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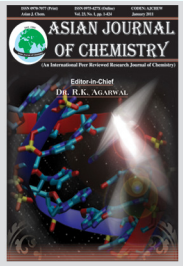

Microwave Assisted Essential Oil Analysis and Antimicrobial Activity of *M. alpestris* subsp. *alpestris*

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The present work describes the chemical composition and antimicrobial activity of the essential oil of *Myosotis alpestris* F.W. Schmidt subsp. *alpestris* (Boraginaceae). The essential oil was obtained from all parts of the plant by microwave distillation and analyzed by GC-FID and GC-MS. Forty-seven components representing 88.2 % of the total oil were characterized and the main components of this species were found to be *n*-nonanal (11.8 %), decanal (10.8 %), *n*-octanal (10.7 %), hexahydrofanesyl acetone (6.6 %), *o*-cymene (3.9 %), tetradecanal (3.7 %), eicosanol (2.2 %) and E-β-farnesene (2.0 %). The isolated essential oils of *M. alpestris* subsp. *alpestris* was tested for antimicrobial activity against the bacteria *Escherichia coli*, *Yersinia pseudotuberculosis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Bacillus cereus*, *Mycobacterium smegmatis* and the fungus *Candida albicans* and *Saccharomyces cerevisiae* at maximum essential oil concentration in hexane of 1000 µg/mL and they showed only antibacterial activity against fungus bacteria.

Key Words: *Myosotis alpestris* subsp. *alpestris*, Microwave, Essential oil, Antimicrobial activity, GC-MS.

INTRODUCTION

Myosotis L. (Boraginaceae) represented with more than 25 taxa, one of which endemic, in Turkish Flora[1,2]. It is a widespread genus in the world with annual and perennial members. *Myosotis alpestris* F.W. Schmidt is represented with a subspecies (*M. alpestris* subsp. *alpestris*) in Turkey. It is quite variable species with many local forms which greatly influenced by the habitat conditions. This subspecies is widespread in Turkey on rocks, slopes, earthy or grassy places (1000-3500 m)[1]. In Anatolia, whole plant material has been used as aphrodisiac and reported to have a healing effect on eye diseases[3].

Essential oils in plant are complex mixtures of volatile compounds, which are present at low concentrations. Before such substances can be analyzed, they have to be extracted from the plant. Several extraction processes have been used and recently, a microwave distillation has been developed for extracting volatile products[4-7].

Previous phytochemical studies on the genus Myosotis species have shown the isolation and identification of a number of compounds, such as alkaloids, anthocyanins, phenolcarboxylic acids and flavonoids[8-10]. In our best of knowledge, no published record has been found for the volatile chemical composition and antimicrobial activity of the essential oil of *M. alpestris* subsp. *alpestris*. Hence, the systematic research was carried out by the microwave extraction of the essential oil constituents of the whole plant in a modified Clevenger-type apparatus.

EXPERIMENTAL

Myosotis alpestris F.W. Schmidt subsp. *alpestris* (Boraginaceae) was collected in Karadag, Akçaabat-Trabzon, on the grassy places (at heights of *ca.* 1650 m) in the north-eastern part of Turkey in May, 2009. The plant was authenticated by Terzioglu[1,2]. Voucher specimen was deposited in the Herbarium of the Faculty of Forestry, KATO (KATO: 12463), Karadeniz Technical University, Turkey.

Microwave distillation apparatus and procedure: Microwave distillation[4-7] was performed at atmospheric pressure with a Milestone DryDIST microwave apparatus using a fixed power of 600 W at 110 ºC (45 min). Temperature was monitored by an external Infrared (IR) sensor. The fresh whole plant (120 g) of *M. alpestris* subsp. *alpestris* was grounded into small pieces, then placed in a round bottom

flask (2 L) with 50 mL water and submitted to microwave distillation using a modified clevenger-type apparatus (cooling column outside the apparatus) with cooling bath (-15 ºC) system (45 min) (yield (v/w): 0.06 %). The obtained oils were extracted with HPLC grade *n*-hexane (0.5 mL) and dried over anhydrous sodium sulphate and stored at -5 ºC in a sealed brown vial.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis: GC-FID and GC-MS analyses were done as described previously[11].

Antimicrobial activity assessment: All test microorganisms were obtained from the Hifzissihha Institute of Refik Saydam (Ankara, Turkey) and were as follows: *Escherichia coli* ATCC 25922, *Yersinia pseudotuberculosis* ATCC 911, *Pseudomonas aeruginosa* ATCC 43288, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 43251, *Bacillus cereus* 702 Roma, *Mycobacterium smegmatis* ATCC607, *Candida albicans* ATCC 60193 and *Saccharomyces cerevisiae* RSKK 251.

Agar well diffusion method: Simple susceptibility screening test using agar-well diffusion method[12] as adapted earlier[13] was used. Each bacterium was suspended in Mueller Hinton (MH) (Difco, Detroit, MI) broth. The yeast like fungi was suspended in yeast extracts broth. Then the microorganisms were diluted approximately 10^6 colony forming unit (cfu) per mL. For yeast like fungi, Sabouraud Dextrose Agar (SDA) (Difco, Detriot, MI) were used. They were "flood-inoculated" onto the surface of Mueller Hinton agar and Sabouraud Dextrose agar agars and then dried. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer and 50 µL of the extract substances were delivered into the wells. The plates were incubated for 18 h at 35 ºC. The *Mycobacterium smegmatis* was grown for 3-5 days on Mueller Hinton agar plates at 35 ºC[14]. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Ampicillin (10 µg), streptomycin (10 µg) and fluconazole (5 µg) were standard drugs.

RESULTS AND DISCUSSION

The essential oil which was extracted by microwave was investigated by GC-FID and GC-MS technique with HP-5 column[15-22]. A total of 47 compounds were identified in the oil of *M. alpestris* subsp. *alpestris* on the basis of a typical library search (NIST, WILEY), reference compounds (*n*-undecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, *n*-octadecane, *n*-nonadecane, *n*-eicosane, *n*-docosane, *n*-tricosane, *n*-tetracosane and *n*-pentacosane) and literature data[10-14] with selecting only components showed matches exceeding 85 %, which represented about 88.2 % of the essential oils in *M. alpestris* subsp. *alpestris*. The general chemical profile of the essential oils, the percentage content and retention indices of the constituents are summarized in Table-1. The main components of the oil were *n*-nonanal (11.8 %), decanal (10.8 %), *n*-octanal (10.7 %), hexahydrofanesyl acetone (6.6 %), *o*-cymene (3.9 %), tetradecanal (3.7 %), eicosanol (2.2 %) and E-β-farnesene (2.0 %).

In addition, 14 terpenoid compounds from the oil of *M. alpestris* subsp. *alpestris* were detected. Some of them could be readily identified by their characteristic mass spectra in the oil[15]. Hexahydrofanesyl acetone (6.6 %), *o*-cymene (3.9 %), E-β-farnesene (2.0 %), *cis*-phytol (1.5 %), 3Z-cembrene A (1.1 %), verbanone (1.0 %) and spathulenol (1.0 %) were major terpenoids with the total ratio of 21.6 %.

TABLE-1
IDENTIFIED COMPONENTS IN THE ESSENTIAL OIL OF *M. alpestris* subsp. *alpestris*

Exp. RI[a]	Lit. RI	Compounds	Area[b] (%)
958	960	Benzaldehyde	1.1
988	990	Decene	0.3
1000	999	*n*-Octanal	10.7
1027	1026	*o*-Cymene	3.9
1043	1042	Benzene acetaldehyde	5.1
1070	1070	*cis*-Sabinene hydrate	0.2
1071	1068	*n*-Octanol	1.0
1101	1100	Undecane[c]	0.2
1103	1101	*n*-Nonanal	11.8
1162	1162	2E-Nonanal	2.6
1203	1202	Decanal	10.8
1212	1209	Verbanone	1.0
1247	1245	Carvacrol methylether	2.2
1300	1300	Tridecene	0.6
1399	1400	Tetradecane[c]	0.4
1409	1409	Dodecanal	0.7
1458	1457	E-β-Farnesene	2.0
1485	1489	E-β-Ionene	0.2
1497	1500	α-Muurolene	0.2
1500	1500	Pentadecane[c]	0.2
1512	1510	Tridecanal	0.3
1578	1578	Spathulenol	1.0
1580	1583	Caryophyllene oxide	0.6
1599	1600	Hexadecane[c]	0.3
1613	1613	Tetradecanal	3.7
1653	1653	α-Cadinol	0.4
1677	1676	*trans*-Asarone	0.7
1711	1714	Pentadecanal	3.2
1761	1760	Benzyl benzoate	0.2
1774	1774	Pentadecanol	0.1
1800	1800	Octadecane[c]	0.3
1815	1815	Hexadecanal	1.2
1846	1847	Hexahydrofanesyl acetone	6.6
1890	1891	Ethyl linoleate	0.9
1899	1900	Nonadecane[c]	0.3
1950	1949	3Z-Cembrene A	1.1
1990	1991	Eicosene	0.3
2000	2000	Eicosane[c]	0.4
2098	2100	Heneicosane	0.7
2115	2113	cis-Phytol	1.5
2199	2200	Docosane[c]	1.3
2215	2218	Neophytadiene	0.2
2222	2225	Z,Z-9,12-Octadecadienoic acid	0.6
2283	2284	Eicosanol	2.2
2298	2300	Tricosane[c]	1.9
2400	2400	Tetracosane[c]	0. 4
2500	2500	Pentacosane[c]	2.6
Total isolate			88.2

[a]Compounds are listed in order of elution. RI (retention index) values are calculated from retention times relative to that of *n*-alkanes (C_6-C_{32}) on the non-polar HP-5 column. [b]Percentages obtained by FID peak-area normalization. [c]Identified by authentic samples.

The chemical class distribution of the essential oil components are reported in Table-2. The compounds are classified into four classes, which are terpenoids, aldehydes, hydrocarbons and others (Table-2). The qualitative and quantitative determination of essential oil of *M. alpestris* subsp. *alpestris* showed

TABLE-3
SCREENING FOR ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL IN *M. alpestris* subsp. *alpestris* (50 µL)

Sample	Conc. (µg/mL)	Microorganisms and inhibition zone (mm)									
		Ec	Yp	Pa	Sa	Ef	Li	Bc	Ms	Ca	Sc
M. alpestris subsp. *alpestris*	1000	–	–	–	–	–	–	–	–	6	10
Ampicillin	10	10	18	18	35	10	10	15	–	–	–
Streptomycin	10	–	–	–	–	–	–	–	35	–	–
Fluconazole	5	–	–	–	–	–	–	–	–	25	> 25

Ec: *Escherichia coli*, Yp: *Yersinia pseudotuberculosis*, Pa: *Pseudomonas aeruginosa*, Sa: *Staphylococcus aureus*, Ef: *Enterococcus faecalis*, Li: *Listeria monocytogenes*, Bc: *Bacillus cereus*, Ms: *Mycobacterium smegmatis*, Ca: *Candida albicans*, Sc: *Saccharomyces cerevisiae*, (–): no activity.

TABLE-2
CHEMICAL CLASS DISTRIBUTION IN THE ESSENTIAL OIL OF *M. alpestris* subsp. *alpestris*

Constituents	Area (%)	NC[a]	Major component	Area (%)	RI
Terpenoids					
Monoterpene hydrocarbon	3.9	1	*o*-Cymene	3.9	1027
Oxygenated monoterpenes	1.2	2	Verbanone	1.0	1212
Sesquiterpene hydrocarbons	2.2	2	E-β-Farnesene	2.0	1458
Oxygenated sesquiterpenes	2.7	4	Spathulenol	1.0	1578
Diterpene hydrocarbon	1.1	1	3Z-Cembrene A	1.1	1951
Oxygenated diterpene	1.5	1	*cis*-Phytol	1.5	2115
Terpene related compounds	9.0	3	Hexahydrofanesyl acetone	6.6	1846
Aldehydes	51.2	11	*n*-Nonanal	11.8	1103
Hydrocarbons	10.4	16	Pentacosane	2.6	2500
Others	5.0	6	Eicosanol	2.2	2283
Total	88.2	47			

[a]NC: Number of compounds.

that major constituents were aliphatic aldehydes (51.2 %), terpenoids (21.6 %) and hydrocarbons (10.4 %). Generally, the number of volatile hydrocarbon compounds is greater than other volatiles present in the oil of *M. alpestris* subsp. *alpestris*.

The antimicrobial activity for the essential oil of *M. alpestris* subsp. *alpestris* was tested *in vitro* using the agar-well diffusion method[12-14] with the microorganisms as seen in Table-3 at maximum essential oil concentration in hexane of 1000 µg/mL, by using ampicillin and fluconazole as standard antibacterial and antifungal agents[31,32]. The test extracts showed antimicrobial activity against the fungus *C. albicans* and *S. cerevisiae*, but no antimicrobial activity was observed against the bacteria *E. coli*, *Y. pseudotuberculosis*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, *L. monocytogenes*, *B. cereus* and *M. smegmatis* (Table-3).

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