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High-mountain Bulgarian plants – free radical scavenging activity and flavonoid composition

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ABSTRACT

Plants in alpine habitats are exposed to variety of unfavourable conditions. It has been suggested that the combined effect of lower temperature and higher light intensity induces accumulation in the plant cells of more antioxidant components such as phenolic compounds. In the present study six Bulgarian plants growing in alpine habitats - *Anthemis montana* L., *Centaurea nervosa* Rchb. ex Steud. (Asteraceae), *Bartsia alpina* L. (Orobanchaceae), *Knautia arvensis* (L.) Coult. (Dipsacaceae) *Gentianella bulgarica* (Velen.) Holub (Gentianaceae) and *Geum bulgaricum* Pancic (Rosaceae) were examined for flavonoid composition and antiradical properties. The extracts of *G. bulgaricum*, *A. montana* and *K. arvensis* showed the strongest activities for 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radicals and the concentration of their extracts needed for 50% inhibition of radicals (IC_{50}) were found to be as 28.25, 56.08, 58.43 μg/ml, respectively. The methanolic extract of *Geum bulgaricum* has a significantly higher total phenolic content in comparison with other extracts. In the TLC screening nine flavonoid aglycones and six flavonoid glycosides were detected. To the best of our knowledge there are no previous reports regarding the antioxidant potential of the studied species.

Key words: *Anthemis montana*, *Bartsia alpina*, *Centaurea nervosa*, *Gentianella bulgarica*, *Geum bulgaricum*, *Knautia arvensis*

Introduction

Plants in alpine habitats are exposed to variety of unfavourable conditions such as high levels of UV radiation, low atmospheric pressure and great extremes of temperature and humidity. Different studies suggested that the combined effect of lower temperature and higher light intensity induces accumulation in the plant cells of more antioxidant components and phenolic compounds (Wildi & Lütz, 1996; Albert et al., 2009; Zidorn, 2010). That is why in extension of our search for new plants and compounds with antioxidant properties we focused our attention to the high-mountain plants. The highest mountain in Bulgaria and the Balkan Peninsula is Rila, famous for its rich plant diversity. Plant species collected from the alpine region of Rila Mountain are objects of research in the present study.

The antioxidant properties of plant extracts have been extensively evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. This is a quick, reliable and reproducible assay (Marinova & Batchvarov, 2011). DPPH is a purple colored radical that, after being reduced by an antioxidant turns into a yellow product. The degree of discoloration of DPPH is indicative of the antioxidant activity of the crude extracts. It has been established that the antioxidant potential of plant extracts is mainly due to phenolic compounds - flavonoids, tanins, phenolic acids, etc. (Rice-Evans et al., 1997; Katalinic et al., 2006; Gan et al., 2010). Simple, quick reliable and inexpensive procedure that can be used for screening of plant extracts for pharmacologically active substances is the thin layer chromatography (TLC). This analysis is very useful for preliminary study before other instrumental techniques. (Mohammad et al., 2010; Braz et al., 2012).

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The aim of present study was to determine free radical scavenging capacity, total phenolic content and flavonoid components of six high-mountain Bulgarian plants: *Anthemis montana* L., *Centaurea nervosa* Rchb. ex Steud. (Asteraceae), *Bartsia alpina* L. (Orobanchaceae), *Knautia arvensis* (L.) Coult. (Dipsacaceae) *Gentianella bulgarica* (Velen.) Holub (Gentianaceae) and *Geum bulgaricum* Pancic (Rosaceae).

Materials and Methods

Plant material. Plant materials were collected from the Rila Mountain in the alpine region over hut „Ribni ezera" (2230 m asl.) excluding *Knautia arvensis* which was collected in the vicinity of "Suhoto ezero" (2045 m asl).

Extraction procedure. Dry, ground plant material (1 g) was extracted with 80% (3 x 30 mL) methanol by classical maceration for 24 h. After evaporation of the solvent the crude extract was subject to subsequent analysis.

Thin layer chromatographic analysis. The methanol extracts were examined for apolar (aglycones) and polar (glycosides) flavonoids by TLC analysis. The used TLC conditions are presented at Table 1.

Chromatograms were viewed under UV light before and after spraying with ''Natural product reagent A", 1% solution of diphenylboric acid 2-aminoethyl ester complex in methanol. The identification of the compounds was achieved by co-chromatography with authentic markers obtained from Prof. Eckhard Wollenweber.

Determination of total phenolic content Total phenolic content of the methanol extracts was determined by employing the method given in the literature involving Folin–Ciocalteu reagent and gallic acid as standard (Giorgi et al., 2009; Nićiforović et al., 2010). The content of total phenols was presented as mean ± standard divisions (SD) of tree independent analyzes (n=3).

DPPH radical scavenging activity The effect of methanolic extracts on DPPH radicals was estimated according to Stanojević et al., (2009). The IC_{50} values were calculated by Software Prizm 3.00. All of the experiments were carried out in triplicate.

Results and Dscussion

The antioxidant potential of the methanolic extracts of the studied species were assayed by scavenging of DPPH radicals and presented as IC_{50} values (μg/mL) - extract concentration providing 50% inhibition of the DPPH solution (Table 2).

Table1. *Used sorbents and mobile phases in TLC analysis*

Mobile phase	Sorbent
Flavonoid aglycones	
toluene-dioxan-acetic acid (95:25:4, v/v/v)	silica gel
toluene-methylethylketone-methanol (60:25:15, v/v/v)	polyamid
toluene-dioxan-methanol (80:10:10, v/v/v)	polyamid
acetic acid–water (30:70, v/v)	cellulose
Flavonoid glycosides	
ethyl acetate:formic acid:acetic acid: methylethylketone:water (50:7:3:30:10)	silica gel
ethyl acetate:formic acid:acetic acid: water (100:11:11:27)	silica gel
acetic acid–water (15:85, v/v)	cellulose

Table 2. *Free radical scavenging activity and total phenolic content of examined species*

High mountain plants	DPPH scavenging activity IC_{50} (μg/mL)	Total Phenols* (mg/g extract) in GAE
Anthemis montana	56,08	107,01±6,7901
Bartsia alpina	116,6	104,94±9,9108
Knautia arvensis	58,43	110,48±9,7512
Gentianella bulgarica	422	110,45±7,2621
Geum bulgaricum	28,25	233,98±9,29042
Centaurea nervosa	158	112,34±6,2437

* values represent mean ±SD; GAE- gallic acid equivalents

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The extracts of *Geum bulgaricum*, *Anthemis montana* and *Knautia arvensis* showed the strongest activities for DPPH radicals and their IC_{50} values were determined respectively as 28.25, 56.08, 58.43 μg/ml. The lowest activity was established for the extract from *Gentianella bulgarica*. Moderate activity was found for the extracts of *Bartsia alpina* and *Centaurea nervosa*.

The results of the total phenolic content determination in the methanol extracts of the studied samples, evaluated using Folin - Ciocalteu method, are presented at Table 2. The methanolic extract of *Geum bulgaricum* has a significantly higher total phenolic content in comparison with other examined species extracts – 233.98 mg GA/g extract. The amounts of phenolic content of the rest species ranged between 104.94 to 112.34 mg GA/g extract.

The metanolic extracts of the studied species were cheeked for occurrence of apolar (aglycones) and polar (glycosides) flavonoids by TLC. Nine flavonoid aglycones and six flavonoid glycosides were determined. The flavonoid compounds were *identified* by co-*TLC* with authentic standards using three different sorbents (silica gel, polyamid, cellulose) and several combinations of mobile phases (Table 3).

The species of Asteraceae *Anthemis montana* and *Centaurea nervosa* showed the greatest diversity of free flavonoid aglycones. Except simple flavone aglycones - apigenin (**1**) and luteolin (**3**) their 6-methyl derivatives: scutellarein 6-methyl ether (**2**) and 6-hydroxuluteolin 6-methyl ether (**5**) were established in the extracts of both species. Highly methylated structures of luteolin and quercetin such as 6-hydroxuluteolin 6,3'-dimethyl ether (**6**), quercetagetin 3,6-dimethyl ether (**7**), quercetagetin 3,6,7-methyl ether (**8**) and quercetgetin-3,6,3'-trimethyl ether (**9**) were also identified.

Table 3. *Phenolic compounds in the examined species*

Phenolic compounds	***Anthemis montana (folia)***	***Anthemis montana (flower heads)***	***Centaurea nervosa***	***Geum bulgaricum***	***Gentianella bulgarica***	***Knautiaarvensis***	***Bartsia alpina***
Flavonoid aglycones							
apigenin (**1**)	trace	trace	trace				
scutellarein 6-methyl ether (**2**)	×						
luteolin (**3**)	×	×	×	trace		×	×
luteolin 3-methyl ether (**4**)		trace	×				
6-hydroxuluteolin 6-methyl ether (**5**)	×		×				
6-hydroxuluteolin 6,3'-dimethyl ether (**6**)	×		trace				
quercetagetin 3,6-dimethyl ether (**7**)	trace		trace				
quercetagetin 3,6,7-methyl ether (**8**)	×						
quercetgetin 3,6,3'-trimethyl (**9**)	×						
Flavonoid glycosides							
luteolin 7-O-glycoside (**10**)	×	trace	×			×	×
apigenin 7-O-glycoside (**11**)	trace		trace				
luteolin 8-C glucoside *orientin* (**12**)						×	
luteolin 6-C-glucoside *isoorientin* (**13**)					×	×	
kaempferol 3-O-glycoside *astragalin* (**14**)	trace	trace		trace		trace	
quercetin 3-O-glucoside *isoquercetrin* (**15**)	trace			××			×
Phenolic acids							
chlorogenic acid (**16**)	××	×	×	×		××	
caffeic acid (**17**)	×	×	×	×	×	××	×

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Luteolin 3'-methyl ether (**4**) were detected in the extract of *Centaurea nervosa* and in the extract of the flowers of *Anthemis montana*. These results supported that the formation of 6-hydroxyflavone and 6-hydroxyflavonol methyl ethers is typical for the species of Asteraceae (Wollenweber & Valant-Vetschera, 1996; Valant-Vetschera & Wollenweber, 2007). The received data for flavonoid composition of *Anthemis montana* are in accordance of previously reported flavonoids for species of genus *Anthemis* (Williams et al., 2001, Bohm & Stuessy, 2001 Wollenweber & Mayer, 1991). To the best of our knowledge there are no previous reports regarding the flavonoid composition of *Anthemis montana*. The extracts of other species: *Knautia arvensis*, *Bartsia alpina* and *Gentianella bulgarica* with regard to flavonoid aglycones (apolar compounds) contain only simple flavonoid - luteolin (**3**).

Concerning to flavonoid compounds with polar properties (glycosides) TLC analysis revealed the presence of six flavonoid glycosides and certain other unknown phenolic compounds.

The extract of *Knautia arvensis* displayed the most complex flavonoid profiles. Glycosides of luteolin were detected: luteolin 7-O-glycoside (**10**), luteolin 8-C-glucoside (**12**) and luteolin 6-C-glucoside (**13**). The present results are in accordance with data reported by Moldoch et al. 2011 for occurrence of flavone 6-C-glycoside of the aerial parts of *Knautia arvensis*. However several substances with TLC behavior of flavonoids remained unidentified.

The presence of luteolin 8-C-glucoside (**12**) of *Gentianella bulgarica*, luteolin 7-O-glycoside (**10**) of *Bartsia alpina*, quercetin 3-O-glucoside (**15**) and kaempferol 3-O-glycoside (**14**) of *Geum bulgaricum* was confirmed (Taylor &Rumsey, 2003; Janković et al., 2005). In the methanolic extract of *Anthemis montana* flavone and flavonol glycosides were detected (Table 3). This result confirms conclusion of Williams et al., 2001 that genus *Anthemis* is differed of the other taxa of Anthemideae such as *Chrysanthemum*, *Cotula*, *Leucanthemum* which contain only flavone glycosides.

It is important to note that chlorogenic acid was detected in the most of the studied species *Anthemis montana*, *Knautia arvensis*, in especially large amounts of *Geum bulgaricum* and *Centaurea nervosa*. Caffeic acid is abundant in the extract of *Knautia arvensis*. Considering that caffeic and chlorogenic acids are major contributor to antioxidant activity (Wu, 2007; Sato et al., 2011) it may be assumed that the antioxidant potential of examined high-mountain plants is determined largely by the high content of phenolic acids.

Conclusion

Six high-mountain plants were surveyed for their flavonoid profiles, total phenolic content and free radical scavenging activity. All studied species were examined for antiradical potential for the first time. The results revealed that the extracts of *Geum bulgaricum*, *Anthemis montana* and *Knautia arvensis* possess significant free radical scavenging activity that making them promising objects for further more detailed studies. The flavonoid profile of *Anthemis montana* are reported for the first time to the best of our knowledge.

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