Original Research Article

Identification of chemical composition of essential oil and evaluation of antimicrobial effects of ethanolic extract of *Mentha pulegium* on *Staphylococcus aureus* and *Escherichia coli*

Abolfazl Jafari-Sales [1,*], Parisa Hossein-Nezhad [2], Parisa Bolouri [3]

[1]Department of Microbiology, School of Basic Sciences, Kazerun Branch, Islamic Azad University, Kazerun, Iran
[2]Department of Food Science and Technology, Marand Branch, Islamic Azad University, Marand, Iran
[3]Department of Field *Crops*, Faculty of Agricalture, Ataturk University, Erzurum, Turkey

***Corresponding author:**. *Abolfazl Jafari-Sales, Department of Microbiology, School of Basic Sciences, Kazerun Branch, Islamic Azad University, Kazerun, Iran. Email: A.jafari_1392@yahoo.com*

DOI: 10.22034/HBB.2019.10

Received: March 16, 2019; **Accepted:** May 18, 2019

ABSTRACT

Increasing antibiotic resistance in strains of bacterial pathogens increase the attitude of people to find new compounds as an appropriate alternative to antibiotics. In this study, the antimicrobial effects of methanolic extract of *Mentha pulegium* against *S. aureus* and *E. coli* strains were investigated. After collecting the plants, essential oil of aerial parts of the plant was done by distillation with water through the clevenger apparatus. Gas chromatography-mass spectrometry (GC / MS) was used to identify the chemical composition of the essential oil. *M. pulegium* extract was prepared by soxhlet extractor method and their antimicrobial effects were investigated using agar well diffusion and dilution methods. The results of GC / MS analysis showed 22 compounds in *M. pulegium* plant. The results showed that *M. pulegium* methanolic extract prevents the growth of *S. aureus* and *E. coli* bacteria. Also, *S. aureus* showed a higher sensitivity to different concentrations of *M. pulegium* extract. Therefore, after supplementary studies, it could be used as a substitute the treatment of infections.

***Keywords*:** Antibacterial effects, extract, *Mentha pulegium, Staphylococcus aureus, Escherichia coli*, essential Oil, GC / MS

INTRODUCTION

Human society always improve their needs, and in this regard they used their own random experiences to improve their individual and social life. One of these valuable experiences is application of medicinal herbs to treat diseases [1]. Medicinal herbs always are related to humans and plants throughout the development of all civilizations. Although most plant species are known, there is still a long way to discover new and valuable herbal resources [2,3]. In this way, plants could be considered as a useful source to make pharmaceutical analogues [4-7]. One of the most important therapeutic challenges is coping with infectious diseases due to their high prevalence. After recognizing penicillin and expanding its use in treatment, new antibiotics were introduced every day for the treatment of infections. The result was the expansion of clinical use of natural and synthetic antibiotics in the treatment of infections. The excessive use of these antimicrobials lead to increase resistance to different antibiotics in most bacteria [8,9]. Also, these herbal medicines are more popular with people [10]. These reasons are due to the increasing wave of widespread worldwide studies and the introduction of antibacterial effects of various plants in recent years [11]. *Mentha pulegium* is a family of *Lamiaceae* with tranquil, analgesic and antiseptic properties, especially diabetes treatment, found in Europe, Australia, South Africa, the mediterranean and East of Iran [12-13]. This plant is used as a perfume, seasoning and food spice. The antimicrobial properties of this plant are due to potent pulegone and 8-1 cineol compounds [14]. The leaves and branches of this plant are used as food and tea [15]. Although *M. pulegium* oil is very toxic, it is commonly used in fresh and dry form [16]. This plant grows well in mountainous areas and in wet places, and is widely used as a medicinal plant, especially for the improvement of the immune system [17]. The antibacterial effects of *M. pulegium* on gram-positive bacteria are higher than gram negative bacteria, with the highest effect on *S.aureus* [18]. Therefore, this study investigated the chemical composition of the essential oils derived from the aerial parts (leaf, flower and stem) and the antibacterial effects of methanolic extracts of *M. pulegium* on *S. aureus* and *E. coli*.

MATERIALS AND METHODS

In this study, plant samples were collected from natural areas around Marand city in East Azarbaijan province, Eish-Abad village. The samples were cleaned after collection and dried in a large and convenient area. After complete drying of the specimens, aerial parts were prepared for milling. For extraction of 100 g of aerial parts of the plant was used. Essential oil from aerial parts was obtained by hydrodistillation method by using clevenger apparatus. After digestion with sodium sulfate, it was stored in the refrigerator before injection into a GC / MS device at 4 °C. The essential oil sample was injected into the gas chromatography–mass (GC / MS Agilent type-American) after obtaining a thermal planning system [19]. Identification of compounds using the device database and comparing their mass spectrometry were carried out. Soxhlet extractor method was used for extraction, So 60 g of ***M.*** *pulegium* powder with 300 ml of methanol as a solvent was placed in a soxhlet extractor for 8 h, this solvent was evaporated at 40 °C using a rotary machine and evaporated slowly. The extracts were concentrated with 5 % DMSO solvent, concentrations of 20, 30, 50 and 400 mg/ml were prepared for minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and agar well diffusion. The microorganisms were used in this study: *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 (the microbial collections of the University of Tehran). To achieve a concentration of 1.5×10^6 CFU/ml/bacteria per ml, a microbial suspension with a McFarland turbidity standard 0.5 was diluted to 0.01. In order to investigate the antimicrobial activity of methanolic extract of 4 concentrations of 20, 30, 50 and 400 mg/ml of methanolic extract of the plant in DMSO 5 % solvent was prepared. In this study, the antimicrobial activity of the methanolic extract was investigated using agar well diffusion and dilution test. In agar well diffusion method, 500 ml of microbial suspensions of 1.5×10^6 CFU/ml was transferred onto agar medium and cultured in a 3-way sterile swab. Then, pits of diameter 6 mm and 2.5 cm apart were created at the agar surface. Subsequently, 100 µl of concentrations of 20, 30, 50 and 400 mg/ml were injected from methanolic extract into each well. The negative control was obtained using a solution that was used to dissolve the extracts (5 % DMSO) and also used as a positive control for chloramphenicol antibiotics. Then the plates were incubated for 24 h at 37 °C and after a certain time, in terms of forming or not forming a non-growth zone in millimeters, were measured. Using the dilution test method, the minimum inhibitory concentration and minimum bactericidal concentration of methanolic extract were determined. In this method, in order to determine the MIC, methanolic extracts from dilutions of 25.6, 12.5, 25, 50, 100 and 200 mg/ml were obtained in mueller hinton broth medium. Then, to each dilution, 1 ml of active bacterial suspension was added. Besides the tubes, positive control (the culture medium containing bacteria, without extracts) and negative control (non-bacterial culture) were used. Finally, the tubes were incubated for 24 h at 37 °C. After incubation, the tubes were examined for turbidity induced by the inoculated bacterial growth and the last dilution in which no turbidity was observed (no growth) as MIC was considered. Subsequently, all tubes in which no bacterial growth was observed were sampled and determined by cultivating the minimum concentration of MBC in the plate. To reduce the error of the test, each of the above experiments was repeated five times. SPSS software version 18 was used to analyze the data. In order to study the significant difference was found between the results of ANOVA and chi-square and the difference between the groups was significant at the significance level of $p < 0.05$.

RESULTS

The results showed that after the injection of essential oil into the GC / MS machine and comparison the output with the reference index, 22 compounds were identified in the essential oil of the *M. pulegium* species. The percentage of substances (chemical compounds) in the essential oil is shown in Table 1. The results of the antimicrobial study of methanolic extracts of *M. pulegium* extract by Agar Well Diffusion method showed that this extract had a significant inhibitory effect on *S.aureus* bacteria. As the concentration of the extract increases, its inhibitory effect also increases. *E.oli* is also inhibited at higher concentrations of the extract. This study showed that the inhibitory effects of *M. pulegium* methanolic extracts on Gram-positive bacteria were higher than Gram-negative bacteria (Table 2). The values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of methanolic extracts of *M. pulegium* herb against bacteria showed that *M. pulegium* extract in *S.aureus* was superior to *E. coli* in bacterial strain (Table 3). These results indicated that there was a significant difference among the tested bacteria in terms of sensitivity of *M.pulegium* extract ($p < 0.05$). In other words, the highest susceptibility to the methanolic extract of *M. pulegium* in *S.aureus* and the least susceptibility to *E.coli* were observed.

Table 1: Chemical compounds of essential oil of *M. pulegium*

No	Compounds	Percentage of compounds (%)
1	Pulegone	51.17
2	Piperitenone	13.84
3	Isomenthone	5.04
5	1,8 cineole	4.08
6	Menthone	3.41
7	Cis Salvene	3.14
8	Piperitenone Oxide	2.91
9	8-hydroxy-D-p- menth-3-one	2.24
10	Delta Terpineol	2.11
11	Endo Borneol	2.01
12	3-Octanol	1.31
13	β-caryophyllene	1.74
14	2,2-dimethyl propylidene	1.27
15	Carvacrol	1.12
16	16-n-buthyl tetra hydropyridine	0.94
17	Limonene	0.91
18	β-pinene	0.51
19	Myrcene	0.3
20	α-pinene	0.3
21	Sabinene	0.1
22	α-Terpineol	0.1
Total Identified Constituents		98.45

Table 2. Diameter of bacteria inhibition zone (mm) in different concentrations of *M. pulegium* methanolic extract by well diffusion method (mean ± SD)

Extract concentration / Bacteria	20 mg/ml	30 mg/ml	50 mg/ml	400 mg/ml	Negative control	Positive control
Staphylococcus aureus	9.06±1.14	14±0.70	19.6±0.83	25.2±1.30	--	20
Escherichia coli	8±0.70	12.6±0.54	15.8±0.83	19.8±1.09	--	26

Table 3. MBC / MIC test for bacteria at different concentrations of *M. pulegium* methanolic extract

Extract concentration / Bacteria	MIC	MBC
Staphylococcus aureus	6.25	12.5
Escherichia coli	12.5	25

DISCUSSION

In this study, 98.4 % of the essential components of *M. pulegium* essential oil were identified, the most important being Pulegone (51.2 %), an antimicrobial formulation. Derwich et al. identified 97.3 % of the active compounds of *M. pulegium* essential oil, of which the most effective compounds were piperitone (35.6 %) and piperitenone (21.1 %) [20]. Shahmohamadi et al. in 2011 [21], Sbayou et al. in 2014 [22], and Sadeghi et al. in 2016 [23], reported pulegone 69.2 %, 78.1 % and 36.7 %, respectively, as the most effective ingredient derived from *M. pulegium* Essential oil. Mahboubi and Haghi analyzed the essential oil of Mentha pulegium L. plant, piperitone (38.0 %), piperitenone (33.0 %), α-terpineol (4.7 %) and pulegone (2.3 %) showed the most effective compounds of this essential oil [24]. The difference with the findings of this study could be attributed to the different separation zone, weather and soil type. Also, by transferring a plant from its natural habitat to crop conditions, there will be significant changes in the effective compositions of the plant and the yield of the essential oil. Increasing pathogen microorganisms and their resistance to a wide range of antibiotics, along with the economic and social problems lead to develope of studies on the production of herbal medicines. Therefore, screening such plants may lead to the discovery of new effective compounds that could inhibit pathogen microorganisms. Compounds that can inhibit the growth of pathogenic pathogens or make them hostile to toxic cells without toxicity which considered as candidates for the production of new antimicrobial drugs. As a result, there is a critical need for research into new antimicrobial agents with promising natural activities to provide alternatives to common antibiotics [25]. Sadeghi et al, examined the essential oil of *M. pulegium*, that the most effective compounds of this plant are pulegone, piperitone and 1-8 cineole. The percentage of these compounds before flowering several times after flowering [26]. Some researchers proved that the active ingredients contained in *M.pulegium* have antibacterial, antifungal, and antioxidant effects [27]. Ehsani and Mahmoudi evaluated the essential oil of *M.pulegium* on *S.aureus* and *L.monocytogenes* showed that this essential oil has significant effects on both bacterial pathogens [28]. In a study in 2018, Ghorbani et al, reported the antibacterial effect of ethanolic extract of *M.pulegium* on *S. aureus, E.coli, Klebsiella* and *S. typhimurium* bacteria. The ethanolic extract of *M.pulegium* has the highest bactericidal potency on *S. aureus* bacteria, which increases the bactericidal capacity by increasing the concentration of the extract [29]. Javanmard and Mahdavi studied the ethanolic extract of *M.pulegium* extract, which showed an antimicrobial effect on *S. aureus* compared to *E.coli* [18]. This can be because of the presence of the lipopolysaccharide layer of gram-negative bacteria that prevent the passage of large hydrophobic molecules, since most of the compounds of the extracts have hydrophobic nature, so they cannot enter the active regions of the Gram-negative bacteria; therefore, the bacteria Negative is more resistant than Gram-positive bacteria to these extracts [30] and since most of the effective compounds in the essential oil and extracts have hydrophobic nature, it can be concluded that these materials are not likely to enter and access the active sites within the Gram-negative bacteria, For this reason, Gram-negative bacteria usually exhibit higher resistance to these compounds than gram-positive species [18]. Mahmodi et al. and Cetin et al. in a separate study in 2011, identified the *M.pulegium* herb effect on Gram-positive bacteria such as *S. aureus* [14, 31]. Also, the results of the present study showed that methanolic extracts of *M. pulegium* extract have an inhibitory effect on *S.aureus* and *E.coli* bacteria, in which Gram positive bacteria are more susceptible than to Gram-negative bacteria, as a result, it is consistent with the findings of Amrita et al. in 2009 [32] , and Riahi et al. in 2013 [33]. In another study, which Ghasemian et al. found with *M.pulegium* extract, they obtained similar results to the results of this study [34]. Therefore, the effect of *M. pulegium* extract on S.*aureus* and *E.coli* showed that the role of pulegone compounds and Cineole 1, 8 in this field is very important [14]. Differences in findings with other studies by researchers could be attributed to the effect of different extraction methods. Also, the extent of antibacterial effects of *M.pulegium* extract are directly related to the environment and climatic conditions of this plant.

CONCLUSION

Considering the cheap and affordable, as well as the considerable effect of methanolic extract of *M. pulegium* on the bacteria studied, this extract could be considered as an appropriate alternative for the production of new natural herbs after further studies on laboratory animals with at least side effects.

REFERENCES

[1]. Ahvazi M, et al. Introduce some of the medicinal plants species with the most traditional usage in east mazandaran region. *JMP*, 2012; 4(44): 164-75.

[2]. Digrak M, et al. Antibacterial and antifungal activities of turkish medicinal plants. *Pharm Biol*, 2001; 39(5): 346-350.

[3]. Skaltsa HD, et al. Composition and antibacterial activity of the essential oils of Stachys Candida and S. Chrysantha from Southern Greece. *Planta Medica*, 1999; 65(3): 255-56.

[4]. Jafari-Sales A, et al. Evaluation of antibacterial activity of ethanolic extract of Malva Neglecta and Althaea Officinalis L. on antibiotic-resistant strains of *Staphylococcus aureus*. *J Biol Today World*, 2015; 4(2): 58-62.

[5]. Jafari-Sales A, et al. Evaluation of antibacterial activity of essential oil of Ziziphora Clinopodioides and Achillea Wilhelmsii on antibiotic-resistant strains of *Staphylococcus aureus*. *IMMINV*, 2017; 2(2): 49-56.

[6]. Mobaiyen H, Jafari Sales A, Sayyahi J. Evaluating antimicrobial effects of Centaurea plant’s essential oil on pathogenic bacteria: *Staphylococcus aureus*, *Staphylococcus Epidermidis*, and *Escherichia coli* isolated from clinical specimens. *JFMS*, 2016; 5(4): 479-87.

[7]. Sales AJ. Evaluation of antibacterial activity of ethanol extract of Lavandula Stoechas L. plant on antibiotic-resistant strains of *Staphylococcus aureus*. *J Curr Res Sci*, 2014; 2(6): 641.

[8]. Sales A, Bagherizadeh Y, Malekzadeh P. Evaluation of the antimicrobial effects of essential oil of Reseda Lutea L. on pathogenic bacteria: *Staphylococcus aureus*, *Staphylococcus Epidermidis*, and *Escherichia coli*. *Arch Clin Microbiol*, 2017; 8(3): 1-5.

[9]. Sales AJ, Shadbad NN, Kaleybar VP. The investigation of the antibacterial effects of ethanol extract of Cichorium Intybus L. on antibiotic-resistant *Staphylococcus aureus* strains. *Bull Env Pharmacol Life Sci*, 2015; 4: 161-64.

[10]. World Health Organization, Traditional Medicine. 2002.

[11]. Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev*, 1999; 12(4): 564-82.

[12]. Duarte MCT, et al. Anti-Candida activity of Brazilian medicinal plants. *J Ethnopharmacol*, 2005; 97(2): 305-11.

[13]. Jouad H, et al. Ethnobotanical Survey of medicinal plants used for the treatment of diabetes, cardiac and renal diseases in the north centre region of Morocco (Fez–Boulemane). *J Ethnopharmacol*, 2001; 77(2-3): 175-82.

[14]. Mahmodi R, et al. Phytochemical properties of Mentha Longifolia L. essential oil and its antimicrobial effects on Staphylococcus aureus. *Armaghane Danesh Bimonthly J*, 2011; 16(5): 400-12.

[15]. Tuzlacı E. Türkiye'nin Yabani Besin Bitkileri Ve Ot Yemekleri. 2011; Alfa.

[16. Ritchason J. The little herb encyclopedia: The handbook of natures remedies for a healthier life. 1995.

[17]. Marzouk B, et al. Chemical composition and antimicrobial activity of essential oils from Tunisian Mentha pulegium L. *JFAE*, 2008; 6(1): 78.

[18]. Javanmard R, Mahdavi S. Investigating antibacterial effect of Thyme (Thymus Persicus) and Pennyroyal (Mentha Longifolia) alcoholic and aqueous extracts against isolated bacteria from domestic cheeses. *JFUMS*, 2018; 8(3): 911-17.

[19]. Zargari A. Handbook of medical plants. 6th Edition.Tehran: Tehran University Publications; 1997, 1-154.

[20]. Derwich E, Benziane Z, Taouil R. GC/MS analysis of volatile compounds of the essential oil of the leaves of Mentha pulegium growing in Morocco. *Chem Bull Poli Univ*, 2010; 55(69): 103-6.

[21]. Shahmohamadi R, Sariri R, Rasa M, Ghafoori H, Aghamol M, Nasuti S, Tahery M. Chemical composition and antimicrobial activity of flowering aerial parts Mentha Pulegium from Gilan. *Pharmacol Online*. 2011; 3: 651-59.

[22]. Sbayou H, Ababou B, Boukachabine K, Manresa A, Zerouali K, Amghar S. Chemical composition and antibacterial activity of artemisia herba-alba and mentha pulegium essential oils. *J Life Sci*, 2014; 8(1): 35-41.

[23]. Sadeghi E, Mohammadi A, Jamilpanah M, Bashiri M, Bohlouli S. Antimicrobial effects of Mentha pulegium essential oil on Listeria monocytogenes in Iranian white cheese. *J Food Qual Hazards Control*, 2016; 3(1): 20-24.

[24]. Mahboubi M, Haghi G. Antimicrobial activity and chemical composition of Mentha pulegium L. essential oil. *J Ethnopharmacol*, 2008; 119(2): 325-27.

[25]. Zhang X, et al. Three supplementary methods for analyzing cytotoxicity of *Escherichia coli* O157: H7. *J Microbiol Methods*, 2016; 120: 34-40.

[26]. Sadeghi E, et al. Antimicrobial effects of Mentha pulegium essential oil on Listeria Monocytogenes in Iranian white cheese. *J Food Qual Hazards Control*, 2016; 3(1): 20-24.

[27]. Yadegarinia, D, et al. Biochemical activities of Iranian Mentha piperita L. and Myrtus Communis L. essential oils. *Phytochemistry*, 2006; 67(12): 1249-55.

[28]. Ehsani, A, Mahmoudi R. Effects of Mentha Longifolia L. Essential oil and Lactobacillus Casei on the organoleptic properties and on the growth of *Staphylococcus aureus* and *Listeria Monocytogenes* during manufacturing, ripening and storage of Iranian white-brined cheese. *Int J Dairy Technol*, 2013; 66(1): 70-76.

[29]. Mazaher Ghorbani M, Ahmady-Asbchin S. Evaluation of antibacterial properties of Coriander, Oregano, Fennel, Thyme and parsley extracts, on pathogenic bacteria *Staphylococcus aureus* (ATCC 33591) , *Escherichia coli* (ATCC 23591) , *Klebsiella* (ATCC 10031) and *Salmonella typhimurium*. *JSUMS*, 2018; 25(4): 591-98.

[30]. Noori N, et al. The antimicrobial effect of Zataria Multiflora boiss essential oil against *E. coli* O157: H7 in minced beef meat during refrigerated storage. *Foodhygiene*, 2011; 1(1):1-8.

[31]. ÇETİN, B., S. ÇAKMAKÇI, And R. Cakmakci, the investigation of antimicrobial activity of Thyme and Oregano essential oils. *Turk J Agric For*, 2011; 35(2): 145-154.

[32]. Amrita V, Sonal D, Shalini R. Antibacterial effect of herbs and spices extract On *Escherichia coli*. *eJBio*, 2009; 5(2): 40-44.

[33]. Riahi L, et al. Phytochemistry, antioxidant and antimicrobial activities of the essential oils of Mentha Rotundifolia L. in Tunisia. *Ind Crops Prod*, 2013; 49: 883-89.

[34]. Gasemian KH. Evaluate the antimicrobial activity of aqueous extract of Oregano plant. *Int Biotechnol Congress Iran*. 2011; 442.