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Comparison of chemical composition and antimicrobial activities of *Mentha longifolia* L. ssp. *longifolia* essential oil from two Tunisian localities (Gabes and Sidi Bouzid)

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Abstract - This study was conceived to evaluate the difference in the chemical composition of the essential oil of *Mentha longifolia* ssp. *longifolia* from two ecotypes (Sidi Bouzid and Gabes) as well as the difference of the composition of the oils extracted from the leaves and stems. The antimicrobial activity was also tested against 16 human pathogenic microorganisms. The chemical composition of the hydrodistilled essential oils of *Mentha longifolia* ssp. *longifolia* were analysed by GC and GC/MS system. Remarkable differences were recorded between the percentages of the few constituents from leaves and stems and between plants from the two geographical provinces. The chemical analysis of the essential oil obtained from leaves and stems showed the presence of 34 compounds. The most important ones were consecutively: 1,8-cineole (5.6-10.8%), menthone (20.7-28.8%), terpineol-4 (3.1-4.9%), menthol (19.4-32.5%), pulegone (7.8-17.8%) and piperitone (2.2-3.3%). These major components occur in different amounts depending on the organs (leaves or stems) and the geographical origin of the plant. The antimicrobial activity of the essential oil was tested using the disc-diffusion assay and minimal inhibition concentration (MIC) values were estimated according to the microdilution method. The results showed that the essential oil of *Mentha longifolia* ssp. *longifolia* had great potential of antimicrobial activity against all 8 bacteria and 8 yeast species tested. The (MIC) for bacteria was ranging from 0.195 to 3.12 x 10^3 µg/ml.

Key words: *Mentha longifolia* ssp. *longifolia*, leaves, stems, essential oil, GC, GC/MS, antimicrobial activity.

INTRODUCTION

The *Lamiaceae* family regroups more than 250 genera and 6700 species and includes many well-known herbs and garden plants such as lavender, rosemary, thyme and mint. Many different mints, different species, hybrids and special selections grow all over the world. The genus *Mentha* has a wide distribution on all continents, except in South America and Antarctica (Chambers, 1992). *Mentha* is a cosmopolitan genus with about 25 to 30 species such as *M. arvensis, M. piperita, M. spicata, M. pulegium and M. longifolia* that are related to temperate regions (Ali *et al.*, 2002). In Tunisia, the *Mentha* genus is represented by 5 species: *M. pulegium, M. longifolia, M. spicata, M. aquatica and M. rotundifolia* which are well represented in the north of Tunisia (Pottier-Alapetite, 1981).

Leaves, flowers and stems of *Mentha* spp. are frequently used in herbal teas or as additives in commercial spice mixtures for many foods to offer aroma and flavour (Kothari and Singh, 1995; Moreno *et al.*, 2002). In addition, *Mentha* species has been used as a folk remedy for treatment of nausea, bronchitis, flatulence, anorexia, ulcerative colitis, and liver complaints due to its antinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue, and anticatharrhal activities (Cowan, 1999; Iscan *et al.*, 2002; Moreno *et al.*, 2002). Furthermore, it is well-documented that the essential oils from some *Mentha* species including *M. spicata*, *M. piperita*, *M. arvensis*, *M. rotundifolia*, *M. suaveolens* and *M. pulegium* possess strong antimicrobial properties (Kaur and Kapoor, 2002; Daferera *et al.*, 2003).

In previous studies, it has been demonstrated that the content of essential oil of medicinal plants like *Mentha* species containing antimicrobial and other biological activities may change based on the differences in cultivation, origin, vegetative stage and growing seasons of the plants (Marotti *et al.,* 1994; Faleiro *et al.,* 2002; Younis *et al.*, 2004). This species is frequently cultivated in the Tunisian gardens thanks to its therapeutic use. It is answered in the wetlands and meadows of the water courts.

During the last years, it had recourse to the use of the medicinal plants like ingredient of food, because certain herbs are bactericidal and have broad-spectrum of activity against Gram-positive and Gram-negative bacteria (Dorman and Deans, 2000; Friedman *et al.*, 2002; Baydar *et al.*, 2004). Moreover, the multiple drug/chemical resistance in both human and plant pathogenic microorganisms have been developed due to indiscriminate use of commercial antimicrobial drugs/chemical commonly used in the treatment of infectious diseases (Loper *et al.*, 1991; Davis, 1994; Service, 1995). On the other hand, food borne diseases are still a major problem in the World, even in well developed countries, like USA (Mead *et al*., 1999).

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The purpose of this study was to characterise the chemical components of the essential oils extracted by hydrodistillation process from leaves and stems of *Mentha longifolia* ssp. *longifolia* harvested from two Tunisian localities (Sidi Bouzid and Gabes) and to evaluate their antibacterial and antifungal activities.

MATERIALS AND METHODS

Plant material and extraction of essential oils. Plant population of *Mentha longifolia* ssp. *longifolia* was collected in May 2006 from tow Tunisian localities. The first population was collected from south of Gabes city (Dkilet Tougen, South of Tunisia) and the second plant population was collected from province of Gatrana (Sidi Bouzid, Centre of Tunisia). The species was identified according to the flora of Tunisia (Pottier-Alapetite, 1981).

The leaves were separated from stems and used for analyses. They were dried at room temperature for five days, then 100 g of the air dried leaves and stems were subjected to hydrodistillation for 3 h with 500 ml distilled water using a Clevenger-type apparatus according to the European Pharmacopoeia (1975). The oil obtained was collected and dried over anhydrous sodium sulphate and stored in sealed glass vials in a refrigerator at 4-5 °C prior to analysis. Yield based on dried weight of the sample was calculated.

Analyses of the essential oil.

Gas Chromatography. GC HP 5890-series II equipped with: flame ionization detectors (FID), HP-5 (BP-1) (5% phenyl + 95% dimethylpolysiloxane) 30 m x 0.25 mm ID, 0.25 µm film thickness fused capillary column and HP Innowax (BP-20; polyethylenglycol) 30 m x 0.25 mm ID, 0.25 µm film thickness fused capillary column. The carrier gas was nitrogen (1.2 ml/min). The oven temperature program was 1 min isothermal at 50 °C, then 50-280 °C (BP-1) and 50-220 °C (BP-20) at rate of 5 °C/min and held isothermal for 1 min. The injection port temperature was 250 °C, detector 280 °C. Volume injected: 1 µL of 1% solution (diluted in hexane). Percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction.

Gas Chromatograph-Mass Spectrometry. The analyses of the volatile constituents were run on a Hewlett-Packard GC-MS system (GC: 5890-series II; MSD 5972). The fused-silica HP-5 MS capillary column (30 m x 0.25 mm ID, film thickness of 0.25 µm) was directly coupled to the MS. The carrier gas was helium, with a flow rate of 1.2 ml/min. Oven temperature was programmed (50 °C for 1 min, then 50-280 °C at 5 °C min^{-1}) and subsequently, held isothermal for 2 min. Injector port: 250 °C, detector: 280 °C, split ratio 1:50. Volume injected: 1 µL of 1% solution (diluted in hexane).

Mass spectrometer: HP 5972 recording at 70 eVolts; scan time 1.5 s; mass range 40-300 amu. Software adopted to handle mass spectra and chromatograms was a Chem Station.

Identification of the compounds. The components of the oils were identified by comparison of their mass spectra with those of a computer library (Wiley 275 library). Further confirmation was done by referring to Kovats Index data generated from a series of alkanes (C_9-C_{28}) (Shibamoto, 1987; Adams, 1995).

Microorganisms. The test microorganisms included the following Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* CIP 106510, *Micrococcus luteus* NCIMB 8166, Gram-negative bacteria: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC *27853, Salmonella typhimurium* LT2 DT104*, Listeria monocytogenes* ATCC, *Enterococcus feacalis* ATCC 29212, and yeasts: *Candida albicans* (five strains), *Candida glabrata* (one strain), *Candida sake* (one strain) and *Candida tropicalis* (one strain).

Screening for antibacterial activity. Antimicrobial activity was tested by the agar-disc diffusion method (Perez *et al*., 1999; Erdemoglu *et al*., 2003; Bagamboula *et al*., 2004). All bacterial cultures were first grown on MHI plates incubated at 37 °C for 18-24 h prior to inoculation onto the nutrient agar. One or several colonies of similar morphology of the respective bacteria were transferred into API Suspension medium (bioMérieux, Marcy l'Etoile, France) and adjusted to 0.5 McFarland turbidity standard with a DENSIMAT (bioMérieux).

The inoculums of the respective bacteria were streaked onto MHI agar plates using a sterile swab. A sterile filter disc (diameter 6 mm, Whatman paper No. 3) was placed. The disc was impregnated by the tested essential oils (10 µl/disc). The treated Petri dishes were placed at 4 °C for 1-2 h and then incubated at 37 °C for 18-24 h. Antimicrobial activity was evaluated by measuring the zone of growth inhibition around the discs after 24 h of incubation at 37 °C.

The standard discs (6 mm diameter) of the antibiotics gentamycin (10 µg) was served as positive antibacterial control.

The diameter of the zones of inhibition around each of the discs was taken as measure of the antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

Screening for antifungal activity. The human pathogenic yeast used in this study was isolated from patients suffering from candidosis. These strains were isolated on Sabouraud chloramphenicol agar plates and identified with API ID 32 C test strips (bioMérieux) according to the Manufacturer's recommendations.

For screening the antifungal activity of *Mentha longifolia* ssp. *longifolia* essential oil, the agar-disc diffusion method was used as previously described (Cox *et al.,* 2000). All *Candida* strains were first grown on Sabouraud chloramphenicol plate at 30 °C for 18-24 h prior to inoculation onto the nutrient agar. Several colonies of similar morphology of the clinical yeast were transferred into API suspension medium (bioMérieux) and adjusted to 2 McFarland turbidity standard with a densimat. The inoculum of the respective yeast was streaked onto Sabouraud chloramphenicol agar plates at 30 °C using a sterile swab and then dried. A sterile filter disc, diameter 6 mm (Whatman paper No. 3) was placed in the plate at room temperature for 15 mn. Ten microlitres of the essential oil were dropped on each paper disc (10 µl/disc). The treated Petri dishes were placed at 4 °C for 1-2 h and then incubated at 37 °C for 18-24 h. The antifungal activity was evaluated by measuring the diameter of the growth inhibition zone around the discs.

The susceptibility of the standard drug amphoterecin B was determined using a disc paper containing 20 µg of amphetorecin B. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

Minimum inhibitory concentration (MIC). The MIC values were studied for the bacterial strains which were sensitive to the essential oil in disc diffusion assay. The inoculums of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The essential oils of *M. longifolia* ssp. *longifolia* dissolved in 10% dimethylsulfoxide (DMSO) were first diluted to the highest concentration

(100 mg/ml) to be tested, and then serial two-fold dilutions were made in a concentration range from 0.097-100 mg/ml in 5 ml sterile test tubes containing nutrient broth. MIC values of *M. longifolia* ssp. *longifolia* essential oil against bacterial strains were determined based on a micro-well dilution method (Zgoda and Porter, 2001; Gulluce *et al*., 2004*,* 2007). The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µl of the inoculum. A 100 µl aliquot from the stock solutions of *M. longifolia* ssp. *longifolia* essential oil initially prepared at the concentration of 100 mg/ml was added into the first wells. Then, 100 µl from their serial dilutions was transferred into eleven consecutive wells. The last well containing 195 µl of nutrient broth without compound and 5 µl of the inoculum on each strip was used as the negative control. The final volume in each well was 200 µl. Gentamycin at the concentration range of 0.122-125 µg/ml was prepared in nutrient broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer. The essential oil tested in this study was screened two times against each organism. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of the microorganisms. Solvent blanks and positive controls were also included. Negative controls with the DMSO 10% were carried out. All tests were performed in triplicate.

Statistical analyses. Results were analysed using the Statistica software. Data were subjected to analysis of variance (ANOVA) and the means of measured traits relative to root growth were compared between lines with Duncan multiple range test.

RESULTS AND DISCUSSION

Chemical composition of *Mentha longifolia* ssp. *longifolia* essential oil

In this study, we intend to compare the chemical composition of essential oils of the *Mentha longifolia* ssp. *longifolia* plants collected from two different Tunisian regions. We also searched to highlight the differences of the essential oils compounds between the leaves and the stems of these plants.

Mentha longifolia ssp. *longifolia* do not posses the same amount of oil from the different parts. In fact the total yield of essential oils obtained from leaves and stems from S1 (Plants from Sidi Bouzid) were 1.6% (w/w) and 0.1% (w/w) respectively. Concerning the second province S2 (Plants from Gabes), the oil yield was 3.3% (w/w) in leaves and 0.2% (w/w) in stems.

The oils obtained were light yellow, liquid at room temperature, their odours were agreeable. The composition of the volatile oils extracted by hydrodistillation from the different parts of the plant was reported in Table 1 together with the Kovats' indices (KI) calculated for each compound, the percentage of composition and the identification methods. The constituents were arranged to their elution on the polar HP-20 M and apolar HP-5 capillary columns.

The analysis through GC and GC-MS of the plants essential oils of the *Mentha longifolia* ssp. *longifolia* tested in this work showed that the chemical composition of these oils was the same in the two organs and for the two provinces. Table 1 showed the presence of at least 34 constituents including the minor ones. The analysis indicated that the major compounds of these essential oils were: menthol (19.4-32.5%), menthone (20.7-28.8%), pulegone (7.8-17.8%), 1,8-cineole (5.6-10.8%), terpineol-4 (3.1-4.9%), and piperitone (2.2-3.3%). The stems of the Gabes locality were particularly rich on menthone (28.8%) comparatively to the leaves (26.9%) from the same origin. No differences in the percentage of the main components were observed for both two organs from the Gabes locality. In contrary, we found that the leaves from Sidi Bouzid were interestingly rich on menthol (32.5%) with a high percentage of pulegone (17.8%) comparatively to percentage of the same component found in the stems (7.8%).

The comparison of the chemical composition of the essential oil obtained from *Mentha* species demonstrates strong differences between countries (Table 2). Indeed, Peréz Raya and colleagues (1990) studied the chemical composition of *Mentha longifolia* and reported that diosphenol (47.7%) and rotundifolone (33.2%) were the main components. In fact, diosphenol is the precursor of a large number of mint essential oil components (Hendriks *et al*., 1976). The differences in the chemical composition of the species belonging to *Mentha* genus growing around the world could be related to the environmental factors influencing their biosynthesis (Franz *et al*., 1984; Voirin *et al*., 1990). Gulluce *et al*. (2007) studied the antimicrobial and the antioxidant of Turkish *M. longifolia* L. ssp. *longifolia* (oil and methanol extract) and found 45 components and *cis*-piperitone epoxide (18.4%), pulegone (15.5%), piperitenone oxide (14.7%), menthone (7.9%), isomenthone (6.6%), thymol (6.6%), carvone (4.9%), *trans*-piperitone epoxide (4.1%) and β-caryophyllene (2.6%) were the dominant ones. Younis and collaborators (2004) showed the presence of 22 compounds in the aerial parts of *M. longifolia* ssp. *schimperi* grown in Sudan, the major ones were carvone (67.3%), limonene (13.5%), 1,8-cineole (5.4%), menthone (2.9%), linalool (2.8%) and isomenthone (1.2%). The proportion of the 1,8-cineole and the menthone in the oil obtained from Gabes were more important than those from Sidi Bouzid. However, the terpineol-4 and the menthol were more represented in the oil of the plants harvested from S1. The properties of the soil and the climate factors (temperature, pluviometry, altitude) probably caused these important differences between the two chemotypes. This hypothesis is supported by Younis and colleagues (2004) who claimed that the oil composition could apparently, be attributed to factors related to ecotype and environmental influences. Furthermore, Marotti *et al.* (1994) and Faleiro *et al*. (2002) showed that the composition of essential oils from the same species can differ between harvesting seasons and between geographical localities.

According to Table 3, the identified components were terpenes (monoterpene and sesquiterpene) belonging to four different families of organic compounds. The results showed that 88.3% of total compounds of the essential oils were monoterpenes and 11.7% were sesquiterpenes. They were organised as follow: 56.6% monoterpenes hydrocarbons, 36.6% monoterpenes oxygenateds and 6.6% ethers. But the height percentage of these compounds is reserved with the monoterpenes oxygenated (75 to 83.3%). Younis *et al.* (2004) showed that oxygenated monoterpenes comprised 81.5% while monoterpene hydrocarbons comprised 14.7% of the total oils from *M. longifolia*. The oil was characterised by the high percentage of C6-oxygenated p-menthone compounds. We noted that the monoterpenes compounds represent the major part of the total constituents in both leaves and stems independently of the geographical sources. In Iran, Nasrin *et al.* (2004), found that the major components in essential oils of *Mentha pulegium* were pulegone (37.8%), menthone (20.3%), piperitenone (6.8%), and p-mentha-1,8-dien-2-one (5.1%). These finding were the same reported for several oils extracted from *Mentha* genus.

Oumzil *et al.* (2002) showed that the chemical analysis of the essential oils of *M. suaveolens* contains mostly monoterpenes bearing an oxygen function at C3 where as *Mentha spicata*

TABLE 1 - Kovats' indices and percentage composition of the essential oils from leaves and stems of *Mentha longifolia* ssp. *longifolia* from two regions of Tunisia

	Compound	Kovats index (KI)		Sidi Bouzid		Gabes		Identification
		HP-5	HP-20 M	L	S	L	S	method
1	Tricyclene	924	1015	0.2*	0.7	0.3	0.9	MS, KI
2	α-Thujene	928	1020	1.6	0.5	1.6	1.7	MS, KI
3	α-Pinene	937	1026	0.6	1.1	0.9	0.5	MS, KI
4	α-Fenchene	950	1044	0.4	0.9	0.7	0.4	MS, KI
5	Camphene	952	1052	0.9	1.3	1.4	1	MS, KI
6	β-Pinene	980	1115	0.5	0.5	0.7	0.5	MS, KI
7	Sabinene	973	1118	tr	tr	tr	tr	MS, KI
8	α-Phellandrene	1008	1154	tr	tr	0.1	tr	MS, KI
9	β-Myrcene	992	1157	0.4	0.3	0.6	0.4	MS, KI
10	α-Terpinene	1015	1189	tr	tr	tr	tr	MS, KI
11	Limonene	1016	1196	0.6	0.7	0.4	0.2	MS, KI
12	1,8-Cineole	1021	1208	5.6	7.2	10.8	9.0	MS, KI
13	*cis*-Ocimene	1040	1230	tr	tr	tr	tr	MS, KI
14	γ-Terpinene	1057	1235	tr	tr	0.2	0.2	MS, KI
15	*trans*-β-Ocimene	1050	1251	tr	tr	tr	tr	MS, KI
16	p-Cymene	1023	1261	tr	tr	tr	tr	MS, KI
17	α-Terpinolene	1084	1273	0.3	0.7	0.1	0.6	MS, KI
18	Menthone	1139	1456	20.7	24.3	26.9	28.8	MS, KI
19	Menthofurane	1147	1473	tr	0.7	0.4	1.1	MS, KI
20	Isomenthone	1164	1481	1.1	1.2	1.0	0.9	MS, KI
21	Linalool	1370	1544	0.4	0.4	0.1	0.2	MS, KI
22	β-Caryophyllène	1098	1571	0.5	0.9	0.2	0.3	MS, KI
23	Terpineol-4	1178	1581	4.9	4.9	3.2	3.1	MS, KI
24	Neomenthol	1156	1594	1.7	1.2	2.0	1.8	MS, KI
25	Menthol	1171	1612	32.5	31.6	20.1	19.4	MS, KI
26	Pulegone	1228	1645	17.8	7.8	15.6	16.2	MS, KI
27	Piperitone oxide	1230	1700	tr	0.2	tr	0.3	MS, KI
28	Germacrene D	1489	1722	0.9	0.7	1.5	1.4	MS, KI
29	Piperitone	1286	1730	2.2	3.0	3.3	3.1	MS, KI
30	Bicyclogermacrene	1486	1744	0.3	0.2	0.4	0.3	MS, KI
31	Carvone	1237	1750	1.2	0.8	1.9	1.4	MS, KI
32	δ-Cadinene	1510	1772	0.2	0.3	0.3	0.3	MS, KI
33	Piperitenone	1315	1909	1	0.3	0.8	0.6	MS, KI
34	Caryophyllene oxide	1575	2008	0.6	1	0.9	1.2	MS, KI
	Total identified			97.2	93.6	96.7	95.6	
	Yield (g/100 g dry weight)			1.6	0.1	3.3	0.2	

The components and their percentages are listed in order of their elution on polar column (HP-20 M). L: leaves; S: stems. * Percentage; tr: trace (< 0.1%).

produces almost exclusively monoterpenes bearing an oxygen function at C6 such as carvone (Lupien *et al*. 1999). McConkey *et al.* (2000) found that the amounts of menthone or menthol components from *Mentha piperita* were related to the age of the plant while. The ethers were weakly found in both number of constituents and proportions. The sesquiterpenes identified were represented by 3 hydrocarbons and only one ether compound.

These divergences in chemical composition of the essential oils harvested from plants belonging to *Mentha* genus may be due to a difference in the levels of biosynthetic enzymes (Gershenzon *et al.,* 2000).

Antibacterial and antifungal activity of *Mentha longifolia* essential oil

The antimicrobial activities of *Mentha longifolia* ssp. *longifolia* essential oil against 16 microorganisms tested in the present study and their potency were qualitatively and quantitatively assessed by the presence or absence of inhibition zone diameter; and MIC values. The results were given in Table 4. The results showed that the essential oil of *Mentha longifolia* ssp. *longifolia* had substantial of antimicrobial activity against 8 bacteria and 8 yeasts species tested. The antibacterial and antifungal activity of 4 essential oils of the various stocks is presented in Table 4.

This study showed that the essential oil from leaves seems to be most active than the stems ones. Indeed, the highest diameters of growth inhibition zone were obtained when the essential oils from leaves were tested. In fact, the essential oils from the leaves were more active on all the microorganisms tested except for *Enterococcus feacalis* (Sidi Bouzid locality) and *Salmonella typhimurium* (Gabes locality). The essential oil from the leaves of Sidi Bouzid plants was more active against all Gram-positive and Gram-negative bacteria and the yeast tested in the present work comparatively to the results obtained with the essential oil from the same organ collected in the second locality according to the statistical analyses (Table 4).

The same remark can be allotted for the essential oil of the stems with some exceptions. These results can be explained by the difference between oils like the variation of the percentage in

TABLE 2 - Variation in the chemical composition of the essential oil extracted from *Mentha longifolia* collected from different countries

Origin	Main components	References
Sudan	Carvone (76.9%), trans- and cis-carveols (8.8% and 2.2%), trans- and cis-dihydrocarveols (5.4% and 3.4%)	Banthorpe *et al*., 1980
Italy	Piperitenone	Maffei *et al*., 1988
Spain	Diosphenol (47.7%) and rotundifolone (33.2%)	Pérez Raya *et al*., 1990
Sinai	1,8-Cineole (28.8%), cts-piperitone oxide (15.4%) and piperitone (13.8%)	Fleisher and Fleisher, 1991
Jordania	Pulegone (over 70%)	
Yugoslavia	trans-Dihydrocarvone (18.3%), isomenthone (11.5%), piperitone (8.2%) and β-caryophyllene (7.3%)	Mimica-Dukic *et al*., 1991
	Isomenthone (42.3%), methone (11.9%) piperitone (5.2%) and pulegone (4.9%)	
	Menthofuran (38.3%), 1,8-Cineole (10.1%), β-caryophyllene (10.9%) and T-cadinol (7.1%)	
	cis- and trans-isomers of dihydrocarvone (15.9% and 30.6%)	Matovc and Lavadinovlc, 1999
	Pepiritone (38.8%), menthone (11.2%), pulegone (4.9%), Neo-menthol (3.5%), isomenthone (3.1%)	Mimica-Dukic *et al*., 2003
Lithuania	Piperitenone oxide (44.2-57.2%), 1, 8-cineole (15.4-8.4%), myrcene (10.0-6.3%), limonene (4.3-3.5%) and germacrene D (1.5-4.1%).	Venskutonis *et al*., 1996
Greece	Piperitone oxide	Kokkini *et al*., 1998
France	Menthone (60%), pulegone (10%) and 1,8-cineole (8.5%)	Fraisse *et al*., 1998 Vidal *et al*., 1985
	Piperitone oxide isomer (60%), piperitenone oxide (15%), α-muurolol (6%) and 1,8-cineole (3%	
	Carvone (57%), 1,8-cineole (12.6%) and limonene (7.01%)	
Israel	piperitenone	Fleisher *et al*., 1998
Morocco	Piperitenone and piperitone	Ghoulami *et al*., 2000
Iran	Piperitone (43.9%), limonene (13.5%) and frans-piperitol (12.9%)	Rasooli *et al*., 2002
Croatia	Carvone, piperitenone oxide, limonene and β-caryophyllene	Mastelic *et al*., 2002
Tunisia	Menthol (31.6-32.5%), menthone (20.7- 24.3%), pulegone (7.8- 17.8%), 1,8-cineole (5.6- 7.2%), terpineol-4 (4.9%), and piperitone (2.2 a 3. %)	This study
	Menthone (26.9- 28.8%), menthol (19.4- 20.1%), pulegone (15.6- 16.2%), 1,8-cineole (9- 10.8%), terpineol-4 (3.1- 3.2%), and piperitone (3.1- 3.3. %)	

the dominant compounds and the difference in their distribution according to organic families.

The essential oils investigated showed better activity against bacterial species than against fungi. *Pseudomonas aeruginosa* was found to be the most resistant strain, as none of the essential oils was active against this strain. Knobloch *et al*. (1987) showed that terpene alcohols such as linalool exhibit strong antimicrobial activity, especially pronounced on whole cells, while hydrocarbon derivatives possess lower antifungal properties, as their low water solubility limits their diffusion through the medium. Griffin *et al*. (2000) have shown that hydrocarbons tend to be relatively inactive regardless of their structural type, and this inactivity is closely related to their limited hydrogen capacity and water solubility. Ketones, aldehydes and alcohols are active but with differing specificity and levels of activity, which is related to the present functional group but also associated with hydrogen-bounding parameters in all cases. Previous results showed that greater antifungal potential could be ascribed to the oxygenated terpenes (Jansen *et al*., 1987; Knobloch *et al*., 1987; Adams *et al.*, 1995).

For the determination of MIC, the essential oil obtained from the leaves of *M. longifolia* collected from Sidi Bouzid was

TABLE 3 - Number and proportion of identified components classified by organic family compound

Family	No.	Percentage of monoterpenes				No.	Percentage of sesquiterpenes			
		Sidi Bouzid		Gabes			Sidi Bouzid		Gabes	
		L	S	L	S		L	S	L	S
Hydrocarbons	17	5.9	5.9	7.2	5.7	3	1.5	1.8	1.9	1.9
Oxygenateds	11	83.3	75.9	75	75.7	-	-	-	-	-
Ethers	2	5.6	7.5	11.1	10	1	0.6	0.9	0.8	1.1
Total	30	94.9	89.5	93.4	91.6	4	2.2	2.8	2.8	3.0

No.: number of identified components; L: leaves; S: stems; -: not found.

TABLE 4 - Antibacterial and antifungal activity of essential oil of *Mentha longifolia* ssp. *longifolia* harvested from tow provinces of Tunisia against human pathogenic bacterial and yeast strains

Microorganisms	Inhibition zone (diameter; mm ± standard deviation)				
	Essential oils (10 µl) from Sidi Bouzid		Essential oils (10 µl) from Gabes		Antibiotics
	Leaves	Stems	Leaves	Stems	Gen (10 µg/disc)
Gram-positive bacteria					
Staphylococcus epidermidis CIP 106510	15.33 ± 0.57d	12.7 ± 0.57c	12.33 ± 0.57d	12.66 ± 0.57d	21.33 ± 0.58a
Staphylococcus aureus ATCC 25923	17 ± 1e	18 ± 0e	17 ± 1.7e	13.66 ± 0.57e	32.67 ± 0.58e
Micrococcus luteus NCIMB 8166	19 ± 1f	18.7 ± 0.57e	17 ± 1e	14.33 ± 0.57e	27.67 ± 1.53c
Gram-negative bacteria					
Listeria monocytogenes ATCC 19115	17.67 ± 0.57e	13 ±1c	17 ± 1e	12.66 ± 0.57d	37.67±0.58f
Enterococccus feacalis ATCC 29212	11.33 ± 0.57c	14.7 ± 0.57d	10.33 ± 0.57c	9 ± 0b	26 ± 1b
Pseudomonas aeruginosa ATCC 27853	9.33 ± 0.57b	NAa	NAa	NAa	30.33 ± 0.58d
Escherichia coli ATCC 35218	26 ± 1f	19 ±1e	23 ± 0f	18.33 ± 0.57f	27.33 ± 0.58bc
Salmonella typhimurium LT2 DT104	8 ± 0a	8 ± 0b	8 ± 0b	10 ± 0c	21 ± 1a
Yeast strains					AmB (20 µg/disc)
Candida albicans 1	15.33 ± 0.43b	12.33 ± 0.57cd	15 ± 0c	10 ± 1ab	23 ± 1d
Candida albicans 2	17.33 ± 0.43c	13 ± 0 de	16.66 ± 0.43d	11 ± 1bc	24 ± 0e
Candida albicans 3	15.33 ± 0.43b	11.66 ± 0.57bc	13 ± 0.75b	11 ± 1bc	20 ±0a
Candida albicans 4	14.66 ± 0.87b	10.66 ± 0.57ab	15 ± 0c	12.66 ± 0.57d	19.33 ± 0.57a
Candida albicans 5	15 ± 0.75b	13.66 ± 0.57ef	12.33 ± 0.43b	9.33 ± 0.57a	21 ± 0b
Candida glabrata	12.33 ± 0.43a	10 ± 1a	10 ± 0a	8.66 ± 0.57a	22 ± 0c
Candida sake	18.33 ± 0.43cd	14.33 ± 0.57f	14.66 ± 0.43c	12 ± 1cd	19.33 ± 0.57a
Candida tropicalis	19.33 ± 0.43d	13.66 ± 0.57ef	16.33 ± 0.43d	13.33 ± 0.57d	24 ± 0.57e

Gen: Gentamycin; AmB: Amphotericin B; NA: not active.
Means followed by the same letters are not significantly different at $P = 0.05$ based on Duncan's multiple range test.

chosen according to the results obtained agar diffusion method. Indeed, the MIC values for bacterial strains, which were sensitive to the essential oil of *M. longifolia* ssp. *longifolia*, were comprised between 0.195 and 3.125 x 10^3 µg/ml (Table 5). This test showed the degree of sensitivity of the bacteria tested with respect to the essential oil of *Mentha longifolia* ssp. *longifolia*. The lowest values of MIC were obtained when Gram-positive bacteria were tested (195 µg/ml for *Staphylococcus epidermidis* and *Micrococcus luteus* and 781 µg/ml for *Staphylococcus aureus*). The highest values of MIC were noted for *P. aeruginosa* (3125 µg/ml), *Salmonella typhimurium* (1562 µg/ml) and *Enterococcus feacalis* (1562 µg/ml). The Gram-positive bacteria are more sensitive than the Gram-negative bacteria. These results are in agreement with the literature (Cosentino *et al*., 1999; Sahin *et al*., 2002; Karaman *et al*., 2003).

In 2003, Mimica Dukic and colleagues demonstrated that *Mentha aquatica*, *M. piperita*, *M. longifolia* exhibited a strong antibacterial activity against several Gram-positive and Gram-negative bacteria including *E. coli*, *Salmonella typhi*, *Salmonella enteriditis*, *Sarcina lutea*, *Shigella sonei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis*. The diameters of the zone of inhibition growth were ranging from 14.4 mm (*E. coli* strain) to 33 mm (*Staphylococcus aureus* strain). These three *Mentha* species possess a notable fungistatic and fugicidal activity. In fact, *M. longifolia* possess the lowest minimum fungicidal concentration (8 µl/ml) against *Candida albicans* strain A117 comparing the bifonazole (25 µl/ml). The essential oil of *Mentha longifolia* L. subsp. *capensis* collected from South Africa was particularly rich on menthone (50.9%), pulegone (19.3%) and 1,8-cineole (11.9%). This oil was similar to that reported by Fraisse *et al*., 1985. The Iranian oil of *M. longifolia* was active on *E. coli* and *Staphylococcus aureus* (Rasooli and Rezaei, 2002).

Gulluce *et al*. (2007) showed a strong antimicrobial activity of the essential oil extracted from *M. longifolia* L. ssp. *longifolia* against 30 microorganisms including Gram-positive and Gram-negative bacteria, yeast (*Candida albicans*) and fungi with a diameters of zone of inhibition growth ranging from 8 mm (*B. subtilis*, *Burkholderia cepacia* and *Salmonella enteritidis*) to 22

TABLE 5 - The MIC values *Mentha longifolia* ssp. *longifolia* of the essential oil against the bacterial strains tested in micro-dilution assay (essential oil of leaves of plants from Sidi Bouzid province)

Microorganisms	Essential oil (x 10^3 µg/ml)	Gentamycin (µg/ml)
Staphylococcus epidermidis CIP106510	1.56	31.25
Staphylococcus aureus ATCC25923	0.78	15.62
Micrococcus luteus NCIMB 8166	0.19	3.90
Escherichia coli ATCC 35218	0.78	3.90
Listeria monocytogenes ATCC19115	0.19	1.95
Enterococcus feacalis ATCC 29212	1.56	7.81
Pseudomonas aeruginosa ATCC 27853	3.12	62.5
Salmonella typhimurium LT2 DT104	1.56	15.62

mm (*Bacillus macerans*), 28 mm for *Candida albicans*-A117 and from 12 mm for *Trichophyton rubrum* to 35 mm for *Aspergillus flavus* fungi. The minimal inhibitory concentrations from this oil were ranging from (15.62 µg/ml) for *Bacillus macerans* and *Staphylococcus aureus* strains to (62.5 µg/ml) for *Bacillus subtilis*, *Salmonella enteritidis*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Enterococcus* spp. and *Clavibacter michiganense* strains. The MIC obtained were comprised between 31.25 µg/ml against fungi (*Trichophyton* spp., *Sclorotinia tabacinum* and *Aspergillus spp.*) and 125 µg/ml against yeast (*Candida albicans* A117). These results confirm the antimicrobial effect of the *Mentha longifolia* ssp. *longifolia* against a large list of microorganisms including bacteria, fungi and yeast. Our results demonstrate that *Mentha longifolia* ssp. *longifolia* has a strong and broader spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria. These results were in accordance with those reported by Gulluce *et al*., 2007.

According to the results obtained in this study and the data collected from the bibliography, two chemotypes of *Mentha longifolia* ssp. *longifolia* were identified: chemotype I rich in menthol (Sidi Bouzid) and chemotype II rich in menthone (Gabes).

CONCLUSION

In conclusion we noted that whether the organs (stems or leaves) in *Mentha longifolia* ssp. *longifolia*, the chemical composition of the essential oils was the same (34 compounds) and contained six major components such as: menthol (32.5-19.4%), menthone (28.8-20.7%), pulegone (17.87-8%), 1,8 cineole (10.8-5.6%), terpineol-4 (4.9-3.1%), and piperitone (3.3-2.2%) but we noted that the amount of each major component is directly related to the organ of the plant and the geographical sources.

The study revealed significant antibacterial and antifungal activity of investigated *Mentha longifolia* ssp. *longifolia* essential oils. In deed, the agar diffusion method indicated a strong activity of *Mentha longifolia* ssp. *longifolia* essential oils against pathogenic microorganisms (fungi and bacteria species). In the same way the values of the MIC obtained are very significant. Given the commercial value of essential oils from *Mentha*, these data suggest that *Mentha longifolia* ssp. *longifolia* may be used as a source of commercialised essential oil in Tunisia and as to enhance the food safety as this oil have an antibacterial and antifungal activities.

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