



Chemical composition and antimicrobial activity of essential oils from Tunisian *Mentha pulegium* L.

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Abstract

The aim of this study was to describe the chemical composition of Tunisian *Mentha pulegium* L. essential oils from Monastir and to test their antibacterial activity. The essential oils, obtained from fresh and dried aerial parts by hydrodistillation, were analysed by gas chromatography and mass spectrometry. The leaf extracts were used to test the antimicrobial activity against nine hospital bacteria and five reference strains. The minimum inhibitory concentrations and the minimum bactericidal concentrations were determined by sub-culture at TSA agar plates which were incubated at 37°C during 18-24 h. Thirty four compounds were identified. All the oils were found to be rich in oxygen monoterpen hydrocarbons. The oils tested displayed antimicrobial activities. *Aeromonas hydrophila*, *Vibrio cholerae* non-O1 and *Enterococcus faecalis* were the most sensitive strains. Gram-positive strains are more susceptible to the essential oils from the fresh leaves collected at the vegetative state than from the dried ones.

Key words: *Mentha pulegium* L., Lamiaceae, essential oils, chemical composition, antimicrobial activity.

Introduction

Despite the medical discoveries of different medicines and advanced ways of treatment, statistics have shown that the number of patients is increasing in industrial countries as well as in developing ones. This is due to chemical drugs used in healthcare, agriculture and diets. This soaring in medicines urges us to look for natural sources, such as aromatic plants, rich in efficient products.

The family of Lamiaceae (Labiatae) contains an extremely wide variety of aromatic plants mainly in temperate countries. Among this rich array of plants yielding essential oils (EOs), the genus *Mentha* holds pride of place. It is very widely distributed around the Mediterranean basin, in America and in occidental Asia ¹⁻⁴. This genus is represented in Tunisian flora by five species. One of them is *Mentha pulegium* L., interesting plant by its EOs, growing in humid places: on mountains at an altitude about 700 m and on plains. It is a perennial plant that has always green leaves. The flowers, with a corolla typically gamopetalous bilabiate, are grouped into small bunches. The fruit is a small hard tetra-achene. The secretory system is located in the leaves and in the stems ³.

Mentha pulegium L. dried and fresh leaves are largely used in traditional medicine. Infusions and decoctions of aerial parts are used as carminative, digestive, antispasmodic, anti-inflammatory, expectorant and for the treatment of colds, head aches, hepatic injuries and asthma ^{2,5-7}.

Mentha pulegium L. oils have been the subject of considerable

studies. They have antifungal ⁸, insecticidal ⁹, antiparasitic, spasmolytic and antioxidant activities ². The qualitative composition of *Mentha pulegium* L. oils depends on the geographical origin and the specific ecological sites from which plant material is collected for distillation. Also the quantitative composition of oil can vary greatly. For example, essential oils extracted from *Mentha* grown in Uruguay contained isomenthone 38% and pulegone 34% ¹⁰, while pulegone content was 76% in samples from North Carolina ¹¹. Furthermore, essential oils analysed in Germany contained neomenthol 35.4%, menthone 15.3% and isomenthone 10.3% ¹², but piperitone (70%) was the main component in oils of *Mentha* plants grown in Austria ¹³. Oil samples analysed in Netherlands contained pulegone 73.5%, menthone 13.1% and isomenthone 5.0% ¹⁴, while *Mentha pulegium* oil from Cuba contained pulegone 25.14%, neoisomenthol 20.68% and isomenthone 9.73% ¹⁵.

In this study, we described in detail the chemical composition of the *Mentha pulegium* EOs of dried and fresh leaves and stems at the vegetative state and during the flowering and leaf extracts were tested for their antibacterial activity.

Materials and Methods

Plant material: Plant populations of *Mentha pulegium* L. were collected from Monastir, in central Tunisia, in May (vegetative state) and in July (flowering period). Identification was performed

in the biological laboratory of the Faculty of Pharmacy of Monastir according to the flora of Tunisia³. A voucher specimen (M.P-01.05) has been deposited in this laboratory.

Isolation of the volatile oils: A 100 g of each sample of the fresh and the air dried leaves and stems was subjected to a hydrodistillation for three hours with 500 ml of distilled water using a Clevenger-type apparatus. The oil obtained was separated from the distilled water and was dried by anhydrous sodium sulphate. EOs are volatile and therefore are stored in sealed glass vials in a refrigerator at 4-5°C in order to prevent changes in chemical composition.

Essential oil analysis: The composition of essential oils was investigated by GC and GC-MS. GC analysis was performed in a gas chromatograph (HP 5890) using two fused silica capillary columns, HP5 (non-polar) and Innovax (polar) (30 m x 0.25 mm, film thickness 0.25 µm) and a flame ionization detector (FID). Injector and detector temperatures were set at 240°C and 280°C, respectively. The oven temperature programme was 50°C for 3 min, then 50-280°C at 9°C/min and finally 280°C for 3 min. Nitrogen was the carrier gas at a flow rate of 1 ml/min. The samples were injected as 0.1 µl of 1% solution diluted in hexane in the split mode. The percentage of the constituents was calculated by electronic integration of FID peak areas and normalized without the use of response factor correction.

The essential oils were analysed by GC-MS using a HP 5972/A mass spectrometer operating at 70 eV in the same conditions as described above, except that the carrier gas was helium at 20 p.s.i. The identification of compounds was confirmed by comparison of their retention indexes (determined relatively to the retention times of a series of *n*-alkanes) and with those of authentic standards of the Wiley library search routines¹⁶, based on fit and purity of mass spectra¹⁷.

Determination of antibacterial activity

Organisms: Fourteen strains were chosen for investigation of which five were reference bacteria: Gram-positive *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* NCIMB 8166 and Gram-negative *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella enteritidis*, *Serratia marcescens*, *Shigella flexneri* and *Vibrio cholerae* serogroup non-O1. All organisms were stored at -70°C in glycerol Mueller-Hinton broth. Fresh subcultures were used for each experiment.

Diffusion method: The disc diffusion method is used as a preliminary essay for antibacterial activity prior to more detailed studies. The determination of bacterial susceptibility was done by diffusion on Mueller-Hinton agar. The pH of Mueller-Hinton agar was adjusted at 7.2-7.4. Preparation of inoculums and inoculation by flooding method were realized according to the standard method described by Kirby and Bauer. The Mueller-Hinton agar was covered completely by bacterial suspension (2-3 ml), however, excess fluid was removed as entirely as possible by aspiration. The plates were incubated at +37°C during 15 min. Impregnated discs with essential oil were applied to the bacterial

surface. The discs had to adhere completely to the agar surface and should be arranged in such a way that the overlapping of the inhibition zones is avoided and that the distance between the discs and the plate edges does not exceed 15 mm. The plates were inverted and incubated after 15 min at 37°C for 18-24 hours.

MIC and MBC determinations: The minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevents visible growth¹⁸⁻²⁰. The minimum bactericidal concentration (MBC) was determined as a concentration where 99.9% or more of the initial inoculum is killed^{19,21}.

We have tested the leaves EOs isolated at the vegetative state because we can collect them all long the year. Each of them was dissolved in Tween-80 at 5%¹⁹ and 10 µl of each bacterial suspension was inoculated. The MIC and MBC concentrations were determined only on reference strains by serial dilution with Mueller-Hinton (M-H) broth. The CFU/ml was adjusted at (1-5) × 10⁵ CFU/ml¹⁵ in (M-H) for all strains tested. All samples were incubated at 37°C during 18-24 h. To confirm results of MIC and MBC, the experimental suspensions were sub-cultured in TSA agar plates which were incubated at 37°C during 18-24 h²². We have used this method because it is more sensitive than the agar dilution²³.

Results and Discussion

Yields in essential oils: The hydrodistillation of fresh and dried leaves and stems gave colourless oils with yields reported in Table 1. The essential oils content increased at the flowering state. The leaves contained more essential oils than the stems because the secretory system is most abundant in the leaves.

Table 1. Yields in essential oils in relation with the organ and the vegetation stage.

State of plants	Leaves		Stem	
	Fresh	Dried	Fresh	Dried
Vegetative state	1.34	3.58	0.33	0.29
Flowering state	1.50	3.75	0.50	0.75

Composition of essential oils: The general chemical profiles of the tested oils, the percentage content of the individual components, the retention indices and the chemical class distribution of the oil compounds are summarized in Table 2.

In all tested oils, thirty four components were identified which represented 89.60-96.47% of the total detected constituent. The major components of the leaves were menthol (46.60-49.86%), 1,8 cineole (13.53-17.31%), menthone (11.13-12.34%) and pulegone (3.76-4.78%). Other components were present in amounts less than 3% (Table 2). In particular, oxygen monoterpene hydrocarbons were the most abundant compound group (82.18-84.64%).

In the oils of the stems, menthol (40.57-51.61%) was the major component followed by menthone (7.32-20.04%), pulegone (4.08-6.97%) and 1,8-cineole (11.10-18.46%). Similar to leaves oxygen monoterpene hydrocarbons are also the most abundant chemical group. From the obvious results, it is evident there are many qualitative similarities between tested oils, although the amounts of some corresponding compounds are different.

In regard to the previously reported contents of the essential oils, it is interesting to point out that there are no significant quantitative differences between fresh and dried organs and between vegetative and flowering states.

Table 2. Essential oils composition of *Mentha pulegium* L. from Monastir represented in %.

N°	Compound	Retention indices HP-20M	Leaves				Stems			
			Vegetative state		Flowering state		Vegetative state		Flowering state	
			Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried
1	tricyclene	1015	1.01	0.53	0.72	0.33	0.80	0.45	tr	0.53
2	α -thujene	1027	tr	tr	tr	tr	tr	tr	tr	tr
3	α -pinene	1030	tr	tr	tr	tr	tr	tr	tr	0.17
4	α -fenchene	1060	1.09	1.24	1.26	0.86	0.56	0.90	1.56	0.86
5	camphene	1071	1.42	0.55	1.49	1.11	0.73	1.09	1.43	1.00
6	β -pinene	1115	tr	0.88	tr	tr	tr	tr	tr	tr
7	sabinene	1124	tr	tr	tr	tr	tr	tr	tr	tr
8	α -phellandrene	1162	1.28	1.79	1.48	1.23	tr	1.06	2.35	1.16
9	β -myrcene	1168	0.75	0.92	0.75	0.54	0.85	0.58	1.37	0.61
10	α -terpinene	1186	0.09	0.32	0.18	0.15	tr	0.11	1.11	0.21
11	limonene	1196	tr	1.29	0.95	1.03	0.23	0.70	1.48	0.79
12	(Z)- β -ocimene	1235	tr	tr	tr	tr	tr	tr	tr	tr
13	γ -terpinene	1245	0.16	tr	0.17	0.21	0.15	0.13	0.10	tr
14	p-cymymene	1269	tr	tr	tr	tr	tr	tr	tr	tr
15	(E)- β -ocimene	1250	tr	tr	tr	tr	tr	tr	tr	tr
16	α -terpinolene	1286	0.18	tr	0.13	0.08	0.13	0.12	tr	0.15
Monoterpene hydrocarbons			5.98	7.53	7.13	5.55	3.44	5.14	9.40	5.48
17	1,8-cineole	1213	15.94	17.31	15.02	13.53	11.10	12.35	18.46	12.08
18	piperitone oxide	1692	1.43	0.46	0.89	0.90	1.12	0.96	0.05	1.01
19	terpin-4-ol	1601	0.66	0.48	0.49	0.48	1.33	0.55	0.54	0.63
20	menthol	1613	46.60	48.56	49.12	49.86	40.57	47.29	51.61	48.79
21	neomenthol	1602	tr	0.35	0.34	0.40	0.29	0.60	0.45	0.29
22	α -terpineol	1708	0.27	0.27	0.25	0.22	tr	0.27	0.14	2.26
23	pulegone	1654	4.78	3.76	4.34	3.85	6.97	5.24	4.08	4.65
24	carvone	1715	0.13	tr	tr	tr	tr	0.18	tr	2.48
25	menthone	1473	11.13	12.34	11.54	12.02	20.04	10.13	7.32	13.09
26	menthofurane	1488	0.43	0.39	0.39	0.38	0.76	0.40	0.29	0.51
27	isomenthone	1492	0.49	0.44	0.55	0.43	0.95	0.65	0.20	0.96
28	linalool	1548	0.06	tr	tr	tr	tr	tr	tr	tr
29	piperitone	1758	0.28	0.28	0.13	0.10	tr	tr	0.09	tr
Oxygen monoterpene hydrocarbons			82.19	84.64	83.06	82.18	83.13	78.63	83.21	86.75
30	germacrene D	1703	1.70	1.82	1.84	1.49	1.17	1.91	1.30	0.50
31	β -caryophyllene	1595	0.52	0.48	0.53	0.57	0.62	0.54	0.47	0.55
32	bicyclogermacrene	1710	0.08	tr	tr	tr	tr	tr	tr	tr
33	γ -cardinene	1732	0.82	1.00	0.82	0.84	1.76	1.24	0.09	tr
Sesquiterpenes			3.12	3.30	3.19	2.89	3.54	3.70	1.86	1.05
34	caryophyllene oxide	1997	0.97	1.00	0.93	0.83	1.07	2.13	0.96	tr
Oxygen sesquiterpenes			0.97	1.00	0.93	0.83	1.07	2.13	0.96	tr
Total			92.26	96.47	94.42	91.46	91.19	89.60	95.43	93.28

Tr: <0.001

Comparison of these tested oils with data that have been published on the oil composition of other samples of *Mentha pulegium* L.; there are some quantitative and qualitative differences. These chemical differences can be most probably explained by the existence of different chemotypes. So the geographical distribution of this plant influenced significantly the chemical composition of its essential oils.

However, we think that it is interesting to do other studies using steam distillation because this process seems to be more suitable since it gives better yields. The extraction method by controlled instantaneous decompression must also be tested because it seems more rapid and more selective²⁴. The extraction by means of liquid carbon dioxide under low temperature and high pressure produces a more natural organoleptic profile, but the method is much more expensive²⁵. The difference in organoleptic profile indicates a difference in the composition of oils obtained by solvent extraction as opposed to distillation and this may also affect anti-microbial properties. EOs extracted by hexane have been shown to exhibit greater anti-microbial activity than the corresponding distilled EOs²⁶.

Antibacterial activity: For this essay, we have used the essential oils obtained from leaves because they are the most used in traditional medicine^{2,5-7}. From the obvious results (Tables 3 and 4), it is evident that essential oils from fresh leaves showed a better activity against Gram-positive strains tested but there are no important differences between extracts from fresh and dried organs against Gram-negative.

Aeromonas hydrophila, *Vibrio cholerae* non-O1 and *Enterococcus faecalis* with MIC values of 0.3 and 1.25 $\mu\text{l ml}^{-1}$ and MBC values of 1.25 and 2.5 $\mu\text{l ml}^{-1}$ respectively to oils from fresh leaves and dried ones were the most sensitive strains. So, *Mentha pulegium* L. oils from leaves can be suggested as an agent of conservation in the cosmetic and/or food industries, and as an active compound in medical preparations.

The antibacterial effect could be probably associated with the presence of high concentration of oxygen monoterpene hydrocarbons. We note that extracts from fresh and dried leaves, having no important differences in the rate of the major compounds signalled, have indicated different activities against the same

Table 4. MICs and MBCs ($\mu\text{l ml}^{-1}$) of *Mentha pulegium* L. oils from Monastir.

Bacterial species	Fresh leaves	Dried leaves
<i>Enterococcus faecalis</i> ATCC 29212		
MIC	0.3	1.25
MBC	1.25	2.25
<i>Staphylococcus aureus</i> ATCC 25923.		
MIC	125	160
MBC	2.5	250
<i>Micrococcus luteus</i> NCIMB 8166		
MIC	31.25	67
MBC	67	125
<i>Escherichia coli</i> ATCC 35218		
MIC	160	180
MBC	250	250
<i>Pseudomonas aeruginosa</i> ATCC 27853		
MIC	125	125
MBC	250	250

Gram-positive strains. These results let us conclude that the bacteriostatic activity is not related only to the major components of the essential oils. This suggests that the minor components are critical to this activity and may have a synergistic effect or potential influence. Burt²⁷ thinks that they enable the major compounds to be more easily transported into cells. However, some studies have shown that whole EOs have a greater antibacterial activity than the major components mixed²⁸.

Most studies agree that EOs are slightly more active against Gram-positive than Gram-negative strains^{20,29,30}. Gram-negative organisms are less susceptible because they possess an outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering³¹. This is in agreement with our results of MBC and MIC on reference strains *Pseudomonas aeruginosa* and *Escherichia coli* by comparison with *Enterococcus faecalis*.

Other studies showed that Gram-negative bacteria are more sensitive to the essential oils than the Gram-positive ones³². This confirms our results obtained with *Aeromonas hydrophila*, and *Vibrio cholerae* non-O1 by the disc diffusion method.

Study of the antimicrobial activity of EOs from flowers and roots is necessary to have a complete knowledge on all the organs of Tunisian *Mentha pulegium* L. It is important to test other biological activities such as antioxidant properties and to investigate whether any correlation exists between them.

Table 3. Screening results for antimicrobial activity of the *Mentha pulegium* L. essential oils from leaves by the disc method.

Bacterial species	Vegetative state		Flowering state	
	Inhibition zone (mm)		Inhibition zone (mm)	
	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves
<i>Staphylococcus aureus</i> ATCC 25923	16	11	18	12
<i>Micrococcus luteus</i> NCIMB 8166	15	13	14	12
<i>Enterococcus faecalis</i> ATCC 29212.	17	14	18	15
<i>Pseudomonas aeruginosa</i> ATCC 27853	11	11	11	11
<i>Escherichia coli</i> ATCC 35218	12	12	11	10
<i>Aeromonas hydrophila</i>	20	20	20	19
<i>Citrobacter freundii</i>	13	14	15	15
<i>Enterobacter cloacae</i>	14	14	13	13
<i>Klebsiella pneumoniae</i>	10	10	10	12
<i>Proteus mirabilis</i>	10	12	14	13
<i>Salmonella enteritidis</i>	12	13	12	15
<i>Serratia marcescens</i>	11	14	12	13
<i>Shigella flexneri</i>	15	16	16	16
<i>Vibrio cholerae</i> non-O1	16	19	18	20

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