Chemical Composition and Antimicrobial Activity of Flowering Aerial Parts *Mentha Pulegium* from Gilan

Rezvan Shahmohamadi, Reyhaneh Sariri*, Mahdi Rasa, Hosein Ghafoori, Mahmudreza Aghamali, Somaieh Nasuti and Mohamad Tahery

Department of Biology, University of Guilan, Rasht, Iran

Summary

The chemical composition and antimicrobial activity of the essential oil of *Mentha pulegium* L., was investigated. Analysis of essential oil using gas chromatography-mass spectrometry (GC-MS) revealed at least 15 components. The chemical composition showed some similarity with essential oil of *M. pulegium* from other regions. It was found that pulegone was the major component with 69.22% of the essential oil and menton accounting for 18.98%. The antimicrobial activity of essential oil was then evaluated against four bacteria important in food industry. According to the results, essential oil from *M. pulegium* showed its highest antibacterial activity on *S. mutans* followed by *K. oxytoca*, *P. aeruginosa* and *E. coli*. According to our literature survey, this is the first report on the quantitative composition and antibacterial activity of the essential oil from *Mentha pulegium* L. grown in Gilan, a northern province of Iran.

Keywords: *Mentha pulegium*; Essential oils; Chemical composition; Antimicrobial activity GC-Mass.

Introduction

Many herbs and spices have been traditionally used as perfume, adding flavor to foodstuff and medicines, and for their preservative properties (1). By increasing awareness about the side effect of chemical preservatives, public demand and scientific interest in the use of these natural antimicrobials and antioxidants for food preservation is increasing rapidly. Moreover, there are new concerns about the increasing occurrence of new food-borne disease caused by pathogenic microorganisms (2) and the emergence of resistant microorganisms that are associated with food borne diseases (3).

Essential oils are volatile mixtures of organic compounds obtained from different parts of the plants by steam or hydro-distillation (4). More than 3000 essential oils have been extracted from various plants some of which have been used in pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (1).

It should be emphasized that most of beneficial properties including antimicrobial activity depend on chemical composition of the essential oil. In practice, two or three major components are always present at high concentrations while other components are only in trace amounts (5). In many cases, the essential oil possesses higher antimicrobial activity than expected considering its major antimicrobial components (6), indicating some that minor components could also play important role.

Mentha pulegium is one of the Mentha species known as pennyroyal, a native herb of Asia and near East (7). *Mentha pulegium* L. has been traditionally used as antiseptic for treatment of cold, sinusitis, cholera, food poisoning, bronchitis and tuberculosis (8). Its possible effects as antiflatulent, carminative, expectorant, diuretic, and antitussive (9) have also been investigated. It has been reported that essential oil of *Mentha pulegium* L. exerts abortifacient effect in rat myometrium (10), cytotoxic activity against different human cell lines as well as antioxidant activity (11).

Although chemical composition of the essential oils from *Mentha pulegium* grown in different regions of the world have been reported (12, 13), their antimicrobial activity has not been compared with synthetic antibiotics.

In the present study, we have used GC–MS to determine the chemical profile of essential oil obtained from *M. pulegium* grown in province of Gilan, south of the Caspian Sea. The antimicrobial activity of the oil was then investigated against four selected microorganisms and compared with known antibiotics.

Materials and methods

Collection and preparation of plant

Mentha pulegium L. samples were collected during the months of April, June and August 2011 in Sarvan part of Rasht located at about 40 km from the Caspian Sea in Gilan Province, North of Iran.

The taxonomic identity was confirmed by comparing specimen with those of known identity collected in the Herbarium of the Department of Biology, University of Guilan. Fresh leaves were transferred to the biochemistry research laboratory, washed, drained and air dried for two weeks in the absence of light at room temperature. The dried samples were weighed, marked and stored in opaque paper bags.

Preparation of essential oil

Dried samples were subjected to steam distillation for 3 h using a Clevenger-type apparatus. The supernatant was separated by decantation after 50% NaCl was added. The essential oil was collected, dried over anhydrous sodium sulfate and stored in sealed glass vials at 4 °C in the dark until used.

Gas chromatography/mass spectrometry (GC/MS) analysis

GC-MS analysis of the essential oils was performed by high resolution gas chromatography, 6890N(G1530N), USA coupled to a Saturn 2000 ion trap mass spectrometer (Varian, Walnut Creek, CA, USA) in the electron impact ionization mode (70 eV). Mass acquisition mode was adjusted to 40 to 400 m/z. A HP5LS fused-silica capillary column (30 m × 0.025 mm) was used for analysis. The range of oven temperature was 70-250 °C and it was programmed so that the initial temperature was 50 °C held for 2 min, then raised to 250 °C at 10°C/min and remained at this temperature for 3 minutes. Helium gas was used as the carrier gas at a constant flow rate of 2 ml/min.

Injector and MS transfer line temperatures were set at 250 °C and 280 °C, respectively. Diluted samples in methanol (v/v) of 1.0 µl were injected in the ratio of 1:100. Analysis of each sample was performed in triplicate, taking into account their mean value.

Identification of each separated component was based on their retention indices (KI) relative to those of a homologous series of n-alkanes (C8-C20) (Fluka, Buchs/sg, Switzerland) and matching their recorded mass spectra with those stored in the spectrometer database (NIST MS Library v. 2.0) and the bibliography (14).

Selection of microorganisms and preparation of growth medium

Antimicrobial studies were carried out using four microorganisms one Gram-positive: *Streptococcus mutans* (PTCC1543) and three Gram-negative: *Klebsiella oxytoca* (PTCC1402), *Escherichia coli* (PTCC1553), and *Pseudomonas aeruginosa* (PTCC1558) bacteria. All strains were obtained from Persian Type Culture Collection (PTCC) centre in Tehran. They were all maintained frozen at -80 °C.

óThe bacterial vials were opened as instructed by PTCC and the strains were grown in nutrient Broth followed by incubation at 37 °C for 4 hours. Disk diffusion method was used to compare *in vitro* antibacterial activity of *M. pulegium* ethanolic essential oil with known antibiotics. In practice, broth subcultures were prepared by inoculating, with one single colony from a plate, a test tube containing 5 mL of sterile nutrient Broth. The tubes were then incubated at 37 °C for at least 24 hours (15).

The bacterial suspension had concentrations of 10^8 CFU/mL on Mac Farland scale. Each suspension was spread on Muller Hinton Agar medium by sterile swabs. Whatman filter paper disks (diameter 6 mm) dipped in samples of essential oil and ampiciline (Padtan Teb™ Iran) were placed on the agar surface. The diameter of inhibition zone was measured after incubation of all plates at 37°C for 24 hours. The inhibition zones (including disk diameter) less than 10 mm were negative. Zone calculation was the average of three measurements. The t-test was performed with the GraphPad PRISM® software (GraphPad Software, Inc., San Diego, CA, USA) and $p \leq 0.05$ differences were considered as significant.

Minimum inhibitory and minimum bactericide concentrations

The 96 plate micro-dilution Broth method was used to obtain minimum inhibitory concentration (MIC) of essential oils against selected bacterial strains (16). In practice, 50 µl of Muller Hinton Broth medium was added to each well, 50 of essential oil was then added to the first well of each raw and dilutions were done to the 6^{th} well. Finally, 50 ml of diluted microbial suspension (10^8 CFU/mL) was added to all wells. The positive and negative wells contained microbial suspension plus Muller Hinton Broth medium and essential oil plus Muller Hinton Broth medium respectively. The optical density was immediately read using ELISA reader (Stat Fax 2100) at 630 nm. A second OD measurement was performed after 24-h-incubation at 37°C. The lowest concentration of essential oil in which no bacteria was grown and the OD started to reduce was taken as MIC.

To evaluate minimum bactericide concentration (MBC), the components of every well with no bacterial growth were separately grown on Hinton Agar medium and incubated at appropriate temperature for 24 h. The lowest concentration at which bacteria failed growing was considered as MBC (16). Each measurement was repeated three times and the mean value was taken as the result.

Results

Although 15 components were clearly identified by the GC mass analysis (Table 1), but the oil was a complex mixture of much more chemicals. As indicated in Table 1, among all the compounds found in oil, pulegone and menthone were the most abundant comprising 69.218% and 18.97% respectively. The elution time and percent of each component are also shown in this table. We found that 3.5 ± 0.2 grams of essential oil could be obtained from each 100 grams of fresh leaves of *M. pulegium* (3.5%).

Table 1. GC Mass analysis of essential oil from *Mentha pulegium* showing the percent of 15 most clearly identified chemicals.

No.	Identified compounds	Retention time (minutes)	Percent of dry weight
1	α-pinene	3.06	0.25
2	β-pinene	3.64	0.32
3	3-Octanol	3.87	1.21
4	Methyl cyclohexene	4.72	0.40
5	Menthone	6.31	18.98
6	Iso-menthol	6.44	1.65
7	Iso- pulegone (cis)	6.62	2.50
8	Dodecane	6.87	0.53
9	Pulegone	7.67	69.22
10	Iso- pulegone (trans)	7.92	0.95
11	8-hydroxy-D-p- menth-3-one	6.27	2.97
12	2, 2-dimethyl propylidene	8.48	0.36
13	Piperitenone	8.99	0.13
14	1-methyl-2-propyl cyclohexane	16.19	0.28
15	16-n-buthyl tetra hydropyridine	16.36	0.23
Total identified compounds (%)			**97.94**

As shown in Table 1, pulegone (69.22%), menthone (18.98%), 8-hydroxy-D-p- menth-3-one (2.97%), cis iso- pulegone (2.5%), iso-menthol (1.65%) and 3-octanol (1.21%) were the most abundant individual compounds respectively found in essential oil of *M. pulegium* from Gilan.

It was also revealed that essential oil obtained from *M. pulegium* growing in Gilan Province was highly active against four common Gram-positive and Gram-negative bacteria as compared to effective known antibiotics (Table 2). The antibacterial activity of the oil was evaluated by observing the presence of inhibition zones and zone diameter, and recorded as MBC and MIC values. Results are compared to the effect of ethanolic extracts in Table 3.

Table 2. Mean diameter (mm) of inhibition zone for the growth of various bacteria in the presence of *M. pulegium* essential oil.

Strains tested	Dilutions			
	1	1/2	1/4	1/8
K. oxytoca	25	18	15	11
E. coli	15	14	10	--
P. aeruginosa	19	19	17	14
S. mutans	30	25	21	15

Table 3. Growth inhibition zones (mm) for different bacteria at a concentration gradient of *M. pulegium*, disk diameter 6.0 mm.

Strains tested	Concentrations of *M. pulegium* extracts (mg/ml).						
	1.50	3.00	6.25	12.50	25	50	100
K. oxytoca	--	--	--	--	9	12	14
E. coli	--	--	--	--	--	--	--
P. aeruginosa	--	--	--	--	--	10	13
S. mutans	12	14	16	17	18	21	22

The results of *in vitro* antimicrobial activity using the filter paper disk agar diffusion technique showed that essential oil of *M. pulegium* from Gilan was almost effective on both Gram positive and Gram negative bacterial strains depending on their concentrations (Tables 2 and 3). However, the Gram negative strain, *S. mutans* was the most sensitive to growth inhibition by oil and Gram negative strains showed different inhibition zone

mostly affected by the concentration. Among Gram negative bacteria, *K. oxytoca* was more sensitive to dilution, so that at 100% dilution was almost inhibited similar to *S. mutans*. On the other hand, while *E. coli* remained unaffected by any concentration of the extract, the concentrations above 50 mg/ml affected two Gram negative strains, i.e. *K. oxytoca* and *P. aeruginosa*. At lower concentrations such as 25 mg/ml, in addition to the Gram positive *S. mutan*, one of the Gram negatives, *K. oxytoca* also showed growth inhibition. We observed that both ethanolic extracts and essential oil of *M. pulegium* from Gilan were more effective on all bacterial strains when the plant samples were obtained during flowering season so that even the growth of *E. coli* was inhibited (data are not shown in this paper and will be published shortly). However, we observed that *E. coli* was the most resistant bacterial strains to the extracts and essential oil of *M. pulegium* from Gilan used in this study. On the other hand, extracts and oil of *M. pulegium* from Gilan could effectively inhibit growth of Gram positive *S. mutan* in almost any conditions and any concentrations.

Discussion

Aromatic plants have been used widely to extend the shelf life of foods and drugs. However their possible property as antibacterial agents in clinical use is still under investigation. Although there are promising results for the antimicrobial properties of various plant derived products against environmental or food-isolated strains, research on *M. pulegium* is limited, especially if the regional diversity is taken into account.

In this study, essential oils from *M. pulegium* was isolated by hydrodistillation, analyzed by gas chromatography (GC) and GC/mass spectrometry (GC/MS) for their chemical components and tested for their antimicrobial activities against 4 bacterial strains were tested. The methods used were disk diffusion and broth dilution in order to determine the Minimum Inhibitory Concentration (MIC).

Our results showed that essential oil from *M. pulegium* of Gilan was highly active against *S. mutans*, the Gram positive bacterial strain. It also inhibited the growth of some Gram negative bacteria including *K. oxytoca*, *P. aeruginosa* and *E. coli* when tested by disk diffusion. The oil also exhibited increased MIC values (>250 mg/ml) with the dilution method.

Although the essential oil from *M. pulegium* is a suitable candidate for food industry as a natural preservative. However, its antimicrobial activity may be improved if other food preservation methodologies are also used (17, 18). The very interesting and promising fact about using *M. pulegium* in food and food and pharmaceutical industries is its pleasant flavor. Most of plant derived preservatives have the limitation of their strong and unvanted flavor as well as the solubility problems associated with their lipophilic nature (19).

Many secondary metabolites of plants are present in the essential oils of herbal plants and contribute important parts in their medicinal properties (20). It is known today that natural products are very important, harmless and cost effective food preservatives.

However, a broad range of research on the antimicrobial and antioxidant activity as well as their chemical composition is required.

According to a literature survey, it was found that chemical composition of essential oil obtained from M. pulegium of Gilan was almost similar to the effective components of some other plants (13, 21). In most of herbal essential oils which exhibit antibacterial activity, the content of pulegone has been found above 50% indicating its major role in exhibiting antibacterial activity by the plant.

In the essential oil investigated during this research, oxygenated monoterpenes such as iso-menthol, pulegone, menthone (5.9%), iso-pulegone and piperitone were present with a total of above 70%. It has been reported that oxygenated monoterpenes, are potentially active compared to hydrocarbon monoterpenes such as Methyl cyclohexene (22).

It is worth indicating that the antibacterial and antioxidant effects of herbal essential oils may be varied by modifying the extraction conditions, time of plant harvest as well as part of the herb used (5, 23). We are, therefore, investigating further in order to improve the beneficial effects of various herbal oils found in this part of the world.

Conclusions

In this study the chemical composition and antimicrobial activity of the essential oils of *M. pulegium* from a northern province of Iran was revealed by a sensitive GC-Mass method. The quantitative data from GC-mass showed that the most abundant chemicals presented in *M. pulegium* were pulegone and menthone respectively. Therefore, the strong antibacterial effects exhibited by oil must be related to these compounds. Both oil and ethanolic extracts of *M. pulegium* showed antibacterial activity, especially on Gram positive bacterial strains. Considering the parallel study on antioxidant activity of this herb, the results of which would be published soon, we concluded that *M. pulegium* obtained from Gilan province of northern Iran is potent antibacterial and antioxidant natural herb especially during its flowering season. Therefore, our investigations suggested that the herb could be used as a potential natural preservative in food and pharmaceutical industries.

Acknowledgments

The authors highly acknowledge the financial support by University of Guilan.

References

1. Bakkali F, Averbeck S, Averbeck D and Idaomar M. Biological effects of essential oils-A review. Food and Chemical Toxicology 2008; 46: 446-475.

2. Tajkarimi MM, Ibrahim SA and Cliver DO. Antimicrobial herb and spice compounds in food. Review. Food Control 2010; 21: 1199-1218.

3. Gibbons A. Exploring new strategies to fight drug resistant microbes. Science 1992; 29: 1036-1038.

4. Batish DR, Singh HP, Kohli RK and Kaur S. Eucalyptus essential oil as a natural pesticide. Forest Ecology and Management 2008; 256: 2166-2174.

5. Burt S. Essential oils: Their antibacterial properties and potential applications in foods - A review. International Journal of Food Microbiology 2004; 94: 223-253.

6. Lattaoui N and Tantaoui-Elaraki A. Individual and combined antibacterial activity of the main components of three thyme essential oils. Rivista Italiana EPPOS 1994; 13: 13-19.

7. Chalchat JC, Gorunovlc MS, Maksimovlc ZA and Petrovlc SD. Essential oil of wild growing *Mentha pulegium* L from Yugoslavia. Journal of Essential Oil Research 2000; 12: 598-600.

8. Zargari A. Herbal Medicines. Publication of Tehran University Iran. 1990; 14-18.

9. Wojdylo A, Oszmianski J and Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry 2007; 105: 940-949.

10. Ritchason J. The Little Herb Encyclopedia: The Handbook of Natures Remedies for a Healthier Life. 3d ed. Pleasant Grove Utah: Woodland Health Books. 1995; 171.

11. Mahboubi M and Haghi G. Antimicrobial activity and chemical composition of Mentha pulegium L. essential oil. Journal of Ethnopharmacology 2008; 119: 325-327.

12. Derwich E, Benziane Z and Boukir A. GC/MS analysis and antibacterial activity of the essential oil of Mentha pulegium grown in Morocco. Research Journal of Agriculture and Biological Sciences 2010; 6 (3): 191-198.

13. Lorenzo D, Paz D, Dellacassa E, Davies P, Vila R and Cañigueral S. Essential oils of Mentha pulegium and Mentha rotundifolia from Uruguay. Brazilian Archives of Biology and Technology 2002; 45 (4): 519-524.

14. Adams RP. Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Allured Publishing Corporation, Illinois 2001.

15. Alzoreky NS and Nakahara K. Antimicrobial activity of extracts from some edible plants commonly consumed in Asia. International Journal of Food Microbiology 2003; 80:223-230.

16. Baron EJ, Peterson LR and Finegold SM. Diagnostic Microbiology. 1994; 9th ed: 44-51.

17. Kowalchik Claire K and Hylton WH eds. Rodale's Illustrated Encyclopedia of Herbs. Emmaus Pennsylvania: Rodale Press 1998; 412-414.

18. Davidson PM. Chemical preservatives and natural antimicrobial compounds. In: Food Microbiology Fundamentals and Frontiers Doyle MP, Beuchat LR, and Montville TJ Eds. ASM Press 1997; 520-556.

19. Demirci F, Guven K, Demirci B, Dadandi MY and Baser KHC. Antibacterial activity of two Phlomis essential oils against food pathogens. Food Control 2008; 19: 1159-1164.

20. Ramarathnam N, Osawa T, Ochi H and Kawakishi S. The contribution of plant food antioxidants to human health. Trends in Food Science and Technology 1995; 6: 75-82.

21. Benayad N. Les huilles essentielles extraites des plantes medicinales marocaines : moyen efficace de lutte contre les ravageurs des denrees alimentaires stockess Projet de recherche. Rapport d'activité. Faculté des Sciences-Rabat, Maroc 2008.

22. Carson CF and Riley TV. Antimicrobial activity of the major components of the essential oil of Melaleuca alternifolia. Journal of Food Microbiology 1995; 78: 264-269.

23. Rauha JP, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, Pihlaja K, Vuorela H and Vuorela P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. International Journal of Food Microbiology 200; 56: 3-12.