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Chemical Composition and in Vitro Antibacterial Activity of Native *Mentha longifolia* Essential Oil from Ardabil, Iran on *Streptococcus mutans, Lactobacillus rhamnosus and Actinomyces viscosus*

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Background: Most of the population in the different areas of world is affected by bacterial infections responsible for dental caries. Due to the importance of traditional medicines derived from herbs used for dental problems, this study investigated in vitro antibacterial activity of *Mentha longifolia* essential oil from Ardabil, Iran, on *Streptococcus mutans*, *Lactobacillus rhamnosus* and *Actinomyces viscosus*, bacteria that cause tooth decay.
Materials and Methods: The volatile oil of *Mentha longifolia* leaves was extracted by hydrodistillation method using a Clevenger-type apparatus and analyzed by GC and GC/MS system.The antibacterial activity was evaluated by the disk diffusion susceptibility in dilutions of 62.5, 125, 250, 500, 1000 and 2000µg/µl and broth macrodilution test methods.
Results: The oil was particularly rich in Pulegone (31.78%), 1, 8-cineole (15.99%), menthoforan (11.25%), cis-isopulegon (10.5%) and paramenth-3-n-8-l (6.85%). The medicinal plant essential oil could prevent the growth of the bacteria, and the rates of MIC and MBC of native pennyroyal essential oil on *Streptococcus mutans*, *Lactobacillus rhamnosus* and *Actinomyces viscosus* were 110, 165, 80, 120, 450 and 650µg/µl, respectively. The maximum inhibition zone diameter was about 12.2, 27.2 and 4.8 mm, for *Streptococcus mutans*, *Lactobacillus rhamnosus* and *Actinomyces viscosus* respectively, at the concentration of 500µgµl^{-1}.
Conclusion: In this work, the essential oil of medicinal plant containing effective ingredients could prevent the growth of bacteria and may be used as an affordable and available source for medicinal purposes.

Keywords: Streptococcus mutans, Lactobacillus rhamnosus, Actinomyces viscosus, Mentha longifolia

1. Background

Nowaday, one of the most common diseases across the world is dental caries. It is also one of the most expensive health issues to cure. So, it causes lots of trouble for health care providers. In order to reduce the prevalence of dental carries, we need to obtain a more thorough understanding of the role of micro-organisms in dental issues.

(1). *Streptococcus mutans* is facultative anaerobe, gram-positive coccus-shaped acidogenic bacterium commonly one of the first and most important microorganisms in dental plaque, that has essential role in the initiation of dental caries. This bacterium is mostly found between adjacent teeth or in the deep crevices on occlusal (the biting surface) of teeth because of its anaerobic growth. It can thrive at temperature ranging from 18 to 40°C. Ability of *S. mutans* to synthesize extracellular glucan from sucrose by *g*lucosyltransferase is the main pathogenic character of this bacterium (1, 2). *Lactobacillus rhamnosus* is a gram-positive bacterium that was originally considered to be a subspecies of *L. casei*; however, genetic research found it to be a separate species. These bacteria primarily exist in the human digestive system, but can also be found in the human urinary and genital tracts and they have been discovered to be pathogenic in certain circumstances, and especially play a role in the progressive development of dental caries. Some strains of *L. rhamnosus* are also used in various food products such as probiotics (3, 4). Some epidemiological studies have shown that carious lesions in the root surfaces of extracted human teeth have predominantly gram-positive pleomorphic filamentous rods, acidogenic and aciduric bacteria, *Actinomyces viscosus*. Actinomyces (from Greek "actis" ray, beam and "mykes" mucus, fungus) is a genus of the *Actinobacteria* class of bacteria. *A. viscosus* is facultative anaerobe. *Actinomyces* colonies form fungus-like branched networks of hyphae without any endospore (5, 6). Treatment and prevention of dental caries by antibiotics and steroids can change oxidation-reduction potential, weaken lysozyme activity, facilitate the development of allergic reactions and reduce the body resistance to pathogenic factors (7). Additionally, great interest is recently seen in various fields of medical science and herbal medicine, because of the use of plants as medicine from centuries ago (8). The leaves and stems of pennyroyal have medicinal properties. The plant contains tannin, resin materials, sugar and essence (9).

2. Objectives

This work investigated the in vitro antibacterial effect of pennyroyal essential oil in Ardabil, Iran; which is a traditional medicine useful in the treatment of oral and dental diseases, on *S. mutans, L. rhamnosus* and *A. viscosus* that cause tooth decay.

3. Materials and Methods

3.1. Plant material and extraction of essential oil

The plant was collected from Ardabil, North West of Iran. The species was identified according to the flora of Iran. First, the leaves were separated from stems and washed with cold water in order to clean from dust and then dried at room temperature for five days. Next, 100g of dried leaves was subjected to hydrodistillation for 3 hours with 500ml distilled

water using a Clevenger-type apparatus. Finally, the oil was collected and dried over anhydrous sodium sulphate and stored in sealed glass vials in a refrigerator at 4 -5°C. Yields were calculated based on dried weight of the sample (10, 11).

3.2. Analysis of the essential oil

The gas chromatography analysis of the volatile oil was done using a HP 5890-series II equipped with Flame ionization detectors (FID), HP-5 (BP-1) (5% phenyl+95% dimethylpolysiloxane) 30 m×0.25 mm ID, 0.25 µm film thickness fused capillary column and HP Innowax (BP-20; polyethylenglycol) 30m×0.25 mm ID, 0.25 µm film thickness fused capillary column. The carrier gas was nitrogen (1.2 ml mn^{-1}). 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). Constituents' percentages were calculated by electronic integration of FID peak areas without the use of response factor correction. The analysis of the volatile constituents was run on a Hewlett -Packard GC-MS system (GC: 5890-series II; MSD 5972). The fused-silica HP-5 MS capillary column (30m x 0.25mm ID, film thickness of 0.25µm) was directly coupled to the MS. The carrier gas was helium, with a flow rate of 1.2 ml mn^{-1}. The temperature of the oven was set at (50°C for 1 min, then 50-280°C at 5°C/min) and subsequently, held isothermal for 2 minutes. Injector port: 250°C ,detector: 280°C, split ratio 1:50. Volume injected: 1 µl of 1% solution (diluted in hexane): HP 5972 recording at 70 e Volts; scan time 1.5 sec; mass Range 40 - 300 amu. Software was adopted to muddle through mass spectra and chromatograms was a Chem Station. The components of the oils were identified by comparison of their mass spectra with those of a computer library (11-13).

3.3. Preparation and culture of microorganisms

The lyophilized standard strains *Streptococcus mutans* (ATCC: 35668), *Lactobacillus rhamnosus* (ATCC: 7469) and *Actinomyces viscosus* (ATCC: 15987) were prepared from ATCC reference center. In order to prepare bacteria from lyophilized samples, first samples were cultured in a nutrient broth overnight at 30-35°C. Afterwards, the turbidity of samples isolated and purified was tested on BHI Agar Slants with 5% Sheep Blood, MRS Agar and BHI agar, respectively (10).

3.4. Antibacterial susceptibility testing

Antibacterial activity of essential oil was evaluated by the agar-disk diffusion method (ADAM). Overnight bacterial suspensions were first adjusted to 0.5 McFarland turbidity standards (approximate concentration: 1.5×10^{8}cfu/ml). The inoculums of bacterial suspensions were delivered onto Muller Hinton agar plates using a sterile swab, aseptically. Sterile blank disks (diameter 6 mm) were impregnated by 20µl of the essential oil in dilutions of 62.5, 125, 250, 500, 1000 and 2000 µg/µl to optimization. Then impregnated disks were completely dried for 24 hours inside oven at 40ºC. The disks impregnated with the solvents were considered as controls. These disks were placed on Mueller Hinton agar medium containing bacteria by pence in regular intervals. Plates containing bacterial cultures and essential oil were incubated at 37°C for 18-24 hours. Antibacterial activity was evaluated by Clinical and Laboratory Standards Institute (CLSI) guidelines (Oxoid). Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded (14-17).

3.5. Determination of Minimum inhibitory concentration (MIC) of Mentha longifolia

The MIC values were determined for the bacterial strains based on a macrodilution method that was sensitive to the essential oil in disk diffusion assay. The inoculums of the bacterial strains were prepared from overnight cultures. Suspensions were adjusted to 0.5 McFarland standard turbidity. The mix of bacteria and dilutions of the essential oil were incubated at 37ºC for 24 hours in Muller Hinton broth. After this period, the concentration of first tube without turbidity was considered as MIC (bacteriostatic). In the next stage the contents of the non-growth tubes were cultured in plates. The first plate that growth was inhibited in it was Minimum Bactericidal Concentration (MBC), indicating bactericidal activity. Controls were as follows: medium and essential oil without bacterial non growth, medium and distilled water with bacteria showing growth, medium and chlorhexidine (positive control) with bacteria showing non growth (15, 17-19).

4. Results

4.1. Chemical composition of M. longifolia essential oil

In this study, we intend to determine the chemical composition of volatile essential oil of the *M. longifolia* collected from Ardabil region, Iran (Table 1). The oil was light yellow and liquid at room temperature; its odor was acceptable. This study allowed identification of 22 compounds representing 95.47% of the totality of the components of the oil of *M. longifolia*. The oil was particularly rich in pulegone (31.78%), 1,8-cineole (15.99%), menthoforan (11.25%), cis-isopulegon (10.5%) and paramenth-3-n-8-l (6.85%).

Table 1. The major compounds of *M. longifolia* essential oil

Number	Components	Kovats index (KI)		Percentage (%)
		HP-5	HP-20M	
1	α-pinene	935	1028	1.78
2	camphene	961	1056	0.53
3	sabinene	973	1069	0.50
4	2-β-pinene	982	1080	3.12
5	β-myrcene	994	1122	0.48
6	3-octanol	996	1136	0.58
7	1,8 cineole	1032	1219	15.99
8	paramentha 3,8-dien	1070	1259	0.59
9	isopentyl-2- menthylbutanoat	1101	1299	0.89
10	paramenth-3- n 8-l	1151	1477	6.85
11	menthoforan	1166	1537	11.25
12	cis-isopulegon	1176	1601	10.5
13	burneol	1192	1627	0.85
14	neo iso dihydrokarool	1223	1643	1.51
15	pulegone	1247	1717	31.78
16	2-cyclohexane-1-van	1344	1938	3.65
17	1-desen	1352	1945	1.51
18	piperitenone oxide	1367	1949	0.24
19	cis-jasmon	1397	1967	0.38
20	1,4-benzen di amin	1491	1977	0.22
21	spatolnol	1577	2011	0.53
22	caryophyllene oxide	1584	2023	1.74

4.2. Antibacterial activity, MIC and MBC

The results of antibacterial activity of native *Mentha longifolia* essential oil on *Streptococcus mutans*, *Lactobacillus rhamnosus* and *Actinomyces viscosus* by the disk diffusion agar susceptibility test method has been shown in table 2 ($p < 0.05$). The results of broth macrodilution test method presented in table 3 shows that rates of MIC and MBC at concentration of 500 µg/µl ($p < 0.05$).

Table 2. The mean and standard deviation of different concentrations of *Mentha longifolia* essential oil (µg/µl) on the non-growth zone (mm) of bacteria that cause tooth decay

	62.5	125	250	500	1000	2000	control
L. rhamnosus	7.1±0.7*	11.3±1.8	16.3±2.2	27.2±1.9	22.9±1.8	19.8±1.7	0 ±0.0
S .mutans	4.9±0.9	6.9±2.2	8.2±2.6	12.2±2.8	10.1±2.3	8.9±2.1	0 ±0.0
A. viscosus	1.2±0.2	1.9±.0.6	2.9±0.9	4.8±0.9	3.7±0.8	2.6±0.6	0 ±0.0

* level of significance ($p < 0.05$)

Table 3. The amount of (MIC) and (MBC) of *Mentha longifolia* essential oil on bacterial species (µg/µl)

	MIC	MBC
L. rhamnosus	80*	120
S .mutans	110	165
A. viscosus	450	650

*level of significance ($p < 0.05$)

5. Discussion

It has been proven that dental caries is a disease associated with microbial plaque and increases in the presence of bacterium such *Streptococcus mutans*. *S. mutans, L. rhamnosus and A. viscosus* are the most important bacteria in plaque formation. It has been proven that these microorganisms are essential for dental caries at different levels of dentition (10). Thus, eliminating the microbial basis of dental caries is one of the measures to prevent this widespread infection. Because of the increased prevalence of infection in different communities and due to increased resistance to antibiotics, it is important to explore new sources of medicines to deal with this disease. Hence, in this study the chemical compositions and antibacterial effect of essential oil of *Mentha longifolia* was investigated on the main factor of tooth decay. The oil composition showed 22 compounds including α-pinene (1.78 %), camphene (0.53 %), sabinene (0.50 %), 2-β-pinene (3.12 %), β-myrcene (0.48 %), 3-octanol (058 %), paramentha 3,8-dien (0.59), isopentyl-2-menthylbutanoat (0.89), burneol (0.85 %), neo iso dihydrokarool (1.51 %), 2-cyclohexane-1-van (3.65 %), 1-desen (1.51 %), piperitenone oxide (0.24 %), cis-jasmon (0.38 %), 1,4-benzen di amin (0.22 %), spatolnol (0.53 %), caryophyllene oxide (1.74 %) and particularly it was rich in pulegone (31.78%), 1,8-cineole (15.99%), menthoforan (11.25%), cis- isopulegon (10.5%) and paramenth-3-n 8-l (6.85%). In antibacterial susceptibility test, the non-growth zone of bacteria expanded with increase of oil concentration until 500 µg/µl, and dropped at concentrations 1000 and 2000 µg/µl dramatically. The maximum inhibitory zones seen at 500 µg/µl were 27.2, 12.2 and 4.8 mm in *L. rhamnosus*, *S .mutans* and *A. viscosus*, respectively. Far more effect was on *L. rhamnosus*. The rates of MIC and MBC of oil were 110, 165, 80, 120, 450 and 650, µg/µl on *Streptococcus mutans, Lactobacillus rhamnosus* and *Actinomyces viscosus*, respectively.

Pennyroyal plant is one of medicinal plants with edible use. Methanol essential oil has antimicrobial effects on a wide range of gram-negative and gram-positive bacteria and fungi (10, 14). According to previous studies, essential oil of pennyroyal extracted from hydro alcoholic extract has several compounds such as piperitone (60 -80%), 1,8 cineole (2-7%), beta-caryophyllene (5-15%) and flavonoids such as hesperidin and quercitrin (20).

The comparison of the chemical composition of the essential oil obtained from Mentha species demonstrates strong differences between countries. 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. 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 (13). Gulluce and colleagues (2007) studied the antimicrobial and the antioxidant properties of Turkish *M. longifolia L.* ssp. *longifolia* (oil and methanol extract) and found 45 components with 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%) as the dominant ones (14). Younis and collaborators (2004) showed the presence of 22 compounds in the aerial parts of *M. longifolia* ssp. *schimperi* grown in Sudan, the major components 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 (21). 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 the factors related to ecotype and environmental influences (21).

6. Conclusion

In accordance to the results obtained from the present study, the antibacterial effects of *Mentha longifolia* on differentmicroorganisms are important and requires further investigation for mechanism of action. However, due to the interest of the international community in traditional treatments, preparations of products such as antimicrobial mouthwash from these plants appear to be more preferable. The native pennyroyal essential oil in Ardabil inhibited growth of bacteria that causes tooth decay. In studied concentration range, the essential oil also had bactericidal effect on the *Streptococcus mutans*, *Lactobacillus rhamnosus* and *Actinomyces viscosus*.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contributions

All authors contributed extensively to the work presented in this paper.

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