SUPPLEMENTARY MATERIAL

Chemical composition and antimicrobial activity of *Micromeria hedgei* Rech. f. oil from Iran

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Abstract

Micromeria hedgei belongs to the Lamiaceae family is a rare endemic and endangered species that has been used in traditional medicine in Iran. In this regard essential oil composition and antimicrobial activity of wild and cultivated *M. hedgei* was reported for the first time. Essential oils isolated via hydro distillation from the aerial parts of *M. hedgei* were analyzed by a combination of capillary GC and GC–MS. The major constituents were geranial (18.04 and 22.68%), neral (13.81 and 15.99%), geraniol (13.15 and 10.74%), nerol (7.69 and 6.02%), E-caryophyllene (6.52- 3.80%), carvacrol (6.20 and 5.27%), geranyl acetate (5.79 and 3.06 %), caryophyllene oxide (4.73 and 3.88 %), thymol (3.13 and 3.63%), and α -humulene (3.27 and 3.27%) in wild and cultivated *M. hedgei*. Antimicrobial activity of essential oils was investigated by disc diffusion method. Essential oil showed good antimicrobial activity against five medically important pathogens compared with standard antibiotics.

Keywords: *Micromeria hedgei*, essential oil, antimicrobial, geranial, neral

Experimental

3.1. *Plant material*

Aerial parts of *M. hedgei* were collected in April 2015 in full- flowering stage in natural habitat of Bokhoon area Hormozgan province (Near Persian Gulf) Southern Iran and cultivated plants in Medicinal and Aromatic Plants Experimental Garden (MAPEG) of the Estahban branch, Islamic Azad University in Fars province in southwest Iran (Fig S1). The plants were identified and authenticated (voucher no.116) at the herbarium of medicinal and aromatic plants of (IAU), Estahban branch, Fars, Iran. Climatic conditions of *M. hedgei* habitats were determined using the nearest meteorology station (Table S1). The harvested plants in different habitats were dried at room temperature (25°C) for 2 weeks. Then, air- dried plants ground and powdered with mixer for essential oil extraction.

3.2. *Essential oil extraction*

Dried aerial parts were ground into powder (mesh< 35), and 100 g of the powdered tissue was distilled with 1 L of water for 3 h using a Clevenger-type apparatus according to the method recommended in the British Pharmacopoeia (British Pharmacopoeia,1988). The oils were dried over anhydrous sodium sulfate, weighed, and stored in dark glass vials at 4°C prior to analysis and antimicrobial tests.

3.3. *Identification of the Oil Components*

The essential oil composition was determined by GC and GC-MS analysis. The analysis was performed using a gas chromatograph (Agilent Technologies 7890 GC) equipped with a FID detector, using HP-5MS 5% capillary column (30 m × 0.25 mm, 0.25 µm film thicknesses). The carrier gas was Helium at a flow of 1 ml/min. Initial column temperature was 60°C and was programmed to increase at 3°C/min to 280°C. The injector and detector temperatures were set at 280 °C. The split ratio was 20:1. Oil samples (0.2 µl) were injected manually. The percentage compositions were obtained from electronic integration of peak areas without the use of correction factors.

The GC-MS analysis was done on the Agilent Technologies 5975 Mass system. The EI-MS operating parameters were as follows: ionization voltage, 70 eV; ion source temperature, 200 °C. The retention indices for all the components were determined according to the Van Den Doll method using n- alkanes as standard (Van Den Dool and Kratz, 1963). The compounds were identified by comparison

of retention indices (RRI- HP-5) with those reported in the literature and by comparison of their mass spectra with the Willey and mass finder 3 libraries or with the published mass spectra (Adams, 2001).

3.4. Microorganisms

Standard strains of *Candida albicans* (ATCC 10231), the Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 1435), *Bacillus cereus* (ATCC 1247) and the Gram-negative bacterium *Escherichia coli* (ATCC 25922) were all obtained from the Iranian Research Organization for Science and Technology.

3.5. Determination of antimicrobial activity by the disk diffusion method

In vitro antimicrobial activities of the essential oils of *M. hedgei* were evaluated by the disk diffusion method, with determination of inhibition zones (IZ), using Mueller-Hinton agar for bacteria (MHA) and Sabouraud dextrose agar (SDA) for fungi (Baron and Finegold, 1990). Fungal or bacterial suspension were seeded into Petri dishes (9 cm) containing 20 mL of growth medium using a sterile cotton swab. The sterile paper discs (6 mm in diameter) were individually impregnated with 10 µL of the oil and then placed on the agar plates which had previously been inoculated with the tested microorganisms. The plates were inoculated with bacteria incubated at 37°C for 24 h and at 24°C for 48 h for the *C. albicans* strain. All studies were performed in triplicate. Blank disks containing 10 µL DMSO were used as negative controls. Nystatin (30 µg/disk), Tetracycline (30 µg/disk), Ketoconazole (20 µg/disk), and Gentamicin (30 µg/disk) were used as positive reference standards to determine the sensitivity of the microorganisms.

A broth micro-dilution method was used to determine the minimum inhibitory concentration (MIC) according to the National Committee for Clinical Laboratory Standards (NCCLS, 2001). A serial double dilution of the oil was prepared in a 96-well micro-titer plate over the range of 0.02–50.00 µL/mL. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. All determinations were performed in triplicate.

Table S1. Geographical and environmental conditions of *M. hedgei* growing wild and cultivated in Iran

Region	Province	Altitude (m)	Latitude (UTM)	Longitude (UTM)	Temp. ^a (°C)	Rainfall ^b	E.C. ^c	Ph	R.H. ^d	S.T. ^e
Bokhoon	Hormozgan	1600	27°56'41"N	56°16'04"E	23.21	212	4.20	7.79	76.7	Loam
Estahban	Fars	1760	29°71'26"N	54°13'57"E	20.50	300	2.80	7.22	35.9	Sandy loam

^a: Average temperature ^b:Average annual Rainfall ^c: Electrical conductivity ^d: Relative humidity ^e: Soil texture

Meteorological information was obtained from the nearest meteorology station within the study area and the surrounding zone; each value in the mean of 10 years data. Physical and chemical Soil characteristics are based on average of three samples taken from each region.

Table S2. Essential oil constituents of *M. hedgei* growing wild and cultivated in Iran

No	Compound	RI ^a	% Bokhoon (Natural habitat)	% Estahban (Cultivated plants)
1	α -Pinene	932	0.49	0.17
2	Sabinene	972	1.10	0.47
3	β -Pinene	976	2.90	1.02
4	Myrcene	990	0.46	0.37
5	δ -3-Carene	1010	0.10	0.05
6	α -Terpinene	1016	0.06	0
7	p-Cymene	1023	0.36	0.07
8	Limonene	1027	1.81	1.15
9	1,8-Cineole	1030	0.07	0.05
10	(Z)- β -Ocimene	1036	0.07	0.07
11	Benzene acetaldehyde	1042	0.08	0
12	(E)- β -Ocimene	1046	0.34	0.76
13	γ -Terpinene	1057	0.36	0.08
14	<i>cis</i> -Sabinene hydrate	1066	0.08	0.13
15	<i>trans</i> -Linalool oxide	1072	0.11	0.04
16	<i>cis</i> -Linalool oxide	1089	0.10	0.09
17	Linalool	1099	2.23	2.04
18	n-Nonanal	1104	0.26	0.15
19	<i>trans</i> -Pinocarveol	1138	0.60	0.19
20	Menthone	1154	0.51	0.36
21	Pinocarvone	1163	0.21	0.25
22	Rosefuran epoxide	1175	0.19	0
23	Terpinene-4-ol	1177	0.17	0.33
24	α -Terpineol	1191	0.46	0.42
25	Myrtenol	1197	0.42	0.24
26	<i>trans</i> -Carveol	1219	0.10	0.11
27	Nerol	1228	7.69	6.02
28	Neral	1241	13.81	15.99
29	Carvone	1244	0.27	0.13
30	Geraniol	1255	13.15	10.74
31	Geranial	1270	18.04	22.68
32	Thymol	1292	3.13	3.63
33	Carvacrol	1300	6.20	5.27
34	α -Cubebene	1349	0.13	0.32
35	Eugenol	1358	0.23	0.17
36	α -Copaene	1376	2.20	2.29
37	Geranyl acetate	1385	5.79	3.06
38	β -Cubebene	1390	0.28	0.56
39	(E)-Caryophyllene	1419	3.80	6.52
40	α -Humulene	1453	3.27	6.39
41	Germacrene D	1480	0.32	0.12
42	(E)- β -Ionone	1486	0.10	0.11
43	(E,E)- α -Farnesene	1509	0.09	0.14
44	δ -Cadinene	1523	0.93	1.56
45	α -Calacorene	1543	0.13	0.14
46	Spathulenol	1577	0.18	0.09
47	Caryophyllene oxide	1582	4.73	3.88
48	n-Hexadecane	1599	0.33	0.27
49	Humulene epoxide II	1608	1.35	1.02
Monoterpene hydrocarbons			8.05	4.21
Oxygenated monoterpenes			73.9	72.09
Sesquiterpene hydrocarbons			11.15	18.04
Oxygenated Sesquiterpene			6.26	4.99
Others			0.43	0.38
Total			99.79	99.71

	Essential Oil Yield (%W/W)	1.12 ±0.13 b	2.23±0.16 a
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^a:RI, retention indices in elution order from HP-5 column. Data expressed as percentage of total. Essential oil yield was obtained by calculating the average of three experiments ±standard deviation. Essential oil yield was obtained by calculating the average of three experiments ±standard deviation.

Table S3. Essential oil constituents of some *Micromeria* species

Species	Main components	References
<i>M. hedgei</i>	geranial (18.04 and 22.68%), neral (13.81 and 15.99%), geraniol (13.15 and 10.74%), nerol (7.69 and 6.02%), E-caryophyllene (6.52- 3.80%), carvacrol (6.20 and 5.27%), isomenthone, <i>cis</i> -piperitone oxide and pulegone	Present study
<i>M. dichodontha</i>	β-caryophyllene (42.56%)	(Baser et al., 1992)
<i>M. myrtifolia</i>	pulegone (57.2%, 81.3% and 39.6%)	(Özek et al, 1992)
<i>M. fruticosa</i>	pulegone (32.8%) and piperitenone (25.7%)	(Kirimer, 1992).
<i>M. thymifolia</i>	piperitenone oxide (41.8% and 38.7%) and pulegone (15.9% and 13.4%)	(Marinkovic et al., 2002)
<i>M. albanica</i>	Carvacrol	(Marinkovic et al., 2002).
<i>M. juliana</i>	α-pinene (7.2-10.6%), β-pinene (4.9-7%), linalool (4.7-7.6%), β-caryophyllene (2.5-4.2%), α-gurjunene (2.1-6.4%).	(Phokas et al., 1980).
<i>M. juliana</i>	caryophyllene oxide (17.0%), epi-α-bisabolol (12.8%) and linalool (18.1%) and β-caryophyllene (12.5%)	(Mastelic et al., 2005)
<i>M. graeca</i>	isoeugenol (31.5%)	(Tzakou and Couladis, 2001).
<i>M. sinaca</i>	linalool (15.2%), α-pinene (15.0%) and (E)-nerolidol (13.8%)	(Hawary et al., 1991).
<i>M. persica</i>	Pulegone (51.7%), menthone (20.9%) and neomenthol (11.9%)	(Masoudi et al, 2009).
<i>M. brownei</i>	piperitenone (50.6%) and pulegone (29.2%)	(Tucker et al., 1992).
<i>M. fruticosa</i>	thymol (33.1% and 28.6%), γ-terpinene (28.7% and 17.5%), limonene (5.0% and 20.7%), 1,8-cineole (14.2% and 0.2%) and <i>p</i> -cymene (7.0% and 17.5%)	(Gulluce et al., 2004).
<i>M. persica</i>	piperitenone oxide (40–45%), pulegone (9.7–11.8%) and verbenone (8.3–9.4%)	(Sefidkon and Kalvandi 2005).
<i>M. congesta</i>	camphor (9-15%), caryophyllene oxide (4-6%), and <i>trans</i> -verbenol (4-6%)	(Kirimer et al., 1991).
<i>M. cristata</i>	neral (25.3–32.2%) and geranial (26.7–41.3%)	(Tabanca et al., 2001).
<i>M. biflora</i>	borneol (26.0%)	(Mallavarapu et al, 1997).
<i>M. carminea</i>	piperitenone oxide (44%)	(Baser et al., 1995).
<i>M. albanica</i>	verbenol (11.8%), thymol (10.8%) and caryophyllene oxide (10.5%)	(Stojanovic et al., 1999).
<i>M. juliana</i>	Caryophyllene oxide	(Stojanovic et al., 2006).
<i>M. croatica</i>	Pulegone (64.10-66.55%)	(Kremer et al., 2012).
<i>M. cilicica</i>		(Duru et al., 2004)

Table S4. Antimicrobial activity of the essential oil of *M. hedgei*

Microorganism	Plant condition				Standard antibiotics			
	Natural habitat		Cultivated plants		Tetracycline (30 µg/disk)	Nystatin (30 µg/disk)	Ketoconazol (20 µg/disk)	Gentamicin (30 µg/disk)
	IZ ^a	MIC ^b	IZ	MIC				
<i>C. albicans</i>	17 b	3.12 a	21 b	1.56 a	---	17	21	---
<i>S. aureus</i>	19 a	1.56 b	25 a	0.78 b	20	---	20	---
<i>S. epidermidis</i>	19 a	1.56 b	24 a	0.78 b	30	---	18	---
<i>E. coli</i>	18 a	1.56 b	23 a	0.78 b	---	---	---	22
<i>B. cereus</i>	19 a	1.56 b	25 a	0.78 b	20	---	---	---

^a Diameter of inhibition zones (mm) including diameter of sterile disk (6 mm). Essential oil was tested at 10 µL/disk for each tested microorganism. Each value in the table was obtained by calculating the average of three experiments. Values followed by the same letter under the same row, are not significantly different ($p > 0.05$).

^b Minimum inhibitory concentration, values as µg/mL. Lower MIC values indicated the highest antimicrobial activity.

Figure S1. *Micromeria hedgei* growing wild (left) and cultivated (right) in Iran

Natural habitat

Cultivated plant

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