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GC-MS metabolic profiling and free radical scavenging activity of *Micromeria dalmatica*

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***Abstract*:**

Nikolova, M., Aneva, I., Berkov, S.: ***GC-MS metabolic profiling and free radical scavenging activity of Micromeria dalmatica. Biologica Nyssana, 7 (2), December 2016: 159-165.***

Metabolite profile of acetone exudate and methanolic extract from aerial parts of *Micromeria dalmatica* Benth were analyzed by GC/MS. Palmitic and linolenic acids, hentriacontane, amyrin, quercetagetin 3,6,7-trimethyl ether, sucrose were identified among the main components in the acetone exudate. In the methanolic extract more than 100 chromatographic peaks were detected including alkanes, fatty alcohols, fatty acids, organic acids, phenolic acids, saccharides, polyoles, phytosterols and other. Most of the compounds were reported for the first time for the species. Hydromethanolic extract of *M. dalmatica* was studied for *in vitro* antioxidant 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity. The inhibitory concentration (IC_{50}) of extract was calculated to be 21.36 μg/mL. The received result shows high antioxidant potential of *Micromeria dalmatica* extract which provide scientific support for the use of the plant as herbs and spices.

Key words: alkanes, DPPH, fatty acid, carbohydrate, flavonoid, lipid, phenolic

***Apstrakt*:**

Nikolova, M., Aneva, I., Berkov, S.: ***GC-MS profilisanje metabolita i sposobnost hvatanja slobodnih radikala vrste Micromeria dalmatica. Biologica Nyssana, 7 (2), Decembar 2016: 159-165.***

Profilisanje metabolita acetonskog eksudata i metanolnog ekstrakta iz nadzemnih delova vrste *Micromeria dalmatica* Benth. izvršeno je GC/MS analizom. Palmitinska i linoleinska kiselina, hentriakontan, amirin, kvercetagetin 3,6,7-trimetil etar i saharoza identifikovane su kao glavne komponente u acetonskom eksudatu. U metanolnom ekstraktu detektovano je više od 100 hromatogramskih vrhova uključujući alkane, masne alkohole, masne kiseline, organske kiseline, fenolne kiseline, saharide, poliole, fitosterole i druge. Većina jedinjenja pronađena je prvi put za ovu vrstu. *Antioksidantna aktivnost h*idrometanolnog ekstrakta *M. dalmatica* proučavana je *in vitro,* analizom njegove sposobnosti hvatanja *2,2-difenil*-1-pikrilhidrazil (DPPH) slobodnih radikala. Izračunata je inhibitorna koncentracija (IC50) ekstrakta od 21.36 μg/mL. Dobijeni rezultati pokazuju visok antioksidantni potencijal ekstrakta *Micromeria dalmatica*, koji daje naučnu potvrdu za upotrebu ove biljke kao medicinske biljke i začina.

Ključne reči: alkani, DPPH, masne kiseline, ugljeni hidrati, flavonoidi, lipidi, fenoli

Introduction

Micromeria Benth. (Lamiaceae, Nepetoideae) is widely distributed in the Mediterranean region on rocky habitats. In Bulgarian flora the genus is represented by four species that have relatively limited distribution but they are used widely as herbs and spices. *Micromeria* species have been applied for the treatment of diseases of the cardiovascular system, digestive tract, respiratory system (asthma), skin inflammations (Said et al., 2002). The species are reported to have antimicrobial, antioxidant, gastroprotective, hepatoprotective, cytotoxic, anti-inflammatory, anticholinesterase and analgesic activity (Öztürk et al., 2011, Vladimir-Knežević et al., 2011, Herken et al., 2012, Shehab & Abu-Gharbieh, 2012, Abu-Gharbieh et al., 2013, Bukvicki et al., 2015).

Micromeria dalmatica Benth. is Balkan endemic distributed in Bulgaria, Greece, Crit, Crna Gora (Petrova & Vladimirov, 2010). Populations of the species of Bulgarian, Serbian (Montenegro) and Greek origin have been well studied for essential oil composition (Slavkovska et al., 2005, Kostadinova et al., 2007, Karousou et al., 2012). For the essential oil of the species has been reported to possess high antimicrobial efficacy against food spoilage microorganisms (Bukvicki et al., 2015). Flavonoid profile of *M. dalmadca* is characterized by the presence of thymonin (5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone) as a major exudate flavonoid aglycone and acacetin (4'-methoxy-5,7-dihydroxyflavone) derivatives as the most abundant flavonoid glycosides (Tomas-Barberan et al., 1991, Marin et al., 2001).

Metabolite profiling is an analytical method for relative quantitation of a mixture of compounds from biological samples using chromatography and universal detection technologies (GC-MS, LCMS). These techniques allow rapid identification of huge number of metabolites. Metabolite profiling have been used in plant and fungi chemotaxonomy, for screening purposes, quality control and standardization purposes as well as for search of new natural products (Frisvad et al., 2008, Fischedick et al., 2010, Hill & Roessner, 2013, Vrancheva et al., 2014, Rohloff, 2015).

As a part of comprehensive survey of chemical composition and biological activity of Bulgarian species of genus *Micromeria* in the present study the metabolite profile and free radical scavenging activity of *Micromeria dalmatica* Benth were examined.

Material and methods

Plant material

M. dalmatica was collected from Vlahina Mt, a part of West Bulgarian Frontier Mts, in August, 2015. Samples, upper flowering part of the plants were collected at full flowering phase without traces of soil, dust or parts of other plants. After that, samples were air-dried in place with good ventilation, away from direct sunlight.

Acetone exudate

Air-dried, but not ground (3g) plant material of aerial parts of *M. dalmatica* was briefly (2-3 min) rinsed 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.

Methanol extract

100 µg of plant material as well as internal standards of 50 µg of nonadecanoic acid, 50 µg of ribitol and 50 µg of 3,4 dichloro-4-hidroxy benzoic acid were placed in 2 mL Ependorf tubes and extracted with 1 mL of MeOH for 2 h at room temperature assisted by an ultrasonic bath for 15 min at 70 ˚C every 30 min, after that the sample was centrifuged. Aliquot of 800 ul was transferred in other Ependorf tubes and was added of 500 µL H_2O and 500 µL of $CHCl_3$, vortexing for 2 min, and the mixture was centrifuged. The chloroform fraction was separated, evaporated and transmethylated with 2% of H_2SO_4 in MeOH at 60˚C for 18 h, than lipids were extracted with *n*-hexane (2x500 µL) which was dried with anhydrous Na_2SO_4 and evaporated to obtain *lipid fraction*. An aliqote of 100 µL from the aqueous fraction was placed in glass vial and evaporated in a speed-vac to obtain *polar fraction*. The rest of aqueous fraction was hydrolyzed with 0.5 mL of 1N NaOH for 18 h at 60˚C. After acidification to pH 1-2 with conc. HCl, the phenolic compounds were extracted with EtOAc (2x500 µL) which was dried with anhydrous Na_2SO_4 and evaporated to obtain *phenolic fraction*.

The fractions of the methanolic extract as well as 20 mg of the acetone exudate were silylated with 50 µL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 µL of pyridine for 2 h at 50°C.

Metabolite analysis

The GC–MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector operating in EI mode at 70 eV. ADB-5MS column (30 m x 0·25 mm x 0·25 µm) was used. The temperature program was: 100-180 ºC at 15 ºC x min^{-1}, 180-300 20 at 5 ºC x min^{-1} and 10 min hold at 300 ºC. The injector temperature was 250 ºC.

Table 1. Identified compounds in the acetone exudate and methanolic extract of *Micromeria dalmatica* by GC/MS

Compounds	RI	Acetone exudate*	Methanol extract*		
			Lipid fraction	Polar fraction	Phenolic fraction
Alkanes (Hydrocarbones)					
Tridecane	1300		0,13		
Tetradecane	1400		3,42		
Pentadecane	1500		0,31		
Hexadecane	1600		2,84		
Heptadecane	1700		1,20		
Octadecane	1800		1,94		
Eicosane	2000		0,92		
Docosane	2200		0,37		
Tricosane	2300	0,11			
Pentacosane	2500	1,18			
Heptacosane	2700	4,5	2,10		
Octacosane	2800	0,89	3,45		
Nonacosane	2900	0,72			
Triacontane	3000	1,53	4,30		
Hentriacontane	3100	13,13			
Dotriacontane	3200	1,23			
Fatty alkohols					
1-Dodecanol	1560		39,96		4,23
1-Tetradecanol	1757	0,64	0,69		1,52
1-Hexadecanol	1955		3,55		10,61
1-Octadec-9Z-enol trimethylsilyl ether	2125		4,43		8,91
1-Octadecanol	2152	0,13	1,72		5,72
1-Eicosanol	2350	0,09			
1-Docosanol	2546	0,21			
1-Tetracosanol	2741	0,56			
1-Hexacosanol	2938	0,03			
1-Octacosanol	3133	0,04	0,17		
Fatty acids					
Octanoic acid (caprylic acid, 8:0)	1575			0,17	
Tetradecanoic acid, methyl ester** (myristic acid, 14:0)	1721		0,62		
Hexadecanoic acid, methyl ether (palmitic acid, 16:0)**	1922		15,95		
Hexadecanoic acid (palmitic acid, 16:0)	2041	11,23	2,20		9,71
Octadecadienoic acid 9,12-(Z,Z),methyl ester (linoleic acid, 18:2)**	2090		4,16		
Octadecatrienoic acid 9,12,15-(Z,Z,Z), methyl ether (α-linolenic acid 18:3)	2098		8,54		
Octadecanoic acid, methyl ester ** (stearic acid,18:0)	2124		3,52		
Octadecadienoic acid 9,12-(Z,Z) (linoleic acid, 18:2)	2204	2,42			
Octadecatrienoic acid 9,12,15-(Z,Z,Z), methyl ether (α-linolenic acid 18:3)	2211	5,67			
Octadecanoic acid (stearic acid,18:0)	2238	0,10			
Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	2775	4,14			
Organic acids					
Malic acid	1473			1,02	
Erythronic acid	1526			0,12	

Table 1. Continued

Glycerides					
Glycerol	1258	2,88	1,03	15,25	
Hexadecanoylglycerol	2582	4,06			
Monooctadecanoylglycerol	2775	4,14			
Tocoferols					
α-Tocopherol	3122	0,351			
Phytosterols					
β-Sitosterol	3335	2,57	2,17		
Triternes					
Amyrin	3415	13,58			
Polyoles					
Erythritol				2,21	
Arabitol	1706	0,34			
Meso-erythritol	1711	5,87		7,35	
Myo-Inositol	2080	1,22		13,18	
Mannose	1919	0,44			
Galactose	1926	0,14			
2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone (Arabinonic acid, 1,4-lactone)	1624	1,53			
Monosaccharides					
Arabinose	1683			2,39	
Monosaccharide 1	1792			7,78	
Fructose I	1800	1,58		6,69	
Fructose derivative	1805	0,83		3,99	
Fructose II	1837	0,12		5,66	
Fructose III	1855	0,04		6,47	
Glucose	1882	2,94		6,02	
Mannose	1918			7,16	
Monosaccharide 2	1969	2,95		12,40	
Monoaccharide 3	2539	0,25			
Disaccarides					
Disacharide 1	2498			0,18	
Sucrose	2628	4,80		56,96	
Trisaccharides					
Rafinnose	3420			0,68	
Amino acids				0,17	
Glycine	1121	0,21			
Flavonoid aglycones	2628				
Apigenin-4'-methyl ether	3040	0,17			
Quercetagein 3,6,7-trimethyl ether	3400	4,78			
Hydroxycinnamic acids					
Quinic acid	1846				29,74
Caffeic acid *trans*	2131				1,66
Benzoates					
3,5-Bis(trimethylsiloxy)benzoic acid	1999				12,75

Legend: *-Data are expressed as percentage of the total peak area [%]
**-due to the way of processing of the lipid fraction the fatty acid is detected as methyl ester

The flow rate of carrier gas (Helium) was 0·8 mL x min^{-1}. The split ratio was 1:10 1 μL of the solution was injected.

The metabolites were identified as TMSi derivatives comparing their mass spectra and Kovats Indexes (RI) with those of an on-line available plant specific database (The Golm Metabolome Database; http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/home/gmd_sm.html), the NIST 05 database and mass spectra available in the on-line lipid library (http://www. lipidlibrary.co.uk/ms/ms01/index.htm), NIST 05 database and literature data as indicated in **Tab. 1**. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. Then, the spectra of individual components were transferred to the NIST Mass Spectral Search Program MS Search

2.0 where they were matched against reference compounds of the NIST Mass Spectral Library 2005 and the Golm Metabolome Database. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9-C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Free radical scavenging activity

The stable 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of studied samples (Stanojević et al., 2009). Different concentrations (10, 20, 50 100 and 200 μg/mL in methanol) of *M. dalmatica* extract were added at an equal volume (2.5 mL) to methanol solution of DPPH (0.3 mM, 1 mL). After 30 min at room temperature, the Ab values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH antiradical scavenging capacity (\%)} = [1-(Ab_{sample}-Ab_{blank})/Ab_{control}] \times 100$$

Methanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank, while DPPH solution plus methanol was used as a control.The IC_{50} values were calculated by Software Prizm 3.00. All of the experiments were carried out in triplicate.

Results and discussion

Metabolite analysis

Acetone exudate and methanolic extract from aerial parts of *Micromeria dalmatica* were analyzed by GC/MS. In the acetone exudate of the sample 174 chemical peak signals were detected. A part of them was identified as representatives of aklanes, fatty alcohols, fatty acids, triterpenes, flavonoid aglycones, and other (**Tab. 1**). Eight *n*-aklanes were found and they ranged from 23 to 32 carbon numbers. Among them the hentriacontane $C_{31}H_{64}$ was the most abundant (13,13%) that in accordance with previously reported data for *Micromeria cristata* and *M. juliana* (Reddy et al., 2000). Seven fatty alcohols were identified as minor components in the acetone exudate. Palmitic and linoleic acids were presented in the most significant relative amount. Amyrin was detected as major triterpene in the acetone exudate. Quercetagetin 3,6,7-trimethyl ether and apigenin 4'-methyl ether were identified as representatives of flavonoid aglycones. Methyl derivatives of quercetagetin are rarely reported to Lamiaceae family (Grayer et al., 2010), however this is the first time that quercetagetin derivative has been found in the genus *Micromeria*.

In the methanolic extract (lipid, polar and phenolic fractions) of *M. dalmatica* more than 100 chromatographic peaks were detected, including organic, fatty and phenolic acids, saccharides, polyoles, phytosterols, alkanes and other (**Tab. 1**). Unlike the acetone exudate in the lipid fraction of the methanolic extract alkanes with carbon number from 13 to 22 were found. Alkanes with carbon number 30, 28 and 16 are dominant. Six fatty alcohols were identified among them dodecanol was the most abundant (39,96%). Seven fatty acids are found, the main being methyl ether of palmitic acid (16:0) followed by linolenic acid (18:3) and linoleic acid (18:2). This fatty acid composition confirms data previously reported for *Micromeria thymifolia* and *Micromeria albanica* (Ristić et al., 1997). In the polar fraction the main carbohydrate was sucrose, approximately 50%. Many monosaccharides and their derivatives were detected. Among identified phenolic compounds quinic acid was the most abundant followed by 3,5-Bis(trimethylsiloxy) benzoic acid.

Free radical scavenging activity

The DPPH assay has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances and plant extracts (Marinova & Batchvarov, 2011). Hydromethanolic extract of *M. dalmatica* was studied for in vitro antioxidant 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity. The inhibitory concentration (IC_{50}) of extract needed to inhibit 50% of the DPPH radicals was calculated to be 21.36 μg/mL. The received value is similar to that obtained for *Micromeria croatica, M. juliana* and *M. thymifolia* extracts and demonstrate considerable activity to scavenge DPPH radicals (Vladimir-Knežević et al., 2011). The commercial antioxidant butylated hydroxytoluene (BHT) was used as positive control and its IC_{50} value was 12.6 μg/mL.

Conclusion

The present study is first report on GC/MS based metabolite profiling of *Micromeria dalmatica*. Most of the compounds were reported here for the first time for the species. Results obtained on the lipid composition appropriate complements the available literature data on other Micromeria species and can be used in comparative chemotaxonomic analyzes. This is the first report on the occurrence of quercetagetin derivatives in genus *Micromeria*. DPPH assay shows high antioxidant potential of *Micromeria dalmatica* extract.

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