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Mentha L. essential oils composition and in vitro antifungal activity

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ABSTRACT: *The essential oils isolated by hydro-distillation from the leaves of wild growing Mentha piperita and Mentha spicata (Lamiaceae) at the region of Mariovo, Republic of Macedonia were analyzed by gas chromatography with flame ionization detector (GC-FID) and gas chromatography wit mass selective detector (GC-MS). A total of forty six and thirty two different components were identified in the essential oils obtained from M. piperita and M. spicata, respectively, constituting approximately >99% (w/w) of the oils. The major components in the essential oil of M. piperita were menthol (34.3%), L-menthone (18.24%) and isomenthone (5.16%), neoisomenthol (3.48%), pulegone (3.03%) and menthyl acetate (3.01%). The major components in the essential oil of M. spicata were carvone (61.4%) ; limonene (11.87%) and 1, 8 – cineol (5.21%). The antifungal activity of the oils was tested by disc diffusion method and the micro-dilution broth method (MIC) against six plant pathogenic fungi: Alternaria alternata Alternaria solani, Aspergillus flavus, Aspergillus niger, Fusarium solani and Rhizopus solani. The results from the disc diffusion method followed by MIC indicated that M. spicata essential oil showed maximum antifungal activity with larger inhibition zone (20 – 29 mm) and the smallest MIC values (65.8 – 120.3 µg mL-1) against all the strains tested. M. piperita essential oil exhibited good antifungal activity with inhibition zone of 19 and 20 mm and MIC values of 120.3 and 115.4 µg mL^{-1}, respectively against Fusarium solani and Aspergilus flavus and excellent antifungal activity with inhibition zone of 28 and 30 mm and MIC values of 65.4 and 50.6 µg mL^{-1}, respectively against Aspergilus niger and Rhizopus solani.*

Keywords: *Essential oil, hydro-distillation, fungi, gas chromatography*

I. INTRODUCTION

Mints comprise a group of species of the genus *Mentha* which belong to the family Lamiaceae [1]. The genus *Mentha* included more than 25 species, grows widely throughout the certain regions of the world [2]. *Mentha arvensis, M. piperita, M. longifolia* and *M. spicata*, commonly known as menthol mint, peppermint, wild mint and spearmint, respectively, are frequently cultivated in many countries of East Asia, Europe, America and Australia form the production of essential oils [3]. The essential oils and extracts from *Mentha* species have been in use since ancient times for the treatment of many digestive tract diseases and in cuisines [4].

The essential oils of some *Mentha* species, including *M. piperita, M. spicata*, *M. arvensis* and *M. longifolia* have antimicrobial, antioxidant, radical-scavenging and cytotoxic activities [5, 6]. Such multiple biological activities of *Mentha* essential oils are mainly due to the presence of various chemical components, such as menthol, menthone, piperitone oxide, camphor and linalool [7-9].

Mentha species grow on the whole territory of the Republic of Macedonia, mainly as wild plants [10]. *M. piperita* and *M. spicata* are the most abundant species of the genus *Mentha* in the Republic of Macedonia. Many experiments have been conducted for chemical characterization of *M. piperita* and *M. spicata* essential oils in various parts of the world [3, 11-15].

However, no earlier reports are available on the detailed chemical composition of the essential oils from *M. piperita,* and *M. spicata* native to the territory of the Republic of Macedonia. This motivated us to investigate the chemical composition of essential oils obtained from leaves of *M. piperita* and *M. spicata* from the region of Mariovo in the Republic of Macedonia. Antifungal activity of the essential oils against several strains of plant pathogenic fungi was also investigated.

II. MATERIALS AND METHODS

2.1. Sampling

The aerial part of *M. piperita* and *M. spicata* was collected in July 2014 at the mountainous area of Mariovo (Fig.1). Mariovo area is located at the farthest southern part of Macedonia with the coordinates 41°7'20"N 21°48'12"E. The climate is moderate continental with the average annual temperature of 13.9 ^{0}C and precipitation of 7. The average T in July was 21.3 ^{0}C (15 ^{0}C – 27 ^{0}C). Plants were collected from the wield fields at the altitude of 1050 m above the sea level. The plant specimens were identified and authenticated by Dr Mitko Karadelev, taxonomist, of the Department of Botany, University of Agriculture, Skopje, Republic of Macedonia. Further authentication was made by comparison with authentic vouchers of *M. piperita* (12097) and *M. spicata* (23098) deposited in the Herbarium of the Botany Department of the University of Natural Science, Skopje, Republic of Macedonia. The leaf samples were dried at 30 ^{0}C in hot air oven (HERA-therm, Thermo Fisher Scientific, USA) to constant weight.

Figure1. *Sampling area*

2.2. Chemicals

Acetone, homologous series of C9 – C_{24} *n* – alkanes and various reference chemicals used in this study were obtained from Sigma Chemical Co. (St. Lous, USA). All other chemicals (analytical grade) used in this study were purchased from Merck (Darmstadt, Germany).

2.3. Extraction of essential oils

The dried leaves of *M. piperita* and *M. spicata* were grounded prior to the operation and than 100 g of samples were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus [8]. The distilled essential oils were dried over anhydrous sodium sulfate, filtered and stored at 4 ^{0}C until analysis. The yields (g/kg) of the oils were calculated on a moisture free basis.

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2.4. Gas chromatography

2.4.1. Gas chromatography with flame ionization detection (GC-FID)

The essential oils were analysed using a gas chromatograph (2010, Shimadzu, Japan) equipped with flame ionization detector (FID), auto injector (AO 20i) and ZB-5 MS capillary column (30 m x 0.25 mm x 0.25 μm). Injector and detector temperatures were set at 250 ^{0}C and 280 ^{0}C, respectively. Column oven temperature was programmed from 40 ^{o}C to 240 ^{o}C at a rate of 5 ^{0}C /min; initial and final temperatures were held for 1 and 10 min, respectively. The total analysis time was 39 min. Nitrogen was used as a carrier gas with a flow rate of 1.0 mL/ min. A sample of 1.0 μL in *n*-hexane (0.5 mg/mL) was injected using the split mode (split ratio 1: 100). The composition of the components was reported as relative percentage of the total peak area.

2.4.2. Gas chromatography with mass selective detection

GC-MS analysis of the essential oils was performed using a gas chromatograph (2010 plus, Shimadzu, Japan), equipped with a Shimadzu QP-2010 mass selective detector and AOC 5000 auto-sampler (Shimadzu). Compounds were separated on a ZB-5 MS capillary column (30 m x 0.25 mm x 0.25 μm). A 1.0 μL sample was injected in the split mode, with a split ratio of 1:100. An electron ionization system with ionization energy of 70 eV was used. Column oven temperature programme was the same as in GC-FID analysis. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. Mass range was 45–550 *m/z*, while the injector (PTV) and MS transfer line temperatures were set at 250 and 280 °C, respectively. The constituents of the oil were identified by using standard reference compounds and also by matching the mass spectra fragmentation pattern with NIST Mass Spectra Library stored in the GC–MS database.

2.5. Antifungal activity of essential oils

Mentha essential oils were individually tested against six strains of pathogenic fungi: *Alternaria alternata Alternaria solani*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani* and *Rhizopus* solani. The pure fungal strains were obtained from the Microbiology Division of the Institute of Public Health, Skopje, Republic of Macedonia. The isolates were collected from the diseased samples and were cultured for 16 hours at 30^0C on potato dextrose agar (PDA, Oxoid). Purity and identity were verified by the Institute of Microbiology and Parasitology, Faculty of Medicinal Science, Skopje, Republic of Macedonia.

2.5.1. Antifungal activity testing methods

2.5.1.1. Disc diffusion method

The antifungal activity of the *Mentha* essential oils and the principal components menthol, menthone, carvone, limonene and 1,8-cineole were determined by the disc diffusion method [16]. Briefly, 100 μL of suspension in PDA containing 10^4 colony-forming units (CFU) mL^{-1} of fungal spores were spread on PDA medium. The paper discs (6 mm in diameter) were separately impregnated with 15 μL of essential oils or main components and placed on the agar, which had previously been inoculated with the selected test fungi. Posaconazole (30 μg per disc) was used as a positive reference for fungi, while the discs without samples were used as a negative control. Plates, after 1 h at 4 ^{0}C were incubated at 30 ^{0}C for 48 h for fungal strains. Antifungal activity was assessed by measuring the diameter of the growth inhibition zone (IZ) in millimetres including disc diameter of 6 mm for the test organisms compared to controls.

2.5.1.2. Micro-dilution broth method

For minimum inhibitory concentration (MIC), a micro-dilution broth susceptibility assay was used [17]. Micro dilution broth test was performed in sabouraud dextrose broth (SDB, Oxoid). Essential oils were solubilised in dimethylsulfoxide (DSMO), and then diluted in culture media for use. Dilution series were prepared from 0.01 to 30.0 mg mL^{-1} of the essential oils or their chief components in a 96-well micro-titre plate, including one growth control, solvent and one sterility control. 160 μL of sabouraud dextrose broth were added to micro-plates and 20 μL of test solution, respectively. Then 20 μL of 5 x 10^5 cfu mL^{-1} of standard fungal suspension were inoculated onto micro-plates. The plates were incubated at 30 ^{0}C for 48 h. Posaconazole was used as a reference compound for antifungal activities. MIC was calculated as the highest dilution (the lowest concentration of antifungal compound) showing complete inhibition of the tested strains.

2.6. Statistical analysis

All the experiments were conducted in triplicate and the data are presented as mean value ± standard deviation (SD) of triplicate determinations. Statistical analysis of the data was performed by the analysis of variance (ANOVA) followed by Tukey's test with probability level < 0.05 using the Statistical Analysis System (SAS, 2004).

III. RESULTS AND DISCUSSION

3.1. Oil composition

The yield of essential oils was found to be 12.2 g/kg for *M. spicata* and 12.4 g/kg for *M. piperita*, respectively. Our results are in line with those of Hussain *et al.* which reported similar essential oil yield for *M. spicata* (12 g/kg) and *M. piperita* (12.2 g/kg) obtained from cultivated fields in the area of Faisilabad, Pakistan from summer harvest [3]. Milic *et al.* reported higher yield for *M. piperita* essential oil (31.9 g/kg) obtained from *M. piperita* from the cultivated fields in Novi Sad, Serbia [11].

The components found in the essential oils of *M. piperita and M. spicata* are reported in Table 1. We have identified a total of 46 and 32 compounds in essential oils of *M. piperita* and *M. spicata*, respectively. The main constituents in the essential oils of *M. piperita* (>5%) were found to be oxygenated monotherpenes: menthol

(34.3%), L-menthone (18.24%) and isomenthone (5.16%); followed by neoisomenthol (3.48%), pulegone (3.03%) and menthyl acetate (3.01%). The main constituents (>5%) in the essential oils of *M. spicata* were found to be oxygenated monotherpenes: carvone (61.4%) and 1, 8 – cineol (5.21%). Limonene (11.87%) was found to be the most abundant monoterpene hydrocarbon in *M. spicata* essential oil. Twenty components were identified as sesquiterpene hydrocarbons in essential oil of *M. piperita* with a total amount of 10.89% (*w/w*) and seven components in essential oil of *M. spicata* with the total amount of 3.94% (*w/w*), respectively. Oxygenated sesquiterpenes were found in low amounts (< 2 %, *w/w*) in the essential oils obtained from the both *Mentha* species.

In their study Sokovic *et al.* [12] identified 26 components in the essential oil of *M. piperita* with the predominant presence of menthol (37.4%), menthyl acetate (17.4%) and menthone (12.7%). They identified 24 components in the essential oil of *M. spicata* with the predominant presence of carvone (49.5%), menthone (21.9%) and limonene (5.8%). Carvon (59.5%), limonene (10.4%) and 1,8-cineol (6.36%) were the main components of the essential oil of *M. spicata* cultivars from Faisalabad, Pakistan, harvested in the summer period [3]. Menthol (64%), menthyl acetate (9.2%) and menthofuran (8.4%) were found to be the main components in the essential oil of *M. piperita* cultivated in Italy [13]. Contrary to these findings, Yadegarina *et al*. found out considerably differences in the chemical composition of the essential oils of *M. piperita* from east Iran[14]. Namely the main components of the *M. piperita* essential oil were found to be: α-terpinene (19.7%), isomenthone (10.3%), trans-carveol (14.5%), piperitenone oxide (19.3%), and *β*- caryophyllene (7.6%)

There are some reports on the variation in the chemical composition of the essential oils with respect to season [3]. The influence of phenological status and environmental conditions can influence the regulation of the biosynthesis of essential oils [18].

Reports found in the literature for the essential oil composition of M. piperita and M. spicata referred to the cultivated crops [3,7-9,11-15], but we could not find a single report showing the essential oil composition of wild growing M. piperita and M. spicata and biological activity of these two wild grown Mentha species.

Table 1. Composition of the essential oils from leaves of two Mentha species

Monoterpene hydrocarbons		**Composition (%, w/w)**	
Component	**RI**	***M. piperita***	***M. spicata***
α – Pinene	928	2.03 ± 0.23	0.06 ± 0.02
Camphene	950	0.22 ± 0.07	-
β – Pinene	971	2.03 ± 0.43	0.04 ± 0.02
β – Myrecene	989	0.34 ± 0.03	-
p – Cymene	1020	0.41 ± 0.09	-
Limonene	1024	4.54 ± 0.22	11.87 ± 0.45
Oxygenated monoterpenes			
1,8 – Cineol	1028	1.15 ± 0.20	5.21± 0.52
Cis- Sabinene hydrate	1068	-	0.12 ± 0.03
Linalool	1088	0.98 ± 0.24	1.14 ± 0.42
Isopulegol	1140	0.44 ± 0.14	0.01 ± 0.04
L -Menthone	1149	18.24 ± 1.9	2.10 ± 0.24
Isomenthone	1159	5.16 ± 1.1	-
Borneol	1163	-	2.46 ± 0.24
Menthol	1170	34.3 ± 1.5	0.76 ± 0.15
Terpinene -4 -ol	1174	1.82 ± 0.23	0.32 ± 0.09
Neoisomenthol	1184	3.48 ± 0.76	-
α-Terpineol	1186	2.45 ± 0.44	1.05 ± 0.23
Dihydrocarveol	1193	-	1.95 ± 0.22
γ-Terpineol	1195	2.15 ± 0.18	-
cis- Dihydrocarvone	1198	-	2.12 ± 0.34
trans- Dihydrocarvone	1200	-	0.19 ± 0.06
trans – Carveol	1214	-	0.29 ± 0.05
cis – Carveol	1227	0.11± 0.03	1.95 ± 0.09
Pulegone	1235	3.03 ± 0.18	-
Carvone	1240	0.65 ± 0.11	61.4 ± 1.80
Carvon oxide	1242	-	0.18 ± 0.03
Bornyl acetate	1286	-	0.30 ± 0.04

Menthyl acetate	1298	3.01 ± 0.18	-
Isopulegyl acetate	1260	0.22 ± 0.02	-
Piperitenone	1340	0.12 ± 0.01	-
cis-Carvyl acetate	1365	-	0.18 ± 0.03
Sesquiterpene hydrocarbons			
α-Ylangene	1369	0.28 ± 0.03	-
α - Copaene	1375	0.35 ± 0.07	-
β – Bourbonene	1384	1.45 ± 0.12	0.82 ± 0.15
β – Elemene	1388	0.88 ± 0.11	-
cis - Jasmone	1390	0.22 ± 0.04	-
Longifolene	1406	0.35 ± 0.02	-
β – Caryophyllene	1418	1.18 ± 0.22	1.15 ± 0.12
β – Cubebene	1420	0.55 ± 0.05	-
Thujopsene	1423	0.28 ± 0.02	-
Aromadendrene	1438	0.58 ± 0.04	-
α-Caryophyllene	1452	0.55 ± 0.03	0.56 ± 0.04
γ – Muurolene	1477	0.88 ± 0.04	0.22 ± 0.01
Germacrene D	1482	0.51 ± 0.03	0.71 ± 0.06
Ledene	1494	0.48 ± 0.08	-
α – Muurolene	1497	0.68 ± 0.06	-
Cuparene	1503	0.11 ± 0.02	-
Amorphene	1438	0.38 ± 0.04	-
γ-Cadinene	1510	-	0.15 ± 0.02
δ-Cadinene	1521	0.98 ± 0.13	-
α-Cadinene	1537	0.11 ± 0.02	-
Calamenene	1539	-	0.33 ± 0.02
α-Calacorene	1544	0.09±	
Oxygenated sesquiterpenes			
Spathulenol	1575	0.39 ± 0.04	0.11 ± 0.02
Cariophyllene oxide	1580	1.05 ± 0.14	0.82± 0.05
α-Cedrol	1593	0.11 ± 0.02	-
1,10-di-epi-cubenol	1612	-	0.19 ± 0.03
α-Muurolol	1642	-	0.72 ± 0.23
β – Eudesmol	1649	0.12 ± 0.03	-
Total		99.44	99.48
Essental oil content (g/kg)		12.4 ± 1.05	12.2 ± 0.98

3.1. Antifungal activity

The antifungal activities of *Mentha* essential oils and main components were assessed against a panel of plant pathogenic fungi. As seen in Tables 2 and 3, the essential oils of *M. piperita* and *M. spicata* exhibited good antifungal activity against the fungi tested. The results from the disc diffusion method followed by MIC indicated that M. *spicata* essential oil showed maximum antifungal activity with the largest inhibition zones (20 – 29 mm) and the smallest MIC values (65.8 – 120.3 µg mL-1) against all the strains tested. *M. piperita* essential oil exhibited good antifungal activity with inhibition zone of 19 and 20 mm and MIC values of 120.3 and 115.4 µg mL^{-1} , respectively against *Fusarium solani* and *Aspergilus flavus*. *M. piperita* essential oils showed excellent antifungal activity with inhibition zone of 28 and 30 mm and MIC values of 65.4 and 50.6 µg mL^{-1}, respectively against *Aspergilus niger* and *Rhizopus solani*. Among the plant pathogenic fungi tested, *Rhizopus solani* , *Aspergilus* niger and *Aspergilus flavus* were the most sensitive strains. Our results are in good agreement with the findings of Hussain *et al.* [3] who reported that *M. piperita* and *M. spicata* essential oils exhibited good antimicrobial and antifungal activity against a wide range of micro-organisms and fungi. The variation in antifungal activity of Mentha essential oils with respect to species was statistically significant ($p<0.05$) for the srains *Alternaria alternata*, *Alternaria solani* and *Fusarium solani*. This difference could be due to the differences in chemical composition of the oils.

Table 2. Composition of the essential oils from leaves of two Mentha species

Tested fungal strain	*Mentha pirerita*	*Mentha spicata*
Disc diffusion method		
Alternaria alternata	16 ± 1 A	20 ± 1 B
Alternaria solani	13 ± 1 A	26 ± 1 C
Aspergilus flavus	20 ± 2 A	25 ± 2 B
Aspergilus niger	28 ± 1 B	26 ± 2 B
Fusarium solani	19 ± 2 A	25 ± A
Rhizopus solani	30 ± 2 B	29 ± 2AB
Minimum inhibitory concentration (MIC, µg mL^{-1})		
Alternaria alternata	138.6 ± 8.4 D	120.3 ± 6.5 D
Alternaria solani	154.5 ± 6.2 D	76.1 ± 5.2 A
Aspergilus niger	115.4 ± 3.9 C	88.5 ± 3.9 C
Aspergilus flavus	65.4 ± 3.1 A	80.5 ± 4.2 C
Fusarium solani	120.3 ± 4.2 C	97.3 ± 4.4 B
Rhizopus solani	50.6 ± 3.5 B	65.8 ± 3.8 B

*Values are mean ± standard deviation (SD) of three different experiments. Mean values marked with the different letters in the same row represents significant difference at $p < 0.05$, by Tukey's test.

**Diameter of inhibition zone (mm) including disc diameter of 6 mm.

Menthol, menthone, carvone, limonene and 1, 8 - cineole, the major constituents of *M. piperita* and *M. spicata,* were also tested for their potential antifungal activity (Table 3). Posaconazole a triazole antifungal drug was used as a positive reference drug for fungi. Menthol exhibited excellent antifungal activity against *Alternaria solani*, *Alternaria alternata*, *Aspergilus niger* and *Rhizopus solani* (inhibition zone 23 – 31 mm, MIC 36.4 – 76.8 µg mL^{-1}), which was close to the antifungal activity of standard drug for the strains *Alternaria alternata, Alternaria solani* and *Rhizopus solani*. Menthone and carvone showed good antifungal activity against *Aspergilus flavus, Aspergilus niger* and *Rhizopus solani* (inhibition zone 20 – 29 mm, MIC 58.3 – 114.5 µg mL^{-1}). Carvone exhibited better antifungal activity for *Fusarium solani* than menthone. Contrary to these findings limonene and 1, 8-cineol exhibited lower antifungal activity for all tested strains (inhibition zone 10-15 mm, MIC 69.3 – 220.6 µg mL^{-1}).

Table 3. Antifungal activity of pure substances*

Tested fungal strain	Menthol	Menthone	Carvone	1,8-cineol	Limonene	Posaconazole
Disc diffusion method						
Alternaria alternata	26 ± 2 B	17 ± 1 B	21 ± 1A	12 ± 1 A	10 ± 0 A	28 ± 2 C
Alternaria solani	23 ± 2 A	18 ± 1 B	14 ± 2 B	11 ± 1 A	10 ± 0 C	26 ± 2 B
Aspergilus flavus	18 ± 1 B	20 ± 1 A	21 ± 1 A	13 ± 1 A	11 ± 1 A	36 ± 2 C
Aspergilus niger	30 ± 2 A	25 ± 1 AB	26 ± 1 C	14 ± 1 AB	12 ± 1 A	37 ± 1 C
Fusarium solani	29 ± 1 B	23 ±1 AB	29 ± 2 AB	15 ± 0 A	12 ± 1 B	36 ± 2 B
Rhizopus solani	31 ± 1 A	29± 2 A	28 ± 2 A	15 ± 1 C	13 ± 1 A	32 ± 1 C
Minimum inhibitory concentration (MIC, µg mL^{-1})						
Alternaria alternata	76.8 ± 3.5 A	120.6 ± 4.8 C	98.7 ± 5.6 A	180.5 ± 3.5 A	210.8 ± 8.1 B	60.5 ± 2.4 A
Alternaria solani	99.5 ± 1.8 A	127.8 ± 2.6 C	148.6 ± 3.2 B	195.2 ± 4.1 C	220.6 ± 7.3 C	71.4 ± 1.7 A
Aspergilus flavus	115.3 ± 3.8 B	114.5 ± 3.4 BC	102.6 ± 6.9 C	150.8 ± 5.2 D	170.3 ± 5.5 D	10.4 ± 1.3 B
Aspergilus niger	75.4 ± 2.4 C	95.6 ± 4.1 C	80.5 ± 4.5 A	143.4 ± 7.5 B	168.3 ± 6.2 B	10.2 ± 1.8 C
Fusarium solani	65.8 ± 1.8 B	105.4 ± 6.2 D	69.3 ± 3.2 D	130.8 ± 2.8 A	155.6 ± 4.8 C	10.5 ± 2.7 D
Rhizopus solani	36.4 ± 2.6 C	58.3 ± 1.9 B	70 ± 5.5A	129.5 ± 4.2B	149.5 ± 4.3A	12.5 ± 2.9 A

*Values are mean ± standard deviation (SD) of three different experiments. Mean values marked with the different letters in the same row represents significant difference at $p < 0.05$, by Tukey's test.

**Diameter of inhibition zone (mm) including disc diameter of 6 mm

IV. CONCLUSION

The results of the present study indicate that essential oils of *M. piperita* and *M. spicata* possess very good antifungal potential against the plant pathogenic fungi. This is due to the main components of the essential oils: menthol, menthone and carvone. The investigated essential oils of Mentha species may be used for the preservation of the processed foods as well as for pharmaceuticals.

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