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Antimicrobial and Anti-Biofilm Effects of *Mentha piperita* and *Zataria multiflora* on Pathogenic Bacteria

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ABSTRACT

Background: The aim of this study was to investigate the antimicrobial and anti-biofilm effects of *Mentha piperita* and *Zataria multiflora* on some pathogenic bacteria.

Methods: *Mentha piperita* and *Zataria multiflora* essential oils were obtained by using the clevenger device. The bacterial cultures were prepared as standard samples. Finally, antimicrobial and anti-biofilm activity was determined by microdilution method.

Results: The results of this study showed that the lowest inhibitory concentration of essential oil of *Zataria multiflora* was 1.25 mg / ml, while the rest of the bacteria were inhibited at a concentration of 2.5 mg / ml. The lowest and highest inhibitory concentrations were found as 1.25 and 5 mg / ml against *Pseudomonas aeruginosa*.

Conclusion: The results of the study showed that *Z. multiflora* essential oil showed antimicrobial and anti-biofilm activity that could be used to treat infections caused by these bacteria.

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Introduction

Medicinal herbs are the potential sources of long-term medical and therapeutic benefits (1). Herbal Essences are volatile and aromatic compounds that exist in different organs of the plant, and one of the roles that they play in the plants is protecting the plant against plant infections (2). These compounds have different components in their composition, which reduces the resistance of bacteria to fully applied herbal essences. Plant essential oils simultaneously affect different parts of the bacteria, which makes them important in the treatment and absence of significant resistance (3).

Mentha piperita's peppermint, which is described in traditional books as Susanber, is a perennial herb of Lamiaceae, Lamiales and Rosidae (4). The medicinal properties of these plants are antispasmodic, anticonvulsant, anti-inflammatory, antibacterial and anti-fungal (5).

Peppermint also has microbial and anti-cancer properties. The oil of this plant is used in home medicine to relieve migraine, headaches, abdominal pain, anxiety and insomnia (6). Also, the leaves of this plant act as a weak cell and have a sedative effect, and it has a significant effect on reducing skin itching (6). The active ingredients of this plant are resins, flavonoids, phenols, carotene, betaine and tannin (7).

Peppermint are one of the most important mint types (Kumar et al., 2011). This plant is reported from most parts of Iran, especially in the north, northeastern provinces, as well as in the Alborz and other areas (8).

Today it is used as a spice and seasoning at homes and restaurants with foods (9). The antimicrobial activity of this plant has been confirmed against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* (10, 11, 12)

It has also been proven that peppermint oil has been responsible for the destruction of a species of *Salmonella* (13) and it has an inhibitory effect on *Candida albicans* (14).

Zataria multiflora is one of the plants of the Mint family, which is native to Iran, Afghanistan and Pakistan. It is commonly used as an antiseptic, anti-inflammatory and anti-inflammatory medicine in traditional medicine and is widely used as a food flavoring (15). *Z. multiflora* has antimicrobial effect, which is mainly related to its phenolic compounds (16). The higher the amount of phenolic matter in the essential oil, the greater its antimicrobial properties. These materials include caracrole, eugenol and thymol (17).

The thyme extracts inhibit the inherent immunity (18) and inhibit the growth of some microorganisms, such as fungi and bacteria (19). Biofilm is a structure composed of a bacterial population enclosed by an exopoly matrix produced by the bacteria. This attribute enables bacteria to connect to different levels and also increase the inherent resistance to antibiotics (20). In most microorganisms, the goal of biofilm formation is to protect microorganisms from severe environmental conditions, host immunity, bacteriophages, harmful chemicals such as natural antibiotics and nutrient uptake (21).

Recently, due to the behavioral differences of microorganisms, a microorganism has a special focus on this physiologically and pathogenic form in a biofilm (22).

The purpose of this study was to evaluate the antimicrobial and anti-biofilm effects of *M. piperita* and *Z. multiflora* on pathogenic bacteria.

Materials and Methods

Bacterial strain

Six antibiotic resistant pathogenic bacteria were used in this research includes: *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 1611), *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 1189), *Enterococcus faecalis* (ATCC1787), and *Streptococcus pneumonia* (ATCC 1234).

Extract preparation and investigation of the antimicrobial effects of the plant extract

The *M. piperita* and *Z. multiflora* used in this study was gathered from Sistan and Baluchistan province at spring. They were detected to be wind cheese by a researcher from the University of Zabol. The samples were cut and 10 g of the dry plant powder was put into half-liter flasks containing 100ml of methanol. The contents of flasks were shaken 24 hours at room temperature b shaker device with speed of 130 rpm and were kept in ultrasonic carrier for 10 minutes and were filtered with Wattman paper. Separation of solution from extract was done by rotary device and vacuum pump. The obtained extract was weighed and solved in DMSO. The samples were used in fridge at 4 degrees of temperature to be used in antimicrobial tests.

Antibiotic resistance pattern of bacteria

The sensitivity and resistance of bacteria to some traditional antibiotics were done. The antibiotics that were used for this experiment was as follow: carbenicillin (CAR), oxacillin (OXA), amikacin (AMK), cefazolin (CFZ), vancomycin (VAN), ampicillin (AMP), gentamicin (GEN) and azithromycin (AZM). The concentrations of all used antibiotics were 10 micro-gram per milliliter. All antibiotics purchase from Sigma-Aldrich.

The antimicrobial effect of the extract on bacteria

Sensitivity of the bacteria samples with multiple resistances to the wind *M. piperita* and *Z. multiflora* against bacteria was analyzed by dilution method in broths. To this end, seven broths of microtitre plates were injected 100 ml of MHB. The first broth was added 100 ml of the diluted extract. Then, 100 ml of the first broth was transferred to the second one and the same was done to the last broth. 100 ml of the last broth was removed and 100 ml of the microbial suspension with 107 units per ml was added to all

broths. The mixture was kept 24 hours at temperature of 37 degrees. The first broth inhibiting the growth of bacteria after being positioned in the incubator was considered as MIC and for more precision; 10 ml of the light broths was transferred to Moller environment. After 24 hours, the first concentration removing 99.9% of the bacteria was regarded as MBC.

Biofilm formation assay in the presence of the biocides

After performing the procedure described above, the microplate was covered and incubated aerobically for 24 h at suitable temperature. At first, the OD (Optical Density) was measured (600 nm) by using an automated ELISA counter, then, the content of each well of the microplate was aspirated and each well was washed three times with 250 μ L of sterile physiological saline. The remaining attached bacteria were fixed with 200 μ L of 99% methanol per well and after 15 min all of the wells were discarded and left to dry. Then, each well was stained for 5 minute with 0.2 mL of 2% crystal violet. Excess stain was rinsed off by washing the plate slowly with distilled water. After the plate was air dried, the dye bound to the adherent cells was solubilized with 160 mL of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 492 nm by using an automated ELISA counter.

Statistical analyses

The growth was compared at each experiment using analysis of variance (ANOVA) repeated measures (SPSS-16.0 for Windows). The level of statistical significance was set at $p < 0.01$.

Results

The results of this study showed that the lowest inhibitory concentration of essential oil of *Z. multiflora* was 1.25 mg / ml and *P. mirabilis* was inhibited in this concentration while the rest of the

bacteria were inhibited at a concentration of 2.5 mg / ml. The lowest and highest inhibitory concentrations were found to be 1.25 and 5 mg / ml against *Pseudomonas aeruginosa* and *E. faecalis* (Table 1).

The highest lethal concentration is 10 mg/ml, which is seen against *E. faecalis*.

The results of antibiotic resistance studies showed that *S. pneumonia* is resistant to OX-CZ antibiotics, *E. faecalis* is resistant to Ox-CZ-AM-AZM antibiotics, *P. mirabilis* bacteria is resistant to AN-OX-AZN, *S. aureus* is resistant to AN-OX-AZN antibiotics and *P. aeruginosa* bacteria were resistant to all antibiotics (Table 2). The results of this study showed that biofilm formation in essential oil concentrations has decreased compared to control sample. The rate of biofilm formation in *Pseudomonas aeruginosa*-*Staphylococcus aureus* and *Proteus mirabilis* was highest (Table 3).

Previous TB Related history among participants

The history of contact among cases was higher 19(18.6%) than controls 11(10.8%). Similarly, the proportion of participants who heard about MDR-TB among cases 33(32.4%) was lower than controls 68(66.7%). From the study participants 93(91.2%) cases and 70(68.6%) controls had pulmonary TB (Table 2).

First line TB treatment related factors of MDR-TB

The proportion of previous TB infection was 74(72.5%) among cases 8 (7.8%) among controls. Concerning participants' treatment category, 38 (51.4%) of cases and 22.2% of control were return after default, failure retreatment, or relapse (Table 3).

Discussion

In a study by Atai Kachwei et al., effect of *M. piperita* essential oil on *Escherichia coli* - *Salmonella typhimurium* and *Staphylococcus*

aureus - the isolation of meat was explored, and the results showed that the inhibition zone against *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* were 2.91 ± 8 , 5.8 ± 7 and 8.22 ± 1 mm (23).

Izadi et al. investigated the effect of *M. piperita* on bacteria, and the results showed that the inhibitory hole diameter of *M. piperita* essential oil against *E. coli*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Listeria monocytogenes* were 13.63 ± 1.53 , 11.67 ± 1.15 , 9 , $1.53 \pm$ mm; respectively (24).

Alvandi et al. evaluated the antimicrobial properties of *M. piperita*, and the results showed that *M. piperita* herbicides had a inhibitory effect on *Salmonella typhimurium* up to a concentration of 300 milligrams per *Staphylococcus aureus* (25 ppm) (25)

Abdolmaleki investigated the effect of *M. piperita* essential oil on herbivorous fungi, and the results indicated that the inhibitory halo diameter at 1000 ppm against *R. solani*-*F. oxysporum*, *P. drechleri* and *B. sorokinianis* fungal species was equal to 0.85 ± 21.25 , 29.25 ± 1.1 , 6 ± 00 , 6 ± 00 mm (26).

In the study of Amiri and Jompour, the minimum inhibitory concentration of *M. piperita* extract on MRSA, MSSA, *E. coli* and *S. typhimurium* was reported to be 3.25-3.25-25 and 12.5 mg / ml, while the inhibitory hole diameter Peppermint peanuts were 15-21- and 25-mm-14 against these bacteria (27).

Iscan et al. found that the essential oil had a greater antimicrobial effect on gram-positive bacteria than gram-negative bacteria. The antibacterial properties of essential oils seem to depend on their hydrophobic properties and on the walls of the plasmid membrane of the microbes. Increasing the amount of certain ions on or inside the plasma membrane has a large impact on the driving force of the protons, the amount of intracellular ATP, and the overall activity of the microbial cells (including controlling the inflation of live cells, transferring the solubilizing material and regulating the metabolism) (28).

Table 1. Results of minimum inhibitory concentration and minimum concentration of *M. piperita* and *Z. multiflora* against Bacteria.

Bacterial Code	<i>M. piperita</i>	<i>Z. multiflora</i>
<i>S. pneumoniae</i>	2.5/ 5	2.5/ 5
<i>E. faecalis</i>	5/ 10	2.5 / 5
<i>P. mirabils</i>	2.5 / 5	1.25/ 2.5
<i>S. aureus</i>	2.5 / 5	2.5 / 5
<i>E. coli</i>	2.5 / 5	2.5 / 5
<i>P. aeruginosa</i>	1.25/ 2.5	2.5 / 5

Table 2. Antibiotic resistance pattern of bacteria.

Bacterial	CAR	OXA	AMK	CFZ	VAN	AMP	GEN	AZN
<i>S. pneumoniae</i>	S	R	S	R	I	I	S	S
<i>E. faecalis</i>	S	R	I	R	S	R	S	R
<i>P. mirabils</i>	R	R	S	I	S	S	S	R
<i>S. aureus</i>	R	R	S	S	I	S	I	R
<i>E. coli</i>	S	R	S	S	S	S	S	R
<i>P. aeruginosa</i>	R	R	R	R	R	R	R	R

Table 3. Results of Anti biofilm *M. piperita* and *Z. multiflora* against Bacteria.

Bacterial Code	<i>M. piperita</i>	<i>Z. multiflora</i>	Control any essential oil
<i>S. pneumoniae</i>	0.007	0.005	0.013
<i>E. faecalis</i>	0.010	0.007	0.015
<i>P. mirabils</i>	0.011	0.018	0.018
<i>S. aureus</i>	0.010	0.015	0.021
<i>E. coli</i>	0.007	0.004	0.011
<i>P. aeruginosa</i>	0.008	0.012	0.029

Singh et al. (2011) reported that the mint extract had a more effective impact on gram-positive bacteria than gram-negative bacteria. The diameter of the non-growth zone for Gram-negative bacteria of *Klebsiella pneumoniae* and *Escherichia coli* was 12.4 and 5.1 mm, respectively, whereas for gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus pyogenes*, the amounts were 17.2 and 3.1 mm, respectively (29). Fazeli et al. studied the inhibitory properties of *Zataria multiflora* extract on foodborne pathogens, and it was determined that both plant extracts had inhibitory effects on bacterial pathogens such as *Salmonella typhi*, *Escherichia coli* and *Staphylococcus aureus*, and

with increasing concentrations, the inhibitory effect was increased (30).

Atayi et al. investigated the effect of *Z. multiflora* essential oil on growth and production of Shiga toxin-2 *Escherichia coli*, and the results showed that in this study, the MIC and MBC content of *Z. multiflora* was 0.04% and 0.06% respectively (31).

Mirzaei et al. investigated the effect of dill flower extract on methicillin-resistant biofilms of *Staphylococcus aureus*, and the results showed that among 50 strains of *Staphylococcus aureus*, 10 (20%) strains of biofilm were positive. Also, quantitative determination of *icaD* gene expression in selected strains under the influence of Sub MIC concentration of extract showed that

expression of *ica D* gene significantly decreased (32).

Jafari et al. investigated the effect of chlorella vulgaris algae on the formation of *Streptococcus mutans* biofilms, and the results showed that prevention of biofilm formation at concentration of 50 mg / ml and higher was effective in assessing the toxicity of the extract at a concentration of 100 mg / Millet (33).

Rahimpour et al. investigated the effect of Basil essential oil on biofilm production of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

In the study of Hasani et al., The effects of green and black tea extracts on the biofilm formation of *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Shigella flexneri* were measured. The results showed that the inhibitory effect of biofilm formation in black tea was higher than green tea. The concentration of 4.5 mg / ml of black tea extract at 5 mg / ml of green tea extract had a bactericidal effect on these micro-organisms. Among these bacteria, *Proteus mirabilis* was the most susceptible to black tea and *Escherichia coli*, which showed the highest susceptibility to green tea. *Klebsiella pneumoniae* showed the highest resistance to both extracts (34).

In the study of Mohsinipour and Hasanshahian, the antimicrobial effects of pomegranate algae on single and biofilm formulas of six bacteria was explored, and the results showed that in the liquid medium, the MIC test showed a good inhibitory effect on all bacteria (70%). These extracts eliminated biofilm structures by at least 50% and up to 95%. The inhibitory effect of the extract depends on its concentration and solvent type. The highest inhibitory effect was that of biofilm formation on *Staphylococcus aureus* (95.84%) and *Pseudomonas aeruginosa* (51.48%) showed the highest degradation in treatment with extracts of this plant. The highest inhibition of metabolic activity was observed in *Bacillus cereus* (77.13%) (35).

Conclusion

The results of this study confirmed that the extract of *M. piperita* had sufficient inhibitory effect against antibiotic resistant bacteria and may be used as antimicrobial agents.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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