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Investigation of Antibacterial Activity of Ethanolic and Methanolic Extracts of Mentha pulegium L.

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Abstract

***Background*:** With regard to the rapid emerging antibiotic resistance bacteria, plants as one of the most common natural sources of antimicrobial agents can be used as alternative for treatment of infectious diseases. This study was designed to investigate antibacterial activity of *Mentha pulegium L*. (Lamiaceae family).

Materials and Methods: In this experimental study, the antibacterial effect of 4, 8, 16 and 24 mg/disc of alcoholic extracts were assessed using standard disc diffusion method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration **(**MBC) were also determined. Furthermore, the structural changes following to the exposure with these extracts were also investigated in test bacteria.

Results: Both extracts of this plant showed considerable antibacterial activity against some Gram positive (*Bacillus cereus* and *Staphylococcus aureus)* and also Gram negative (*Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis*) bacteria. All of the tested bacteria were resistant to nafcillin. The maximum effects was observed in the case of both ethanolic and methanolic extracts in all concentrations on *P. mirabilis* (25 mm) and the lowest effect was on *P. aeruginosa.* MIC and MBC values of both extracts against *S. aureus* were equal (MIC=MBC=8 mg/mL) and *P. mirabilis* were MIC=4 mg/mL and MBC=8 mg/mL. The SEM analysis revealed deformation and cell wall disruption of affected bacteria.

Conclusion: Based on these results it can be suggested that *M. pulegium* L. is an effective antibacterial plant that can be used as a new source for antibiotic discovery against bacterial pathogens especially food poisoning pathogens such as *S. aureus, B. cereus* and also for treatment of *P. mirabilis* infection.

Introduction

Plants are the most common source of antimicrobial agents. Their application in traditional health remedies is the most popular for 80% of world population in Asia, Latin America and Africa, because plant products have minimal side effects [1]. In recent years, many countries have spent a lot of time and money to develop natural products extracted from plants and produces more effective remedies for their population [1]. Some metabolites that are present in all higher plants usually play an important role in protection of them as antibacterial, antiviral, antifungal and insecticides. They also may attract some insects to favor the dispersion of pollens and seeds [2]. *Mentha pulegium* L. is one of the 20 species of Mentha genus of Lamiaceae family that is commonly known as pennyroyal [3-6].

The Lamiaceae family contains wide variety of aromatic plants mainly in temperate countries which is native in Europe, North Africa, Asia Minor, and near East [7, 8]. This plant is a perennial aromatic herb with 10 to 45 cm height and a 4 angles stem with small green long-stalked leaves that grows in moist damp streams [9]. Medicinal aromatic plants like as *Mentha pulegium* due to their chemical components with therapeutic effects are important for treating human diseases [10]. This plant has been traditionally used due to its antiseptic effect for treatment of cold, sinusitis, bronchitis, cholera, food poisoning and tuberculosis [3]. Microorganisms including gram positive and gram negative bacteria have been recognized as the main causes of various human infections. With regard to the occurrence of multidrug resistant bacteria, it is necessary to discover new antibiotic sources and plants can be potential source for this purpose [1, 10].

The aim this study was to investigate and compare the antibacterial activity of methanolic and ethanolic extracts of *Mentha pulegium* L. against some bacterial pathogens.

Materials and Methods

In this experimental study, plant materials were collected from Dezful, southwest of Iran, and were identified based on taxonomic criteria in department of biology, Shahid Chamran University, Ahvaz, Iran.

The aerial parts (leaves) of collected plants were dried at room temperature for 10 days and then finely powdered using electronic blender. In order to extract preparation 1

g of plant powder was mixed with 10 mL of 80% ethanol or methanol (ethanol or methanol-distilled water, 8:2 v/v) and vortexed for 1 min. Then these samples were centrifuged at 3000 rpm for 15 min and the supernatant was harvested. This process was repeated 3 times and after three days of incubation, solvents were finally removed by evaporation [11, 12].

The test microorganisms used in this study were: Gram-positive bacteria including *Bacillus cereus and Staphylococcus aureus* and Gram-negative bacteria including *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Proteus mirabilis.* These strains were prepared from the bacterial collection present in department of biology.

Antibacterial activity of the ethanolic and methanolic extracts was discovered using standard disk diffusion method (Kirby-Bauer method) against test bacteria [3]. Stock culture of test bacteria were grown in nutrient broth medium (Merck, Germany) at 37ºC for 24 h. A lawn culture of test bacteria with 0.5 McFarland turbidity then prepared on Muller-Hinton agar (Merck, Germany) using sterile cotton swab [13].

Four concentrations of each extract (100, 200, 400 and 600 mg/mL) were prepared and sterile blank paper disks (6 mm diameter) were saturated with each concentration through adding 40 µL of each extract [14]. So the effective doses per discs were as 4, 8, 16 and 24 mg. The prepared discs were placed on lawn cultures and plates remained at room temperature for about 15 min to allow the diffusion of extract into the medium, and then were incubated at 37ºC for 24 h. Discs containing standard antibiotics were also used as control. After incubation the inhibition zone diameter around each disc was measured (mm). In order to determine the possible inhibitory effect of solvents on test bacteria, discs containing 80% ethanol or methanol were also used.

For determining the minimum inhibitory concentration (MIC) of ethanolic and methanolic extracts, a serial 2 fold dilutions of each extract (0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32 mg/mL) was prepared. These dilutions were added to tubes containing 1 mL Muller Hinton broth (Merck, Germany) and 30 µL of bacterial suspension with 0.5 McFarland turbidity was also added and incubated at 37ºC for 24 h. The MIC of the extract was determined for the most sensitive bacterial species determined in disc diffusion method. The lowest concentration of crude extract in broth culture that had inhibited the growth of the test microorganism was considered as MIC [15].

To determine the minimum bactericidal concentration (MBC), a loopful of broth culture in tubes of MIC which did not exhibit any visible growth in the MIC assay was cultured on freshly prepared sterile Muller-Hinton agar and then incubated at 37ºC for 18-24 h. After incubation the highest dilution (least concentration) that did not show any visible growth and colony formation on agar was considered as MBC [15].

For finding the structural changes induced as a result of bacterial exposure to extracts, a sample from those species that had inhibited by extract were prepared for scanning electron microscopy (SEM). These samples were coated by carbon and studied by SEM of central laboratory in Shahid Chamran University.

Results

The results of antibacterial activity assay of ethanolic and methanolic extracts of *M. pulegium* are presented in table 1. These results showed that different concentrations of both ethanolic and methanolic extracts of this plant had significant antibacterial activity against tested Gram positive (*B. cereus* and *S. aureus)* and gram negative (*E. coli, P. aeruginosa* and *P. mirabilis*) bacteria. The maximum effects was observed in the case of both ethanolic and methanolic extracts in all tested concentrations on *P. mirabilis* (inhibition zone diameter 20-25 mm) and the lowest effect was in case of *P. aeruginosa* (inhibition zone diameter 8-11 mm) by the both ethanolic and methanolic extracts. On the other hand, the ethanolic and methanolic extracts did not have antibacterial activity against *K. pneumoniae* and *S. typhi* even in the highest concentration. The methanolic extract of this plant at 24 mg effective dose had more effect on *B. cereus* than the ethanolic extract. The hydro-alcoholic extract of this plant had no antibacterial activity against 2 Gram negative bacteria including *S. typhi* and *K. pneumoniae.* In general there was no significant difference between antibacterial activity of ethanolic and methanolic extracts of this plant against tested bacteria. The results of antibacterial activity of standard antibiotics were shown in table 2. The results showed that all of the tested bacteria were resistant to nafcillin. All concentrations of ethanolic and methanolic extracts of *M. pulegium* showed higher antibacterial activity against *P. mirabilis* comparing to the tested standard antibiotic. Discs containing 80% ethanol and methanol did not have any zone of inhibition.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ethanolic and methanolic extracts for *S. aureus* and *P. mirabilis* were shown in table 3. MIC and MBC values of both ethanolic and methanolic extracts against *S. aureus* were equal (MIC=MBC=8 mg/mL) while for *P. mirabilis* were MIC=4 mg/mL and MBC=8 mg/mL.

As can be obviously found from SEM analysis the hydro-alcoholic extracts of *M. pulegium* L. have bactericidal effects through targeting bacterial cell wall (Fig. 1). The irregular shape of bacteria reveals that cell wall integrity was disrupted or its synthesis was interfered.

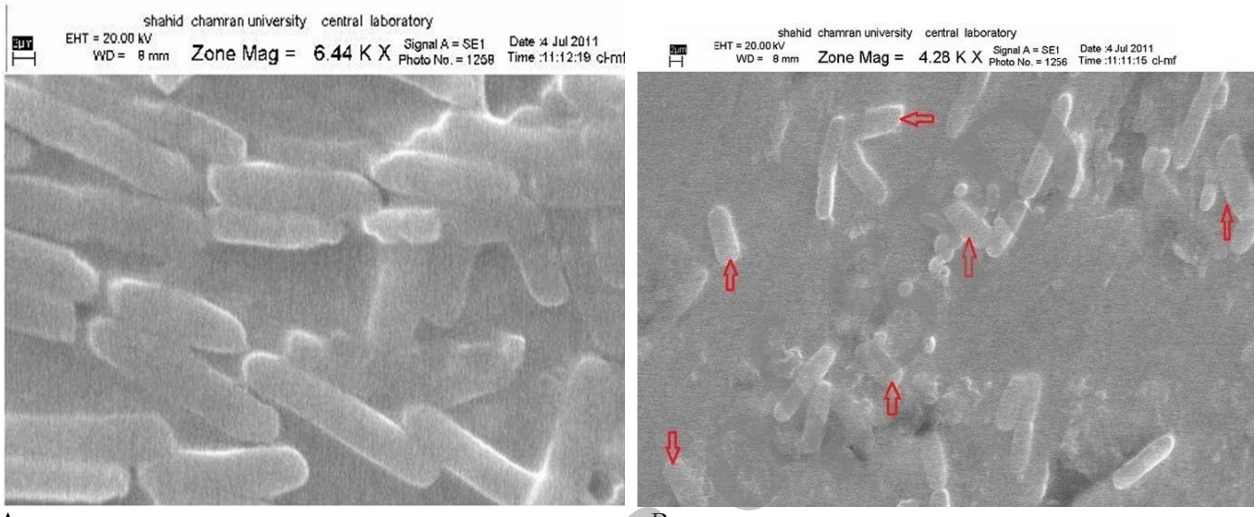
Discussion

Based on the results of this study both extracts of *Mentha pulegium* L. showed considerable antibacterial activity against *B. cereus*, *S. aureus, E. coli, P. aeruginosa* and *P. mirabilis*, that is comparable to the standard antibiotics. The maximum effects was observed in the case of both ethanolic and methanolic extracts in all concentrations on *P. mirabilis* (25 mm) and the lowest effect was on *P. aeruginosa.* MIC and MBC values of both extracts

against *S. aureus* were equal (MIC=MBC=8 mg/mL) and *P. mirabilis* were MIC=4 mg/mL and MBC=8 mg/mL. The SEM analysis revealed deformation and cell wall disruption of affected bacteria.

Mentha pulegium L. is one of the medicinal plants that Iranians used it in traditional folk medicine for treatment of infectious diseases [8]. Aerial parts of this plant contain a wide diversity of secondary metabolites such as: tannins, resins, pectins, bitter principles and essential oils [16]. Hydro-alcoholic extracts and the essential oil of this plant contain polyphenols, flavonoids and the condensed tannins. They are very rich in phenolic compounds [2, 16]. These secondary metabolites can act as defense mechanisms against pathogenic microorganisms [17].

The therapeutic effects of this plant are significantly evaluated by antioxidant activity of the extracts like capacity to eliminate free radical DPPH (diphenylpicrylhydrazyl radical) and by the capacity to eliminate the anion superoxide ($O_2^{\cdot}$) [2]. Phenolic compounds were reported to play an important role in inhibiting autoxidation. The anti-oxidative effects of this extract could be assigned to the presence of some phenolic compounds related to phenolic acids, rosmarinic acid and polyphenols [2, 18].



A B

Figure 1. Structural changes of affected E. coli following exposure to hydro-alcoholic extracts. A: Unaffected bacteria. B: Deformation and shape changing from rod to cocci or irregular shapes

Table 1. Inhibition zone (mm)* of Mentha pulegium L. ethanolic and methanolic extracts at various concentration on pathogenic bacteria

Various concentration of extract (mg/mL)		Ethanolic extract (mg effective dose)				Methanolic extract (mg effective dose)			
Bacterial Spp.		4	8	16	24	4	8	16	24
Gram-positive bacteria	S. aureus	15	16	15	15	15	16	17	17
	B. cereus	R	R	10	12	R	9	12	14
	S. typhi	R	R	R	R	R	R	R	R
	E. coli	12	12	13	14	12	12	13	13
Gram-negative bacteria	P. aeruginosa	R	R	R	10	R	R	8	11
	K. pneumoniae	R	R	R	R	R	R	R	R
	P. mirabilis	20	21	23	25	20	21	23	24

R: Resistant, * (6 mm) diameter of disc

Table 2. Inhibition zone (mm)* of standard antibiotics on tested bacteria

Bacterial Spp. \ Antibiotic disks		NF	CB	NB	DX	CL
Gram-positive bacteria	S. aureus	R	13	30	15	R
	B. cereus	R	7	18	18	R
	S. typhi	R	23	30	26	R
	E. coli	R	R	17	11	R
Gram-negative bacteria	P. aeruginosa	R	R	12	R	15
	K. pneumoniae	R	R	15	R	11
	P. mirabilis	R	15	17	R	R

NF: Nafcillin 1 µg, CB: Carbenicillin 100 µg, NB: Novobiocin 30 µg, DX: Doxycyline 30 µg, CL: Colistin 10 µg

Table 3. MIC and MBC of ethanolic and methanolic extracts of Mentha pulegium L. against S. aureus and P. mirabilis

Bacterial Spp.	Ethanolic extract (mg/mL)		Methanolic extract(mg/mL)	
	MIC	MBC	MIC	MBC
S. aureus	8	8	8	8
P. mirabilis	4	8	4	8

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

In the study of Hassanpouraghdam et al. it was showed that essential oil from *Mentha pulegium* from northern parts of Iran contains 46 components including monoterpenes (78.9%) [16]. Mahbouby and Haghi showed that main oil components (71%) of *Mentha pulegium* were piperitone and piperitenone [3-6]. This study showed that the ethanolic and methanolic extracts of *Mentha pulegium* L. have significant antibacterial activity against Gram positive and Gram negative bacteria. Diameter of inhibition zones showed that hydro-alcoholic extracts of this plants is more effective against gram negative bacteria like as *E. coli* and *P. mirabilis* and also *S. aureus*, but this was not true about *S. typhi* and *K. pneumoniae* because of hydrophobic lipo-polysaccharide in the outer membrane and polysaccharide capsule which can provide protection against different agents [3]. These results are in agreement with the obtained results in the present study.

In contrast Hajlaoui et al. found that methanolic extract of this plant had no significant effect against *E. coli* and S. *aureus* [2]. The possible reason can be due to this fact the growth region of the plants have direct effect on their composition and active constituents. Also differences in climate conditions can affect the physiology of plants and consequently their active materials [12]. The antibacterial mechanism of terpenes is not fully understood but it might be due to the hydrophobic characters of phenolic compound that could potentially impair cellular function and its membrane permeability and integrity [2, 13, 18]. This plant due to the presence of pulegone in its extract has been introduced as a potent insecticide, also [17]. It has been reported that the native *M. pulegium* in Iran contains less than 0.1% pulegone [18]. Impact of ethanolic extract of *M. pulegium* on *S. aureus* in lower concentrations was increased that it could be due to reduction of some inhibitory factors in the extract [15] or related to hydrophobic characters of extract components [3]. Despite some previous records, both ethanolic and methanolic extracts of this plant had considerable antibacterial activity against some pathogenic bacteria. It could be related to the region of plant growth. One important finding in this study was this fact that active components of this plant affect cell wall. As a common rule those antibacterial agents that can disrupt cell wall or inhibit its biosynthesis are of great importance. These agents act as bactericidal agents and so it is unlikely that after their usage the infection relapses. In most medicinal plant studies this step is ignored while finding those medicinal plants that can affect bacterial cell wall can help to find effective bactericidal agents. Finally it should be noticed weather condition is very decisive in producing active substances in plants and hence this plants that grown in different climate area should be screened for bioactive compounds [17].

With regard to these results it can be suggested that *Mentha pulegium* L. is an effective medicinal plant that have high potential to be used as new source of antibiotic against bacterial pathogen. Addition of this plant in foods as a food additive can control food poisoning pathogens such as *S. aureus*, *B. cereus* and also *P. mirabilis* infection. Also have the potential to be applied in treating opportunistic infections due to *P. aeruginosa*.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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