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FLAVONOID COMPOUNDS AND ANTIOXIDANT ACTIVITY OF BULGARIAN SPECIES OF MICROMERIA

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Abstract: Four *Micromeria* species, naturally distributed in Bulgaria were examined: *Micromeria dalmatica* Benth, *Micromeria frivaldszkyana* (Degen) Velen., *Micromeria juliana* (L.) Rchb. and *Micromeria cristata* (Hampe) Griseb. Acetone exudates and methanolic extracts of the species were studied for flavonoid compounds by TLC and HPTLC. Sixteen flavonoid compounds were detected by screening in different TLC systems (different sorbents and mobile phases). Flavonoid aglycones – apigenin, luteolin, apigenin 4'-methyl ether, quercetagetin 3,6,7-trimethyl ether – were identified in acetone exudates of the examined species. Flavonoid glycosides – kaempferol 3-rutinoside, quercetin 3-rutinoside (rutin), quercetin 3-glucoside/hyperoside, luteolin 7-0-glucoside as well as chlorogenic acid – were determined in the methanolic extracts. The methanolic extract of studied species were evaluated also for free radical scavenging activity using DPPH assay. All extracts showed significant antioxidant activity, and the ones of *M. dalmatica* and *M. frivaldszkyana* exhibited the highest activity.

INTRODUCTION

Micromeria Benth. (Lamiaceae, Nepetoideae) is distributed in the Mediterranean basin, Macaronesia and some parts of Asia and Africa from the sea level to 4500 m of altitude (Puppo 2015). Four *Micromeria* species: *M. dalmatica* Benth, *M. frivaldszkyana* (Degen) Velen., *M. juliana* (L.) Rchb. and *M. cristata* (Hampe) Griseb. are part of the natural Bulgarian flora (Assyov et al. 2012). *M. dalmatica* Benth. is a Balkan endemic species and it grows only in Bulgaria, Greece, Montenegro and Serbia. *M. frivaldszkyana* and *M. juliana* are rare species in Bulgaria and are also valuable medicinal plants. They are included in the Red Data Book of Bulgaria and are protected by the Biodiversity Act of Bulgaria.

Up to 2006 year the species of *Micromeria* have been classified into four sections: *Micromeria*, *Pineolentia* P. Perez, *Cymularia* Boiss. and *Pseudomelissa* Benth. (Arabaci et al. 2010). Later on, based of molecular analysis, chromosome numbers, leaf width and smooth leaf margins (Bräuchler et al. 2005) the members of sect. *Pseudomelissa* were transferred to genus *Clinopodium* L. (Bräuchler et al. 2006). Thereby the species *M. dalmatica* and *M. frivaldszkyana* became synonyms of *Clinopodium dalmaticum* (Benth.) Bräuchler & Heubl and *Clinopodium frivaldszkyanum* (Degen) Bräuchler & Heubl, respectively. It is now accepted that the genus *Micromeria* has 54 species, 32 subspecies and 13 varieties (Bräuchler et al. 2008).

Generally *Micromeria* species are used as medicinal plants and spices. Antimicrobial, antioxidant, gastroprotective, hepatoprotective, cytotoxic, anti-inflammatory, anticholinesterase and analgesic activity have been determined for the species of the genus (Said et al. 2002, Herken et al. 2012, Abu-Gharbieh et al. 2013, Bukvicki et al. 2015).

Previous phytochemical studies on flavonoid composition of *Micromeria* species revealed the presence of flavonoid derivatives of acacetin, apigenin and luteolin (Tomas-Barberan et al. 1991, Marin et al. 2001). Thymonin (6-hydroxyluteolin 7,8,3'-trimethyl ether) were found as a major exudate flavonoid of *M. dalmatica*, as well as many other methyl derivatives of luteolin and apigenin in lesser amounts (Tomas-Barberan et al. 1991). Naringenin 4'-methyl ether and naringenin 4'-methyl ether (isosakuranetin) 7-O-rutinoside and acacetin glycosides have been reported for *M. juliana* (Marin et al. 2001). The presence of quercetin, rutin, naringin, chlorogenic acid and rosmarinic acid was established in the methanolic extract of the aerial parts of *M. frivaldszkyana* by thin layer chromatography (Vukelić 2015). The studies of Tomas-Barberan et al. (1988) detected 6-hydroxyflavone glycosides (mainly 6-hydroxyluteolin glycosides) and flavone C-glycosides (apigenin-6,8-di-C-glycopyranoside) in *Micromeria cristata* and other *Micromeria* species.

Flavonoids and phenolic acids are considered to be the major contributors to the antioxidant activity of medicinal plants. The evaluation of free radical scavenging activity of plant extracts has been extensively studied by DPPH (1,1-diphenyl-2-picrylhydrazyl) method, which is a quick, reliable and reproducible assay (Marinova & Batchvarov 2011).

The aim of the present study was to perform a comparative TLC analysis of flavonoid profiles of four *Micromeria* species distributed in Bulgaria and to make an assay of the antioxidant potential of their methanolic extracts.

MATERIALS AND METHODS

Plant material

The material for analysis was collected in natural populations of the species of interest. For the respective species, the details can be summarized as follows: *Micromeria dalmatica and Micromeria cristata* plant material was sampled in the region of Trigrad (Central Rhodope Mts, Southern Bulgaria, geographic

coordinates: 41°37’25.57”N 24°23’58.87”E; *Micromeria juliana* samples were taken in the Mesta River Valley – the vicinities of the village Godeshevo (41°27’52.37”N 24°3’27.25”E) and *Micromeria frivaldszkyana* samples were collected in Central Balkan Mts, Northern of the city of Karlovo (42°41’54.33”N 24°55’37.74”E). The material was air dried and kept separately before the analysis.

Extraction procedure

Acetone exudates: Air-dried, but not ground (1g) plant material of the studied species was rinsed for 2-3 min. with acetone at room temperature to dissolve the lipophilic components accumulated on the surface. The obtained acetone filtrate was then dried using a rotary-evaporator to give a crude extract which was suspended in methanol and then subjected to TLC for non-polar flavonoids.

Methanolic extracts: Air-dried, 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 subjected to further analysis.

Thin layer chromatographic analysis: The methanolic extracts were examined for polar (glycosides) whereas acetone exudates for non-polar (aglycones) flavonoids and by co-TLC with authentic compounds obtained by Prof. Eckhard Wollenweber. The TLC conditions used are presented in Table 1. Chromatograms were viewed under UV=366 nm light before and after spraying with ‘‘Naturstoffreagenz A’: 1% solution of diphenylboric acid 2-aminoethyl ester complex in methanol.

Table 1. TLC conditions used in the analysis of flavonoids

TLC conditions	Adsorbent	Mobile phase
S_1	silica	ethyl acetate/formic acid/acetic acid/ methyl ethyl ketone /water; (50:7:3:30:10 v/v/v/v/v)
S_2	cellulose	15% acetic acid
S_3	silica	toluene/dioxane/acetic acid (50:25:4v/v/v)
S_4	polyamide	toluene/methyl ethyl ketone /methanol (30:25:15 v/v/v)
S_5	cellulose	60% acetic acid

DPPH radical scavenging activity: The effect of methanolic extracts on DPPH radicals was estimated according to Stanojević et al. (2009). Results are presented as IC_{50} values (μg/mL) - extract concentration providing 50% inhibition of the DPPH solution. The IC_{50} values were calculated by Software Prizm 3.00. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Plant materials (aerial parts) of examined species were analyzed for their flavonoid profiles. The acetone exudates were examined for occurrence of apolar (aglycones) whereas methanolic extracts for polar (glycosides) flavonoids by TLC analysis. Seven flavonoid aglycones were detected and four were identified. Apigenin, luteolin and their derivative: apigenin 4'-methyl ether as well as flavonol derivative: quercetagetin 3,6,7-trimethyl ethers were identified by TLC analysis in direct comparison with authentic compounds (Table 2).

Simple flavonoids – apigenin and luteolin were detected in the acetone exudates of *Micromeria cristata* and *M. juliana*. Methyl derivatives of flavonoids were observed of *M. dalmatica* and *M. frivaldszkyana*. Apigenin 4'-methyl ether is a common flavonoid aglycone for the both species. This is the first report for occurrence of the compound in *M. frivaldszkyana*. The acetone exudate of *M. dalmatica* exhibited the most complex flavonoid profile. Additionally, four flavonoids presented in great amounts were detected of the species but only one has been identified. Using three different sorbents (silica gel, polyamide, cellulose) and various mobile phases: S_3-S_5 TLC systems (Table 1), retardation factor (R_f value) and coloring were identical to that of quercetagetin 3,6,7-trimethyl ether. Although the same compound has been reported for species of the genus *Plectranthus* (Grayer et al. 2010) and quercetagetin dimethyl ether-O-hexoside for *Thymus x citriodorus* (Pereira et al. 2013) generally quercetagetin derivatives are very uncommon for Lamiaceae and were never reported for *Micromeria* species.

In the methanolic extracts seven flavonoid glycosides were detected and four of them were identified. These compounds showed TLC behavior (R_f -values and color) in the studied TLC systems (S_1 and S_2), respectively, as kaempferol 3-rutinoside, quercetin 3-rutinoside, quercetin-3-glucoside or hyperoside, luteolin 7-0-glucoside (Table 2).

The comparative analysis of flavonoid profiles of the four species showed that the species are grouped in couples, *M. dalmatica* and *M. frivaldszkyana* forming the first one, and *M. cristata* and *M. juliana* the other. This grouping is in accordance with sectional classification and previously reported data (Tomas-Barberan et al. 1991; Marin et al. 2001; Arabaci et al. 2010). Generally, *Micromeria dalmatica* and *M. frivaldszkyana* exhibited higher relative levels of flavonoids, and are placed in section *Pseudomelissa*, whereas *M. juliana* and *M. cristata*, with lower levels of flavonoids, are placed in section *Eumicromeria*.

Thin layer chromatography determined higher level of chlorogenic acid in the methanolic extracts of *M. dalmatica* and lower – in these o*f M. frivaldszkyana.*

The antioxidant activity was assayed by scavenging of DPPH radicals. All studied extracts exhibited considerable activity to scavenge DPPH free radicals with IC_{50} values below 50 µg/mL (Table 2). The methanolic extracts of *M. dalmatica* and *M. frivaldszkyana* displayed the highest activity. The reported significant antioxidant activity of studied *Micromeria* species is consistent with the previously reported data of M. *juliana* and other *Micromeria* species (Oztürk et al. 2009; Vladimir-Knežević et al. 2011).

Table 2. Flavonoid compounds and free radical scavenging activity of Bulgarian *Micromeria* species

Compounds	***M. dalmatica***	***M. frivaldszkyana***	***M. juliana***	***M. cristata***
	External flavonoid aglycones in acetone exudate			
apigenin			●	●●
luteolin	●			●●
apigenin 4'-methyl ether	●●	●●		
quercetagetin 3,6,7-trimethyl ether	●●			
unknown compound	●			
unknown compound	●			
unknown compound	●			
unknown compound	●			
	Flavonoid glycosides in methanolic extracts			
kaempferol 3-rutinoside	●●	●	●	●
quercetin 3-rutinoside (rutin)	●●	●		
quercetin-3-glucoside or hyperoside	●●			
luteolin 7-0-glucoside			●●	●
unknown compound	●	●		
unknown compound			●	
unknown compound				●
chlorogenic acid	●●	●		
	DPPH radical scavenging activity IC_{50} [µg/mL]			
	27.53±0.78	25.48±4.42	35.98±8.05	38.82±3.97

CONCLUSIONS

Bulgarian species of the genus *Micromeria* showed significant free radical scavenging activity supporting their use as medicinal plants and as spices in the culinary. Flavonoid profiles of the studied species confirmed their taxonomic classification. *Micromeria dalmatica* had the most complex flavonoid composition with the highest amounts of flavonoid content. Some flavonoid substances were reported here for the first time for the species and quercetagetin 3,6,7-trimethyl ether was reported for the first time for the genus *Micromeria*.

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