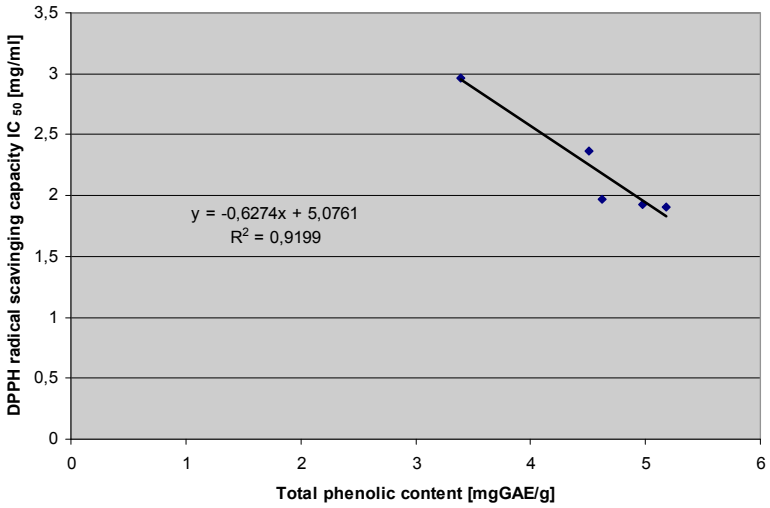


Fig. 1. Correlation between the free DPPH radical scavenging activity (IC_{50}) and the total phenolic contents of *Rhodiola rosea* calli extracts.

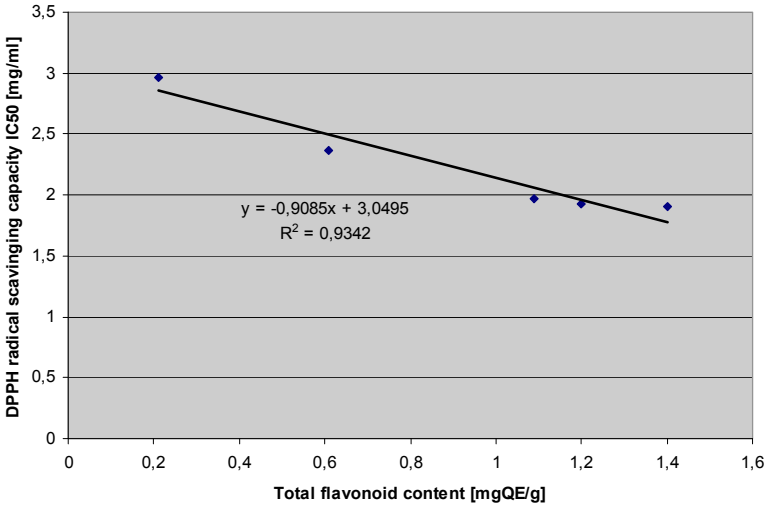


Fig. 2. Correlation between the free DPPH radical scavenging activity (IC_{50}) and the total flavonoid contents of *Rhodiola rosea* calli extracts.