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The constituents of essential oil and in vitro antimicrobial activity of *Micromeria cilicica* from Turkey

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Abstract

The chemical composition of the essential oil of *Micromeria cilicica* (*Labiatae*) that has been used in folk medicine were analysed by GC, GC–MS, ^{1}H NMR and ^{13}C NMR. The totals of 34 components in hydrodistillation, 30 components in steam distillation were detected. The major component characterized in the essential oils was pulegone (66.55, 64.10%) and other main components were determined as *cis-p*-menthone (21.71, 25.31%), *trans-p*-menthone (9.59, 5.59%), nerol (0.35, 2.49%) and 3-octonol (0.81, 0.25%), respectively. Essential oils obtained by hydro and steam distillation and organic solvent extracts of the aerial parts of the plant were investigated for antimicrobial activities on several microorganisms including bacteria and yeast. Moreover, the main constituent of the oil has been tested against the same microorganisms. The extracts and pulegone exhibited a significant antibacterial and antifungal activity. The activities were increased depend on the amount of extracts and pulegone. Pulegone also showed antimicrobial activity, particularly against *Candida albicans* and *Salmonella typhimurium*. Furthermore *Candida albicans* is the most susceptible to pulegone giving two times the effect of nystatin.
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Keywords: *Micromeria cilicica*; *Labiatae*; Essential oil; Chemical composition; Antimicrobial activity

1. Introduction

The genus *Micromeria* is a member of *Labiatae* family. *Micromeria* genus has 14 species and 22 taxa in Turkey and 12 of them are endemic. Also these species are grown naturally in Turkey. *Micromeria cilicica* (*Labiatae*) is an endemic species and grow in open habitats Mediterranean (Davis, 1982). In general, several *Micromeria* species are also used against heart disorders, headache, wounds and skin infections and the most usage of *Micromeria* species are in colds (Baytop, 1984; Ali-Shtayeh et al., 1998). *Micromeria cilicica* is used as a tea and for folkloric medicinal purposes known as "Topuk Çayı" or "Filiskin" in Fethiye, Muğla (Baytop, 1984). Besides, the leaves from some *Micromeria* species are utilized a seasoning for food preparation in Turkey (Baytop, 1984; Kirimer et al., 1993a,b; Harmandar, 1988). Traditional usage of *Micromeria cilicica* is very extensive in this area. The antimicrobial activity and chemical composition of this plant have not been investigated.

Pulegone, isomenthone, *p*-menthone, limonene, linalol, α-pinene, β-pinene, *p*-cymene, α-terpinene, γ-terpinene, α-terpineol, camphene, β-bourbonene and borneol are the most encountered essential components in *Micromeria* species. Essential oil composition of *Micromeria* species such as *Micromeria fruticosa* spp. *serpillifolia* (Kirimer et al., 1993a,b; Harmandar, 1988), *Micromeria nervosa* (Ali-Shtayeh et al., 1997), *Micromeria dalmatica* (Stojakoviç et al., 1990), *Micromeria abysinica*, *Micromeria biflora* (Rovesti, 1952), *Micromeria furiticosa* (Svendsen, 1985), *Micromeria furitucosa* subsp. *barbata* (Kirimer et al., 1993a,b), *Micromeria furitucosa* subsp. *giresunica* (Baser et al., 1996), *Micromeria furitucosa* spp. *brachycalyx* (Kirimer, 1992) and *Micromeria congesta* (Kirimer et al., 1991), *Micromeria varia* (Perez-Alonso et al., 1996), *Micromeria herpyllomorpha* (Perez-Alonso et al., 1996), *Micromeria lachnophylla* (Perez-Alonso et al., 1996), *Micromeria hyssopifolia* (Perez-Alonso et al., 1996), *Micromeria lasiophylla* subsp. *palmensis* (Perez-Alonso et al., 1996) and *Micromeria teneriffae* (Lawrance, 1989), *Micromeria myrtifolia* (Özek et al., 1992), *Micromeria cremnophila* spp. *amana* (Başer et al., 1997), *Micromeria carminea* (Baser et al., 1995) and *Micromeria graeca* (Tzakou and

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Couladis, 2000), *Micromeria dolichodontha* (Baser et al., 1997a,b), *Micromeria thymifolia* (Vladimir-Knezevic et al., 2000), *Micromeria capitallata* (Puri and Jain, 1988) and *Micromeria cristata* subsp. *phrygia* (Tabanca et al., 2001) have been studied.

Nowadays the development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for further attempts to search for new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents (Ali-Shtayeh et al., 1998; Primo et al., 2001). Hence, this in vitro study was aimed at screening the essential oil for its composition and antimicrobial activity evaluating its potential use in treating various infections caused by bacteria and/or *Candida albicans*, and determining whether its use in folkloric medicine use justified.

2. Materials and methods

2.1. Plant material

Micromeria cilicica was collected at the flowering stage in June 2002 from Fethiye-Girmeler region in Muğla. A voucher specimen (Herbarium No.: M-101) has been deposited in the Herbarium of Faculty of Arts and Science of University of Muğla in Muğla.

2.2. Preparation of the organic extracts

The air dried and powdered aerial parts of *Micromeria cilicica* (400 g) were extracted with acetone four times (24 h × 4) at room temperature (25 °C). The extract was evaporated under vacuo to leave a residue extract (5 g), which was taken up in water and extracted in succession with *n*-hexane, chloroform and ethyl acetate, yielding 0.254, 0.837 and 0.252 g of extractives, respectively (Deliorman et al., 2001).

2.3. Isolation of the essential oil

The essential oil of dried aerial parts of *Micromeria cilicica* was obtained by hydrodistillation by using a Clevenger type apparatus and by steam distillation for 4 h. The essential oil was dried with anhydrous sodium sulphate and stored under nitrogen in a sealed vial until required (Kirimer et al., 1993a,b; Harmandar, 1988).

2.3.1. Gas chromatography (GC)

GC analyses of the essential oil were performed using a Shimadzu GC-17 AAF, V3, 230V LV series gas chromatography equipped with a FID and a Optima-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm); the oven temperature was held at 40 °C for 15 min, then programmed to 200 °C at 3 °C/min and held at their temperature for 15 min; injector temperature and dedector temperature was 250 and 270 °C, respectively; carrier gas was He at a flow rate of 1.3 mL/min; Sample size, 1.0 μL; split ratio, 50:1. The percentage composition of the essential oil was determined with Class-GC 10 computer programme.

2.3.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were carried out on a Fisons MD 800 mass spectrometer (ion source 200 °C, RI 70 eV) equipped with a ZEBRON-5 fused silica column (60 m × 0.25 m i.d., 1 μm) programmed at 40–280 °C with a rate of 4 °C/min. Injector temperature 280 °C, Carrier gas, He (20 psi).

2.3.3. Isolation of the major components of essential oil

The essential oil of *Micromeria cilicica* was subjected to column chromatography, using silica gel 60 F_{254} (70–230 mesh) and eluting with *n*-hexane containing increasing amounts of diethyl ether, *p*-menthone and pulegone were eluted from *n*-hexane:diethyl ether fractions (92.5:7.5) and (87.5:12.5), respectively from essential oil. The NMR spectra was recorded on a Varian-200 Spectrometer, using $CDCl_3$ as solvent and TMS as internal standard.

2.3.4. Identification of major components of essential oil

Identification of components of the essential oil was based on GC retention indices and computer matching with the WILEY and NIST Library as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and when ever possible, by co-injection with authentic compounds. The identity of the main components of *Micromeria cilicica* oil was assigned by ^{1}H NMR and ^{13}C NMR at 200 and 50 MHz, respectively. The NMR datas of pulegone and *p*-menthone are in agreement with literature data (Swigar, 1981).

2.4. Antimicrobial activity

2.4.1. Microorganisms and condition for cultivation

Human pathogens, *Staphylococus aureus*, *Micrococcus luteus*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus mutans* and *Candida albicans* were obtained from the culture collection of the Microbiology Department in Muğla University.

The above mentioned bacteria, except *Streptococcus mutans*, were cultured in Nutrient Broth (NB) (Difco) at 37 ± 0.1 °C; *Streptococcus mutans* were cultured in Brain Heart Infusion Broth (BHIB) (Difco) at 37 ± 0.1 °C, *Candida albicans* cultured in Sabouraund Dextrose Broth (SDB) (Difco) at 28 ± 0.1 °C.

Inocula were prepared by adjusting the turbidity of the medium to match the 0.5 Mcfarland Standard Dilutions of this suspension in 0.1% peptone (w/v) solution in sterile water were inoculated on NB, BHIB, SDB to check the viability of the preparation.

The cultures of bacteria were maintened in their appropriate agar slants at 4 °C throughout the study and used as stock cultures.

Table 1
The physicochemical properties of essential oil of *Micromeria cilicica*

Physicochemical property	A	B	Pulegon
d_{20}	0.9716	1.020	0.940
$[\alpha]_D^{20}$	+37.95	+35.54	+25.00
n_{20}^{0}	1.4786	1.4766	1.487

A: essential oil obtained by hydrodistillation, B: essential oil obtained by steam distillation, d_{20}: density at 20 °C, n_{20}^{0}: refractive index at 20 °C, $[\alpha]_D^{20}$: specific rotation at 20 °C.

2.4.2. Antimicrobial assays

The antimicrobial activity of the essential oils and the pulegone were assayed by the standard disc diffusion method (Collins, 1995; Murray, 1995). These materials of *Micromeria cilicica* were injected into sterilized discs of 6 mm in diameter (Schleicher & Schuell).

Mueller Hinton Agar (MHA) (Difco) and Sabouraund Dextrose Agar (SDA) (Difco) sterilized in a flask and cooled to 45–50 °C were distributed to sterilized petri dishes with a diameter of 9 cm (15 ml) after injecting cultures (0.1 ml) of bacteria and yeast and distributing medium in petri dishes homogeneously. Dishes injected with above mentioned materials were located on the solid agar medium by pressing slightly. Petri dishes were kept at 4 °C for 2 h, placks injected with yeast were incubated at 28 °C for 48 h, and the bacteria were incubated at 37 °C for 24 h. On each plate an appropriate reference antibiotic disc was applied depending on the test microorganisms. At the end of the period, inhibition zones formed on the MHA and SDA were evaluated in millimetres. Studies were performed in triplicate, and the developing inhibition zones were compared with those of refence disks.

Table 2
The essential oil constituents of *Micromeria cilicica* (Hausskn. ex P.H. Davis)

Peak no.	Compound	Rt[a]	A (%)	B (%)	Identification methods
1	α-Pinene	933	0.04	–	1, 2, 3
2	3-Methyl-cyclohexanone	952	0.03	0.09	2
3	Sabinene	988	0.05	0.01	1, 2, 3
4	3-Octanol	994	0.81	0.25	1, 2
5	α-Terpinene	1007	*t*	–	1, 2, 3
6	*p*-Cymene	1033	0.01	0.20	1, 2, 3
7	Limonene	1038	0.07	0.04	1, 2, 3
8	1,8-Cineole	1044	0.03	0.03	1, 2, 3
9	3-Ethyl,2-hydroxy cyclopentenon	1060	0.07	0.05	2
10	Fenchone	1097	*t*	0.02	1, 2, 3
11	α-Terpinolene	1098	0.02	–	1, 2, 3
12	β-Thujone	1102	–	0.01	1, 2, 3
13	*cis*-Sabinen hydrate	1110	0.04	0.04	1, 2, 3
14	3-Octanyl acetate	1115	0.01	*t*	1, 2
15	1,2:8,9-Diepoxy-*p*-menthane	1134	*t*	–	2
16	1-Terpineol	1136	–	0.01	2
17	*trans*-Limonene-1,2-epoxide	1137	*t*	–	1, 2
18	*trans* -*p*-Menthone	1155	9.59	5.59	1, 2, 3
19	*cis*-*p*-Menthone	1166	21.71	25.31	1, 2, 3, 4
20	Isopulegone	1170	0.39	0.63	1, 2
21	*trans*-Dihydrocarvon	1172	0.06	–	1, 2
22	Myrcenol	1184	0.01	0.04	1, 2
23	Berbenone	1192	0.01	0.03	2
24	Nerol	1202	0.35	2.49	1, 2
25	Pulegone	1224	66.55	64.10	1, 2, 3, 4
26	7-Hydroxy citronellal	1228	0.01	0.13	1, 2
27	2-Undecenal	1232	*t*	0.02	2
28	Unidentified	1220	*t*	0.01	
29	Bornyl acetate	1246	0.03	0.09	1, 2, 3
30	Piperitenone oxide	1254	*t*	0.01	1, 2, 3
31	Citronellyl acetate	1270	*t*	0.01	1, 2
32	Eurocarvone	1280	0.06	0.23	2
33	Geranyl acetate	1283	0.02	–	1, 2
34	Isoiridomirmesin	1326	–	0.04	1, 2
35	Mintfuranone 1	1364	*t*	0.48	2
36	Mintfuranone 2	1375	–	0.03	2
37	*cis*-Nerolidol	1382	0.01	–	1, 2
38	Elemol	1402	0.01	0.01	1, 2, 3

A: essential oil obtained by hydrodistillation, B: essential oil obtained by steam distillation, 1: co-injection with authentic compounds, 2: MS, 3: literature comparison, 4: ^{1}H NMR and ^{13}C NMR, $t < 0.01$.

[a] In ZEBRON-5 fused silica column.

Table 3
Antimicrobial activity of *Micromeria cilicica* extracts and main compound of essential oil

Extracts	Concentration (μl/disc)	Inhibition zone diameter (mm)									
		Microorganisms									
		S. aureus ATCC 25923	*Micrococcus luteus* NRLL B-4375	*Enterobacter aerogenes* RSKK 720	*Salmonella typhimurium* CCM 5445	*Bacillus subtilis* ATCC 6633	*Bacillus cereus* RSKK 863	*Escherichia coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*Streptococcus mutans* CNCTC 8/77	*Candida albicans* ATCC 1023
Hydrodistilled essential oil	10	9	10	9	–	12	12	12	9	11	28
	25	20	19	15	17	21	17	13	13	19	34
Steam distilled essential oil	10	11	10	14	14	11	13	–	9	–	13
	25	23	16	22	15	16	17	–	15	–	21
Hexan fraction	10	–	13	10	10	9	10	10	10	–	18
	25	15	16	15	14	16	12	16	17	–	26
Chloroform fraction	10	12	–	–	8	11	8	–	–	–	15
	25	14	10	8	11	13	11	9	8	–	19
Ethyl acetate fraction	10	9	8	8	9	8	9	8	8	–	–
	25	11	11	11	11	12	11	10	12	12	–
Pulegone	10	17	11	11	22	11	14	8	9	10	31
	25	22	18	13	23	14	17	11	10	15	44
Reference antibiotics											
Ampicillin (μg)	10	NT	30	–	–	NT	NT	20	NT	22	NT
Penicillin (U)	10	31	31	8	NT	12	24	19	NT	21	NT
Oxacillin (μg)	1	21	22	–	NT	8	15	NT	NT	NT	NT
Gentamicin (μg)	10	NT	NT	NT	NT	NT	NT	NT	16	NT	NT
Nystatin (U)	100	NT	NT	NT	NT	NT	NT	NT	NT	NT	19

NT: not tested.

3. Results and discussion

Yields of 0.88 and 0.55% the essential oils were obtained by hydrodistillation and steam distillation, respectively, based on the dry weight of aerial parts of the plant. Physicochemical properties of both oils are given in Table 1. The physical properties of essential oils are similar to each other.

Thirty-four components of the oil were obtained by hydrodistillation, 30 components of the oil were obtained by steam distillation and were detected by using GC, GC-MS analytical methods. All of the components in the both oils were identified with using GC, GC–MS, ^{1}H NMR and ^{13}C NMR methods. The major components were pulegone (66.55, 64.10%), *cis-p*-menthone (21.71, 25.31%), *trans-p*-menthone (9.59, 5.59%), nerol (0.35, 2.49%) and 3-octanol (0.81, 0.25%), respectively (Table 2).

Most of the essential oil consists of monoterpenoids; this represents 98.86% in essential oil obtained by hydrodistillation and 99.32% obtained by steam distillation. No sesqueterpenes are present in the oil. Essential oils also contain monoterpene hydrocarbons (0.19, 0.25%), sesqueterpenoids (0.02, 0.01%) and the remaining percentage consists aliphatic alcohols, aliphatic aldehydes, aliphatic esters, aliphatic hydrocarbons and aliphatic ketones (0.92, 0.41%).

Antimicrobial activity of five different extractions and pulegone, which is the main component of *Micromeria cilicica*, has been evaluated in vitro against nine bacterial species and one yeast (Table 3) which are known to cause infections in humans. As summarized in the table, pulegon and the extracts exhibited antimicrobial activity against most of the microorganisms. Although, at low quantities (10 μl) the essential oil of *Micromeria cilicica* caused growth inhibition in many bacteria and *Candida albicans*, strong antimicrobial and anticandidal activities were observed at the high concentrations (25 μl) for all the bacteria and *Candida albicans*.

In the present study, pulegone exhibited high antimicrobial activity on the all test bacteria especially *Salmonella typhimurium* and *S. aureus*. In other studies, the antimicrobial activity could be explained by the presence of pulegone (Primo et al., 2001; Flamini et al., 1999).

Compared to reference antibiotics, both compounds showed activities against the test microorganisms (Table 3). This work has also clearly demonstrated the high anticandidal effect of pulegone and the water extract of *Micromeria cilicica* against the yeast *Candida albicans*. This effect is approximately twice that of nystatin's (100 U). This result is in concordance with other studies. Ali-Shtayeh et al. (1997) also found the essential oil of *Micromeria nervosa* to be active against *Candida albicans* (Ali-Shtayeh and Al-Nuri, 1997). Antifungal activities of essential oils from *Micromeria dalmatica*, *Micromeria albanica*, *Micromeria thymifolia* (Marinkovicacute et al., 2002), *Micromeria cristata* (Tabanca et al., 2001) were evaluated. Biological assays showed strong toxicity against fungi of oil from these species. In another study, *Micromeria nervosa* was found to be active against *Candida albicans* (Ali-Shtayeh et al., 1998).

In this study, all of the different extracts showed different antimicrobial activities. Ethylacetate extracts of the plant showed antibacterial activity against all of the bacteria which were tested. But it showed no anticandidal activity.

The results detected in this in vitro study provided evidence that the plant is potentially a rich source of antimicrobial agent against many microorganisms especially *Candida albicans*. Hence, the extracts of *Micromeria cilicica* may be useful as an alternative antimicrobial agent in natural medicine for the treatment of many infectious diseases.

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