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GC-MS Analysis of the Antioxidant Active Fractions of *Micromeria juliana* with Anticholinesterase Activity

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The aerial parts of *Micromeria juliana* (L.) Bentham ex Reichb. were extracted with light petroleum, acetone and methanol, successively. The antioxidant activity of different concentrations of the extracts was evaluated using different antioxidant tests, namely total antioxidant (lipid peroxidation inhibition activity), DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging, ferric reducing power, and metal chelating. Total antioxidant activity was determined using the β-carotene-linoleic acid assay. Unexpectedly, the light petroleum extract exhibited strong lipid peroxidation inhibition activity. The extract was fractionated on a silica gel column and the antioxidant activity of the fractions was determined by the β-carotene-linoleic assay at 25 μg/mL concentration. The fractions that exhibited more than 50% inhibition activity were analysed by GC and GC/MS; thus, the structure of fourteen compounds were elucidated. In addition, acetyl- and butyryl-cholinesterase inhibitory activities of the extracts were also determined *in vitro*. The light petroleum and acetone extracts were found to have mild butyrylcholinesterase inhibitory activity.

Keywords: *Micromeria juliana*, Lamiaceae, antioxidant activity, anticholinesterase activity.

In Turkey, the genus *Micromeria*, family Lamiaceae, has 14 species and 22 taxa, of which 12 are endemic. *M. juliana* (L.) Bentham ex Reichb. grow in open habitats in the Mediterranean region, and in north and west Europe [1].

Several *Micromeria* species have shown medicinal value and are used in popular medicine against heart disorders, headache, wound and skin infections [2a,2b], as antispasmodics, stimulants and expectorants [2c] as well as being effective against intestinal colic [2a,2d,2e]. In general, *Micromeria* species are used for colds and respiratory diseases [2a], and in the perfume industry [3].

The essential oil and/or the activity of nineteen *Micromeria* species growing in Turkey have been investigated previously [2d,4a-4d,5a-5f]. Monoterpenoids, such as pulegone, isomenthone, *p*-menthone, limonene, linalool, α-pinene, β-pinene, *p*-cymene, α-terpinene, γ-terpinene, α-terpineol, camphene, β-bourbonene and borneol were the most encountered components in the essential oils of *Micromeria* species.

The chemical composition of the volatile oil of *M. juliana* has been determined [4d,6a-6c], as well as antioxidant [6d] and its antimicrobial activities [6a]. The antioxidant activity was only determined using the thiobarbituric acid assay as α-tocopherol equivalents. The aim of this present study was to determine the chemical composition of the antioxidant active fractions of the light petroleum extract of *M. juliana* by using GC and GC-MS techniques. Since there are some relationships between antioxidant and anticholinesterase activities in the literature [7a,7b], the anti-cholinesterase activity was evaluated as well.

The light petroleum, acetone and methanol extracts of *M. juliana* were subjected to antioxidant tests namely, lipid peroxidation inhibition (β-carotene-linoleic acid assay), DPPH free radical scavenging, ferric reducing power, and metal chelating activities. In addition, anticholinesterase activities were performed on the extracts.

Interestingly, the light petroleum extract exhibited stronger lipid peroxidation inhibition activity than

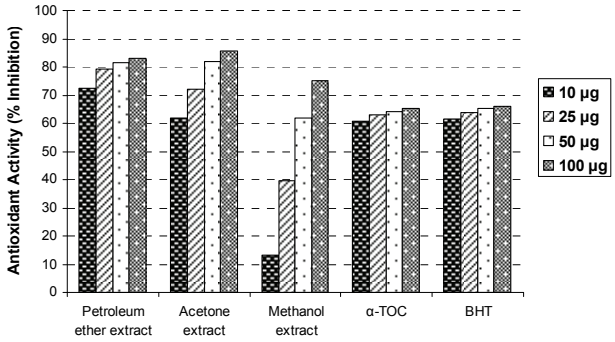

Figure 1: Inhibition (%) of lipid peroxidation by extracts of *M. juliana*, BHT, and α-tocopherol (α-TOC) by the β-carotene bleaching method. Values are mean of n=3. p<0.05, significantly different with Student's t-test.

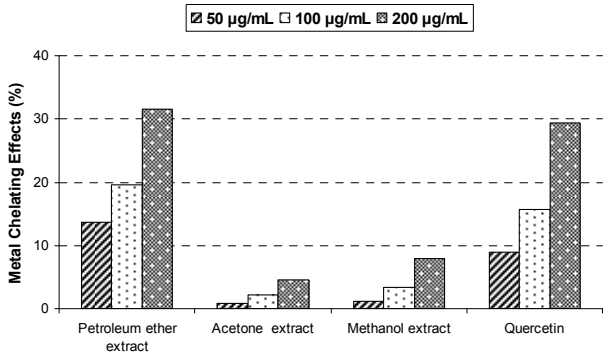

Figure 2: Metal chelation effect of extracts of *M. juliana* and quercetin on ferrous ions. Values are mean of n=3. p<0.05, significantly different with Student's t-test.

BHT and α-tocopherol, which were used as positive standards (Figure 1). This method reveals the level of inhibition of lipid peroxidation, and it is important to understand the type of antioxidant giving H• radicals to the medium to stop the radical degradation [8]. This method is also important to understand the antioxidants which scavenge singlet oxygen causing radicals in lipids.

The light petroleum extract demonstrated the best metal chelating activity among the others tested, being better than quercetin (Figure 2). Transition ions, such as ferrous, and cupric accelerate lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction [9a,9b].

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + {}^{-}OH + {}^{\bullet}OH$$

Therefore, chelating agents known as secondary antioxidants are important for antioxidant activity.

The light petroleum extract showed almost no activity in the DPPH and reducing power assays. Conversely, the methanol extract was found to be the most active extract in both assays (Figures 3 and 4).

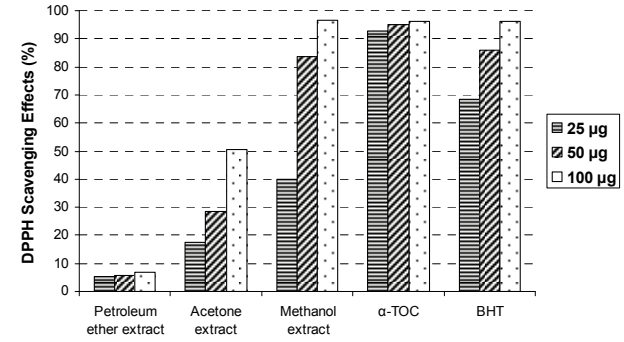

Figure 3: Free radical scavenging activity of extracts of *M. juliana*, BHT and α-tocopherol (α-TOC) by DPPH assay. Values are mean of n=3. p<0.05, significantly different with Student's t-test.

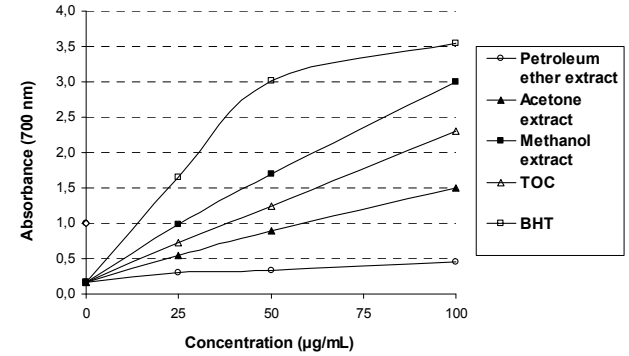

Figure 4: Reductive potential of extracts of *M. juliana*, BHT and α-tocopherol (α-TOC) using spectrophotometric detection of the Fe^{+3}-Fe^{+2} transformations. Values are mean of n=3. p<0.05, significantly different with Student's t-test.

Table 1: Constituents of fraction 1–3.

Compound	No	RI[a]	%	Identification Method
Unidentified		–	2.5	MS
Unidentified		–	58.4	MS, RI
Unidentified		–	1.1	MS
3- Phenethylphenol	(**1**)	1625	1.2	MS, RI
2,6-Diisopropylnaphthalene	(**2**)	1715	2.4	MS, RI
Unidentified		–	3.6	MS
Unidentified		–	3.4	MS
Unidentified		–	1.3	MS
1,3,3- Trimethyl-1- Phenylindan	(**3**)	1877	1.8	MS, RI
Unidentified		–	6.1	MS
Biformene	(**4**)	1897	1.1	MS, Co-GC, RI
Unidentified		–	1.8	MS
Nordehydroabietane	(**5**)	1919	4.7	MS, RI
Abieta-8,11,13-triene	(**6**)	2060	8.6	MS, Co-GC, RI
Dehydroabietinal	(**7**)	2181	1.9	MS, Co-GC, RI
Total identified:			21.7	

[a]: Kovats index on DB–1 fused silica column; [MS]: Mass spectrum; [Co-GC]: Co-injection with authentic compounds; [RI]: Retention Index literature comparison.

Based on the results of the antioxidant activity tests on the extracts, the light petroleum extract was studied for its chemical composition by GC and GC-MS. For this purpose, the extract was fractionated on a silica gel column. Fifteen fractions were obtained after similar fractions had been combined. Total antioxidant activity of the fractions at a concentration of 25 µg/mL was also investigated by the β-carotene-linoleic acid assay. Frs 1-3 (78.4%), Frs 4-5 (72.2%), and Frs 8-9 (71.9%) were found to be active, showing

Table 2: Constituents of fraction 4–5.

Compound	No	RI[a]	Content (%)	Identification Method
Unidentified		–	19.3	MS
Dihydroactinidiolide	(**8**)	1471	7.7	MS, RI
Phytone	(**9**)	1820	60.1	MS, RI
Unidentified		–	12.0	MS
	Total identified:		67.7	

[a]**:** Kovats index on DB–1 fused silica column; [MS]**:** Mass spectrum; [RI]**:** Retention Index literature comparison.

more than 50% inhibition. Therefore, these three were examined by GC-MS.

The yield of Frs 1-3 in the total light petroleum extract was 0.82% (31.90 mg). The chemical constitution of this fraction is shown in Table 1. Seven compounds were identified by GC and GC-MS analyses, of which major ones were abieta-8,11,13-triene (8.6%), nordehydroabietane (4.7%) and abieta-8,11,13-trien-18-al (1.9%).

Biformene (**4**) (=labda-8(20),12,14-triene) has been reported as a constituent of *Dacrydium* and *Helianthus* species [10]. Nordehydroabietane (**5**) (= 18-demethyl abieta-8,11,13-triene), abieta-8,11,13-triene (**6**), and dehydroabietinal (**7**) (= abieta-8,11,13-trien-18-al) are constituents of the oleoresin of *Larix* and *Pinus* species [11a-11c]. Compounds **4**, **5**, **6** and **7** have not been previously reported in *Micromeria* species.

Frs 4-5 yielded 2.60 mg, corresponding to 0.07% of the light petroleum extract. Only four compounds were detected in this fraction, two of which were identified as dihydroactinidiolide (7.7%) and phytone (= hexahydrofarnesyl acetone) (60.1%), which corresponded to 67.7% of the fraction (Table 2).

Dihydroactinidiolide, which showing physiological activity in the cat family, is an important aroma constituent of cigar tobacco and tea [12a]. Phytone, which is an oxidation product of phytol, is widespread in the plant family [12b]. However, both were detected in a *Micromeria* species for the first time.

Frs 8-9 yielded 3.00 mg, corresponding to 0.08% of the fraction. Seven compounds were detected, five of which were identified; this corresponded to 82.8% of the fraction. Cembrene (thunbergen) (27.2%) and eicosane (24.0%) were the major compounds (Table 3).

Thymoquinone (= 2-isopropyl-5-methyl-1,4-benzo-quinone), a biologically important toxic compound, and cembrene (= thunbergen) are constituents of black cumin and *Pinus* species, respectively [13-15]. Both compounds were detected in a *Micromeria* species for the first time. In some investigations, radical scavenging activity was related with anticholinesterase activity [7a,7b] and so this was also determined for the extracts (Table 4). The acetone and light petroleum extracts exhibited moderate butyryl-cholinesterase inhibitory activity, whereas no such acetyl-cholinesterase inhibitory activity was observed in either the light petroleum or methanol extracts. Only the acetone extract demonstrated mild acetylcholinesterase inhibitory activity.

Table 3: Constituents of Fraction 8–9.

Compound	No	RI[a]	%	Identification Method
Thymoquinone	(**10**)	1450	18.9	MS, RI
3,5-Di-*tert*-butil-4-hydroxyacetophenone	(**11**)	1890	1.8	MS, RI
Eicosane ($C_{20}H_{42}$)	(**12**)	1988	24.0	MS, Co-GC, RI
Cembrene	(**13**)	2060	27.2	MS
Heneicosane ($C_{21}H_{44}$)	(**14**)	2106	10.9	MS, Co-GC, RI
Unidentified		–	8.9	MS
Unidentified		–	8.0	MS
	Total identified:		82.8	

[a]**:** Kovats index on DB–1 fused silica column; [MS]**:** Mass spectrum; [Co-GC]**:** Co-injection with authentic compounds; [RI]**:** Retention Index literature comparison.

Table 4: Anticholinesterase activity of *M. juliana* extracts [a].

Samples	AChE assay		BChE assay	
	200 µg	IC_{50} (µg/mL)	200 µg	IC_{50} (µg/mL)
Light petroleum extract	-5.9±4.1	>200	40.9±3.1	>200
Acetone extract	35.3±3.1	>200	52.4±1.8	185.6±1.9
Methanol extract	-7.6±6.8	>200	-6.2±2.3	>200
Galantamine [b]	74.0±0.8	5.0±0.1	75.0±0.6	50.8±0.9

[a] IC_{50} values represent the means ± standard deviation of three parallel measurements (p<0.05). [b] Standard drug (at µM concentration).

In this study, 14 components were identified for the first time from the light petroleum extract of *M. juliana* by GC and GC-MS. Interestingly, the volatile oil components, even the major ones previously revealed [4d,6a-6c], were not identified as compounds that showed antioxidant activity.

In conclusion, the identified constituents were found to have cyclic and/or unsaturated structures. These compounds can stop radical degradation or can contribute to antioxidant activity by either scavenging or converting singlet oxygen to triplet oxygen in the medium [15]. Frs 8-9 contained a phenolic, a paraquinoid, a diterpene and two hydrocarbons. However, further studies are needed to understand the origin of the activity. In particularly, other minor and major components need to be tested for their activity, individually, as well as for their possible synergistic effects.

Experimental

General experimental procedures: GC-MS utilized a Varian Saturn 2100 and were performed at the Department of Chemistry, University of Mugla. Antioxidant activity measurements were recorded on a Shimadzu UV–1601 (Kyoto, Japan), and anticholinesterase activity measurements on a SpectraLab 340PC, Molecular Devices (NY, USA).

Plant material: The aerial parts of *Micromeria juliana* were collected from Marmaris-Mugla, Turkey in June 2005 at 250 m altitude and identified by Dr Tuncay Dirmenci. A voucher specimen was deposited in the Herbarium of the Faculty of Arts and Sciences.

Extraction and fractionation: The dried and powdered aerial parts *M. juliana* (930 g) were extracted with light petroleum, acetone and methanol, successively, at room temperature (24 h x 3). After filtration, the solvent was evaporated to dryness under vacuum. The crude light petroleum extract (3.88 g) was fractionated on a silica gel column (2.5 x 100 cm) by elution with light petroleum (40-60°), followed by a gradient of dichloromethane up to 100%. Using TLC, 16 fractions, coded Frs 1-3 to Frs 33, were obtained after similar fractions were combined. These 16 fractions were subjected to an antioxidant activity test at 25 μg/mL concentration using the β-carotene-linoleic acid assay. The fractions that exhibited antioxidant activity were studied further. Since the polarity was suitable, these 3 fractions were analyzed by GC and GC-MS. Identification of components **1-14** was based on GC retention indices and computer matching with the Wiley and Nist, 2005 Library, as well as by comparison of the fragmentation patterns of the MS with those reported in the literature and when, ever possible, by co-injection with authentic compounds.

Gas chromatography: GC analyses of the antioxidant active fractions were performed using a Shimadzu GC-17 AAF, V3, 230V series gas chromatograph equipped with a FID and a DB-1 fused silica capillary column (30 m x 0.25 id., film thickness 0.32 μm); the initial oven temperature was held at 100°C for 5 min., then programmed to 220°C at 4°C/min and held isothermal for 15 min; injector and detector temperatures were 250°C and 270°C, respectively; carrier gas was He at a flow rate of 1.3 mL/min; sample size, 1.0 μL; split ratio, 1:50. The percentage composition of each fraction was determined with a Class-GC 10 computer programme.

Gas chromatography mass spectrometry: GC-MS analysis of antioxidant active fractions was performed using a Varian Saturn 2100 (Old York Rd., Ringoes, NJ, USA, Quadrupole, EI-mode, 70 eV) equipped with a DB–1 fused silica capillary column (30 m x 0.25 id., film thickness 0.32 μm). For GC–MS detection, an electron ionisation system with an ionization energy of 70 eV was used. Carrier gas was helium (15 psi) at a flow rate of 1.3 mL/min. The oven temperature was held at 100°C for 5 min, then increased to 220°C with 4°C/min increments and held at this temperature for 15 min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. Ion source temperature was 200°C. The injection volume was 0.5 μL, with a split ratio of 1:30. The mass range was from m/z 28 to 650 amu. Scan time was 0.5 sec, with 0.1 interscan delays. Diluted samples (1/100, v/v, in *n*-hexane) of 1.0 μL were injected manually in the splitless mode. The relative percentage of each fraction constituent was expressed as percentage.

Antioxidant activity

Chemicals: Potassium ferricyanide, ferrous chloride, ferric chloride, trichloro acetic acid (TCA), methanol, and quercetin were obtained from E. Merck (Darmstadt, Germany). β-Carotene, linoleic acid, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonicacid)-1,2,4-triazine (Ferrozine), polyoxyethylene sorbitan monopalmitate (Tween-40), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), α-tocopherol (α-TOC), acetylcholinesterase (AChE), butyryl-cholinesterase (BChE), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide, butyrylthiocholine chloride, and galanthamine were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade.

Determination of antioxidant activity with the β-carotene bleaching method: The antioxidant activity was evaluated using the β-carotene-linoleic acid test system [16]. β-Carotene (0.5 mg) in 1 mL of chloroform was added to 25 μL of linoleic acid, and 200 μL of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water, saturated with oxygen, was added by vigorous shaking. Four mL of this mixture was transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was

incubated for 2 h at 50 °C. A blank, devoid of β-carotene, was prepared for background subtraction. BHT and α-tocepherol were used as standards.

DPPH free radical scavenging activity: The free radical scavenging activity was determined by the DPPH assay described by Blois [17,18] with slight modification. DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, a 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this was added to 1 mL of sample solutions in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Antiradical activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x } 100$$

Reducing power: The reducing power of extracts was determined according to the iron(III) reductive assay [19]. Sample solutions in different amounts were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). After the mixture was incubated at 50°C for 20 min, 2.5 mL of TCA (10%) was added and the mixture centrifuged at 1000 *g* (MSE Mistral 2000, London, UK) for 10 min. Supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of ferric chloride (0.1%), and the absorbance was measured at 700 nm.

Metal chelating activity: The chelating activity of extract on Fe^{2+} was measured as reported by Decker and Welch [19,20]. The extract was added to a solution of 2 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The mixture was shaken vigorously and left standing at room temperature for 10 min. After reaching equilibrium, the absorbance was determined at 562 nm.

Anticholinesterase activity*:* Acetyl- and butyryl-cholinesterase inhibitory activities were measured by slightly modifying the spectrophotometric method of Ellman *et al* [21]. Acetylthiocholine iodide and butyryl-thiocholine chloride were used as substrates of the reaction and DTNB for the measurement of the anticholinesterase activity. Sodium phosphate buffer (pH 8.0; 150 µL of 100 mM solution), 10 µL of test compound solution, and 20 µL AChE or BChE solution were mixed and incubated for 15 min at 25 ºC, and 10 µL of DTNB was added. The reaction was then initiated by the addition of 10 µL of either acetylthiocholine iodide or butyrylthiocholine chloride. The hydrolysis of these substrates, shown by the formation of yellow 5-thio-2-nitrobenzoate anion, was monitored spectrophotometrically at a wavelength of 412 nm. Ethanol was used as a solvent to dissolve the test compounds and controls.

Statistical analysis: All data from all activity tests are the average of triplicate analyses. The data were recorded as means ± standard deviations. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by the Student-*t* test, with *p* values of <0.05 being regarded as significant.

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