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ORIGINAL PAPER

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CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF THE ESSENTIAL OIL OF WILD-GROWING *Micromeria thymifolia* (Scop.) Fritsch

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ABSTRACT

Essential oil (EO) from the leaves of wild-growing *Micromeria thymifolia* (Scop.) Fritsch (Lamiaceae) was screened for its chemical composition as well as its possible antioxidant and antibacterial properties. According to the gas chromatography – flame ionization detector (GC-FID) and gas chromatography – mass spectrometry (GC-MS) analyses, twenty-six compounds were identified, representing approximately 99.0% of the chromatographic area. The major component present in the essential oil was pulegone (44.8%), other main constituents were piperitone oxide (14.5%), iso-menthone (9.3%) and limonene (8.0%). The oil was tested for the antioxidant activity in 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay and showed a dose-dependent free radical scavenging activity with EC_{50} value of 6.71×10^4 μg/ml. The antibacterial effect of the essential oil was tested against Gram-negative and Gram-positive bacteria. The essential oil of *M. thymifolia* showed antioxidant potential and antibacterial activity.

Key words: *Micromeria thymifolia*, essential oil, GC-FID, GC-MS, antioxidant, antibacterial

Abbreviations: GC-FID – gas chromatography-flame ionization detector, GC-MS – gas chromatography-mass spectrometry, DPPH – 2,2-diphenyl-1-picryl hydrazyl, EO – essential oil, MIC – minimum inhibitory concentration, MHB – Mueller-Hinton broth, BHI – brain heart infusion, MBC – minimum bactericidal concentration

INTRODUCTION

A great number of plants, especially those defined aromatic, spicy, and medicinal contain chemical compounds exhibiting antioxidant properties [Suhaj 2006]. The *Lamiaceae* family is well-known for the antioxidant properties of its taxa.

The genus *Micromeria* Benth (*Lamiaceae*, *Nepetoideae*) includes more than a hundred species [Šilić 1984], with a wide geographical distribution [Harley et al. 2004, Brauchler et al. 2005].

Micromeria species are reported to have many pharmacological activities including anaesthetic, antiseptic, abortifacient, antirheumatic, and central nervous system (CNS)-stimulant effects. The essential oil and extracts of some species own considerable biological activities [Marinković et al. 2003, Gulluce et al. 2004].

Micromeria thymifolia (Scop.) Fritsch is known as an endemic species of the Balkan Peninsula, usually grows on karst, as well on serpentine in cleavage of fissured rocks [Šilić 1979]. This species among the most original plants on the field of ethno pharmacology, comparing with ethno therapy practice, are being used in fresh, raw or dried condition [Redžić 2007].

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Considering the importance of this species as a medicinal plant, in the present study we carried out determination of the chemical composition of the essential oil (EO), potential antioxidant and antibacterial properties on Gram-negative and Gram-positive bacteria.

MATERIAL AND METHODS

Plant material

The plant material (aerial parts) was collected in Serbia. A voucher specimen (BEOU 16731) has been deposited in the herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

Extraction of essential oil. Essential oil was extraction from fresh plant material. Air-dried aerial parts of plant material was cut up into small pieces and subjected to hydrodistillation for 2 h using Clevenger apparatus to obtain the EO in yield of 0.49%. The oil was light-yellow in color with a density of 0.93 g/cm^3.

Gas chromatographic analysis. Gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometer electron ionization (GC-MS-EI) analyses were performed using an Agilent 7890A GC system equipped with a 5975C inert XL EI/CI MSD and a FID detector connected by capillary flow technology (Agilent Technologies, Santa Clara, California, USA). The separation was achieved using an Agilent HP-5MSI fused silica capillary column, 30 m × 0.25 mm i.d., 0.25 µm film thickness. GC oven temperature was programmed from 60°C to 285°C at a rate of 4°C/min. Helium was used as carrier gas; inlet pressure was 25 kPa; linear velocity: 1 ml/min at 210°C. Injector temperature: 250°C. Injection mode: splitless. Mass spectrometry (MS) scan conditions: source temperature 200°C; interface temperature 250°C; EI energy 70 eV; mass scan range *m/z* 40-550.

Retention indices were calculated using AMDIS software version 2.70 from retention times of *n*-alkane series obtained under the same chromatographic conditions used for essential oil analysis.

Identification of the components was done by the comparison of MS spectra with reference (Wiley, NIST, and ADAMS databases) and by comparison of obtained retention indices with literature values from ADAMS retention indices library. Percentages (relative) of the identified compounds were computed from the GC-FID peak area.

Free radical scavenging activity

The potential antioxidant activity of the *M. thymifolia* EO was measured in terms of DPPH radical scavenging ability, using the stable radical, 2,2-diphenyl-1-picryl hydrazil – DPPH. Concentrations of the EO experimentally found to satisfy the linear dependence of absorbance from the concentration of the remaining DPPH were 20 µl/ml, 50 µl/ml, 100 µl/ml and 200 µl/ml. The volume of 200 µl was mixed with 1800 µl of 100 µM methanolic solution of DPPH and put in the dark for 30 min. The decrease of absorbance a wavelength is 517 nm after the reaction was determined by UV-VIS spectrophotometer Cintra 40 (GBC Scientific Equipment, Melbourne, Australia) for all samples (*Au*). The absorbance of the DPPH radical without antioxidant (with 200 µl of pure methanol) was the control (*Ac*). Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution (keeping in the dark and cooling). All determinations were performed in triplicate. The percentage of inhibition of the DPPH radical by the samples was calculated according to the following equation:

$$\text{DPPH radical scavenging (\%)} = (A_c - A_u / A_c) \times 100$$

where A_c is the absorbance of the control and A_u is the absorbance of the remaining DPPH radical after 30 min reaction with antioxidant. DPPH scavenging activity, half-maximal effective concentration (EC_{50} value) was also determined for butylated hydroxy toluene (BHT) an artificial antioxidant, was used as a reference.

Determination of antibacterial activity. Bacteria and media. Antibacterial activity of the EO was tested against Gram-negative bacteria (*Escherichia coli* SY252 *lpcA*, *Escherichia coli* ATCC25922, and *Escherichia coli* ATCC8739), and Gram-positive bacteria (*Staphylococcus epidermidis* ATCC12228 and *Listeria innocua* ATCC33090).

Bacterial strains were cultivated (24 h at 37°C) in Mueller-Hinton Broth (MHB, Biomedics, Madrid, Spain), with exception of *L. innocua* for which Brain Heart Infusion Broth (BHI, LAB, Lanchasire, UK) was used.

Determination of minimum inhibitory concentration (MIC). Optical density (OD_{600}) of the overnight cultures (O/N) of bacteria was measured (UV-6300pc double beam spectrophotometer, VWR International); bacteria were centrifuge ed for 10 min at 4000 rpm and resuspended in 0.01 M $MgSO_4$ (aqueous solution) to provide 0.5 OD_{600}.

The MIC of *M. thymifolia* EO against tested bacteria was assessed using Microtitre plate-based antibacterial assay [Sarker et al. 2007] which was used for the assessment of MIC values for bacteria. Briefly, in sterile 96 well plates, the concentration gradient of EO was made. In the first row of the microplate, 180 µl of media was mixed with 20 µl EO, while in the remaining rows 100 µl of media was added. The serial dilutions were performed by pipetting 100 µl from row to row in serially decreasing concentrations. To each well, 80 µl of media was added and finally 20 µl of cell suspension prepared as described earlier. For each strain, growth control (media inoculated with bacteria) was prepared. The highest tested concentration of EO was 46.3 mg/ml. For positive control, serial gradients of antibiotics streptomycin or tetracycline (Sigma Aldrich, USA) were prepared with the highest tested concentrations of 200 µg/ml and 50 µg/ml respectively. The plates were wrapped with parafilm to prevent dehydration of plates and incubated at 37°C for 24 h.

After incubation, OD was read at 600 nm (Multiscan FC, Thermo Scientific, USA). Indicator, a water solution of resazurin (0.675 mg/ml, TCI Europe NV, Belgium) was added in each well in final concentration 10% volume. The plates were wrapped with parafilm to prevent dehydration of plates and incubated at 37°C for 3 h.

Antibacterial activity of EO was detected by reading the OD_{600} and by observing the changes in resazurin color. The resazurin is a blue oxidation-reduction indicator which reduced to resorufin by oxidoreductases within viable cells and becomes pink. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest EO concentration with no change of resazurin color was suspected to be MIC [Sarker 2007].

Determination of minimum bactericidal concentration (MBC). The MBC of *M. thymifolia* EO was determined during confirmation test for suspected MIC value, in which wells which didn't have the change of blue color were plated on media (MHB agar for all bacteria with exception of *L. innocua* which were plated on BHI agar respectively).

Statistical analyses. Pearson's correlation analyses were performed by Statistica 6.0 Software (StatSoft, Inc.) with a significance level of $p < 0.05$.

RESULTS

Chemical composition of the essential oil. The results of the essential oil composition of wild *Micromeria thymifolia* are presented in Table 1. Twenty-six compounds were identified from the GC-MS and GC-FID, representing approximately 99.0% of the chromatographic area. The major compound found in volatiles of *Micromeria thymifolia* was pulegone (44.8%) followed by piperitone oxide (14.5%), iso-menthone (9.3%) and limonene (8.0%), (Fig. 1).

Potential antioxidant activity. As, to the best of our knowledge no data about potential for antioxidant activity of EO of *M. thymifolia*. In the present investigation, the free radical-scavenging activity (or potential antioxidant activity) of the EO of wild-growing *M. thymifolia* was determined by the DPPH assay. The EC_{50} value (the concentration of antioxidant required to scavenge 50% of DPPH radical), of the investigated EO was 6.71×10^4 µg/ml. EC_{50} value for BHT, well known artificial antioxidant, obtained by the same conditions of DPPH assay, was 328 µg/ml.

Antibacterial activity. The essential oil of *M. thymifolia* showed antibacterial activity against tested strains (Tab. 2). Higher antibacterial activity of dissolved EO in comparison with concentrated EO against *E. coli* ATCC25922, *E. coli* ATCC8739, and *L. innocua* was detected.

The MIC of EO ranged from 1.4 mg/ml – 4.7 mg/ml for Gram-negative and 4.7 mg/ml – 46.3 mg/ml for Gram-positive bacteria. Among tested Gram-negative bacteria, *E. coli* SY252 *lpcA* was found to be the most sensitive with MIC detected at 1.4 mg/ml of EO. Among the Gram-positive bacteria, *S. epidermidis* was the least sensitive with MIC detected at 46.3 mg/ml. The lowest detected MBC was for *E. coli* SY252 *lpcA* (5.7 mg/ml). For the rest of the tested bacteria, MBC weather had high value (46.3 mg/ml) or was not

Table 1. Volatile constituents of the essential oil of wild-growing *Micromeria thymifolia* (Scop.) Fritsch

No.	Compound[a]	R_t	RI[b]	RI[c]	%
1	*α*-Thujene	5.70	916	924	0.1
2	*α*-Pinene	5.90	919	932	0.9
3	Camphene	6.30	934	946	0.1
4	Sabinene	6.98	970	969	0.3
5	*β*-Pinene	7.10	981	974	2.5
6	Myrcene	7.49	987	988	0.4
7	*p*-Cymene	8.64	1021	1020	0.6
8	Limonene	8.79	1025	1024	8.0
9	(*Z*) -*β*-Ocimene	9.09	1033	1032	0.1
10	*γ*-Terpinene	9.88	1061	1054	1.1
11	Iso-menthone	13.74	1166	1158	9.3
12	Borneol	14.24	1168	1165	0.4
13	Iso-pulegone	14.71	1179	1175	0.8
14	Pulegone	17.59	1237	1233	44.8
15	Carvacrolmethylether	17.71	1243	1241	1.2
16	Piperitone-*trans*- epoxide	18.20	1258	1252	7.7
17	Carvacrol	20.31	1306	1298	1.6
18	Piperitone	22.09	1346	1340	0.2
19	Piperitoneoxide	23.21	1372	1366	14.5
20	*α*-Copaene	23.57	1375	1374	0.2
21	*β*-Bourbonene	23.97	1384	1387	0.1
22	(*E*)-Caryophyllene	25.46	1419	1417	0.5
23	D-Germacrene	28.15	1488	1484	2.5
24	Bicyclogermacrene	28.76	1503	1500	1.3
25	*δ*-Cadinene	29.88	1525	1522	0.1
26	Spathulenol	32.11	1577	1576	0.5
	Total identified				99.8%

[a] Compounds are listed in order of their elution from a HP-5 column
[b] Retention index on HP-5 column, experimentally determined using homologous series of *n*-alkanes
[c] Relative retention index taken from Adams and/or NIST 05 for HP-5 capillary column tr, traces (<0.1%)

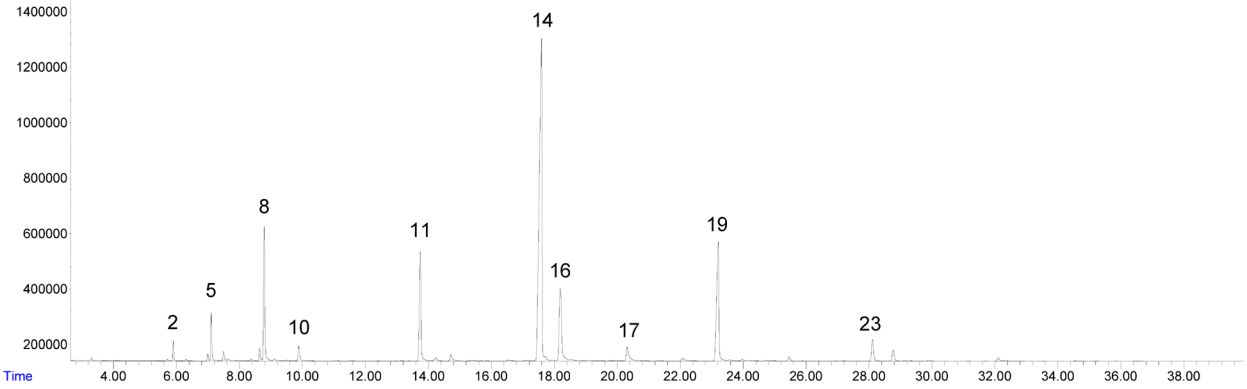

Fig. 1. GC-MS-FiD chromatogram of *Micromeria thymifolia* essential oil with marked component

Table 2. Values of MIC and MBC of *M. thymifolia* EO detected in microtitre plate-based antibacterial assay

Tested microorganism	MIC (mg/ml)	MBC (mg/ml)	Tet[1]/Str[2]/ (µg/ml)
G – bacteria			
Escherichia coli SY252 *lpcA*	1.4	5.7	7.7[2]
Escherichia coli ATCC25922	4.7*	46.3	31.3[2]
Escherichia coli ATCC 8739	2.3*	46.3	2.0[2]
G + bacteria			
Staphylococcus epidermidis ATCC 12228	46.3	/	125.0[2]
Listeria innocua ATCC 33090	4.7*	/	62.5[1]

*Values obtained when EO was dissolved in ethanol in 1 : 9 ratio

Table 3. Pearson's correlation between the EO concentration and OD_{600} of bacteria

Tested microorganism:	Pearson r	p
Escherichia coli SY252 *lpcA*	–0.53	0.0090
Escherichia coli ATCC25922	–0.97	0
Escherichia coli ATCC8739	–0.77	0.0097
Staphylococcus epidermidis ATCC12228	–0.86	0.00060
Listeria innocua ATCC33090	–0.93	0.00210

detected at all. Pearson's correlation analyses indicated a significant linear correlation between the concentration of EO and OD_{600} of bacteria (Tab. 3).

DISCUSSION

The essential oil composition of some *Micromeria* species has been studied previously [Mastelić et al. 2005, Slavkovska et al. 2005]. Comparing our results with those previously reported for this species, we noticed similarities in chemical composition. In our research pulegone appeared as the most abundant, similar to previous studies [Vladimir-Knežević et al. 2000, Duru et al. 2004, Slavkovska et al. 2005]. The study of EOs obtained from *Micromeria fruticosa* [Isa and Ceylan 2007], has shown that they are characterized by a high amount of linalool (30–39%), while the quantity of pulegone was (9–16%).

As reported by Stojanović et al. [1999] the oil of *Micromeria albanica* was rich in monoterpene oxide – piperitenone oxide (44%), while in the work of Marinković et al. [2003] the amount of piperitone oxide was (38.73%). However, in our research, piperitone oxide appeared at significantly lower concentration (14.5%). The composition of EO of some other investigated *Micromeria* species showed minor differences. The major constituent of *Micromeria kosaninii* and *Micromeria juliana* oils was borneol (8.2% and 9.3%, respectively). The oil of *Micromeria parviflora* was marked by a high content of the main constituent spathulenol (29.9%) [Palić et al. 2010]. In the study of Al-Rehaily et al. [2006] major constituents in the EO of *Micromeria biflora* ssp. *arabica* was *trans*-caryophyllene (43.7%).

Due to the production of EOs which showed antimicrobial and antioxidant effects, aromatic plants attracted the attention of many scientists in order to find natural antimicrobial and antioxidant agents. Numerous researches demonstrated the effectiveness of EOs against a wide range of microorganisms, and more recently many EOs have been qualified as natural antioxidants [Bakkali et al. 2008].

The results of study [Formisano et al. 2014] showed that *Micromeria myrtifolia* have a huge potential as an alternative to chemical additives for the food industry due to its antioxidant properties. The significant antioxidant potentials of the acetone extract and the isolated compounds from *Micromeria cilicica* was established by radical-scavenging activity [Öztürk et al. 2011].

Pharmacological and biological properties of essential oils and extracts of some *Micromeria* species are well known [Gulluce et al. 2004, Vladimir-Knežević et al. 2011].

The results obtained in the research of Gulluce et al. [2004] showed that the free radical-scavenging activity of the aqueous methanolic extract of *Micromeria fruticosa* was superior to that of the oil, providing 50% inhibition at a concentration of 70.9 µg/ml, while the oil exhibited weak activity at a higher concentration 98.2 µg/ml. The weak activity of *Micromeria fruticosa* oil is due to the disability of main oil constituents, pulegone, and piperitone to form enough stable radicals, while oils with a high content of phenolic compounds would have significant antioxidant properties. Our results showed lower antioxidant activity of the investigated essential oil (6.71×10^4 µg/ml), comparing to the essential oil of *Micromeria fruticosa* (98.2 µg/ml).

With respect to the risk of food poisoning, antimicrobial activity of *Micromeria thymifolia* EO was tested on bacteria which could be found in food [Nostro et al. 2009, Mihajlov-Krstev et al. 2010, Djenanane et al. 2011].

Considering EO composition, strong antimicrobial activity was expected since EOs of plants, in which pulegone, menthone, and piperitone dominated, such as EOs of *Calamintha nepeta* (L.) *Savi ssp. glandulosa* (Req.) Kitić et al. [2002], exhibited strong antimicrobial activity. In the antimicrobial study carried with EOs from *Micromeria* species, it was assumed that pulegone possesses significant antibacterial and antifungal activity [Marinković et al. 2002, Duru et al. 2004]. Carvacrol which occurs in *M. thymifolia* oil attenuated biofilm formation of *S. epidermidis* [Nostro et al. 2007], and enhanced activity of carvacrol against biofilm of *S. epidermidis* in an acidic environment could be an important instrumentality to control the staphylococcal biofilm in the medical and food environment [Nostro et al. 2012]. Monoterpenic phenol carvacrol could be used to prevent biofilm formation by Gram-negative bacteria uropathogenic *Escherichia coli* and to reduce its virulence [Lee et al. 2017].

Several studies concluded that, as lipophilic agents, monoterpenes execute their action at the level

of the membrane and membrane-embedded enzymes [Uribe et al. 1985, Sikkema et al. 1994], and that outer membrane of the cell wall of Gram-negative bacteria could attribute to the higher resistance to plant extracts and EOs in comparison with Gram-positive bacteria [Mitić-Ćulafić et al. 2005, Marin et al. 2012].

CONCLUSION

The conclusion of our research is that the EO of wild-growing *Micromera thymifolia* has antibacterial activity, and may be useful for food, cosmetics, and other applications according to its antioxidant potential.

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