



The antimicrobial activity of peppermint (*Mentha piperita*) and pennyroyal (*Mentha pulegium*) essential oil on three mastitis-causing pathogens in milk

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ABSTRACT

Bovine mastitis causes a lot of economic losses, and the appearance of resistant strains of bacteria has led to the use of alternative natural bioagents for treatment. It is generally believed that high levels of fat and/or protein in foods may protect bacteria against the effects of essential oils (EOs). The purpose of this paper was to investigate the effect of EOs of *Mentha piperita* (peppermint) and *Mentha pulegium* (pennyroyal) on three bovine mastitis bacteria (*Escherichia coli*, *Streptococcus agalactiae*, and *Staphylococcus aureus*) in milk. Gas chromatography/mass spectrometry was used for the analysis of EOs. Antibacterial effects of the EOs on bacteria were evaluated with minimum bactericide concentration (MBC), minimum inhibitory concentration (MIC), and time-kill assay. Major components of peppermint EO were carvone (63.02%) and limonene (24.48%), and those of pennyroyal EO were pulegone (48.16%), eucalyptol (14.57%), and piperitenone (10.09%). The MIC and MBC were 0.62% and 1.25% for pennyroyal, 0.31-1.25% and 0.62-2.5% for peppermint, 0.31-0.62% and 0.62-2.5% for peppermint and pennyroyal, respectively. At 6-h, the bacterial reduction of treatments compared to the control group was significant for *E. coli* and *S. agalactiae* bacteria. The *S. agalactiae* and *S. aureus* counts significantly decreased in the peppermint and pennyroyal group at 24-h. In conclusion, peppermint and pennyroyal EO showed an antibacterial effect on these three bacteria and can be evaluated as an adjunct or alternative to antibiotics in the treatment of bovine mastitis.

Keywords

Antibacterial effect, mastitis, *Mentha piperita*, *Mentha pulegium*, milk

Abbreviations

EOs: Essential oils
MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration

E. coli: *Escherichia coli*
S. aureus: *Staphylococcus aureus*
S. agalactiae: *Streptococcus agalactiae*

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Introduction

Bovine mastitis is a universal disease caused by many agents and affects the quality and quantity of milk. Mastitis is the cause of most economic losses in dairy cattle in many countries [1]. For a long time, antibiotics have been the most important cure and control program for mastitis. The appearance of resistant strains of common bacteria in lactating animals has been due to the usage of antibiotics [2]. EOs are secondary metabolites of plants and possess antimicrobial activity. After long-term use, no side effects in humans and resistance in bacteria have been observed, making them a potential weapon in the fight against bacterial diseases [3]. Antimicrobial effect of EOs is not due to one specific mechanism because there are several different chemical groups in the structure of EO. Hydrophobicity of essential oils or their components helps them to target the cell membranes of bacteria that contain lipid. This property increases the permeability of membranes, thus contents of cell leak [4].

The *Mentha* species is the most common plant for medicinal and health purposes [5]. Most studies have shown the antibacterial ability of *Mentha pulegium* (pennyroyal) and *Mentha piperita* (peppermint), two of the *Mentha* species, against different bacteria [6,7].

Escherichia coli, *Staphylococcus aureus*, and *Streptococcus agalactiae* cause various diseases. Moreover, these bacteria are the most important causes of bovine mastitis [8]. The most common treatment for mastitis is intramammary antibiotics. Nutrients of milk, such as fat and protein, may interfere with the antibacterial agents and decrease the bioavailability of EOs [9]. It is essential to examine the antibacterial activity of essential oils in milk before using them as an intramammary infusion. In previous studies, we tested the antimicrobial effects of essential oils from four other plants on these bacteria in milk [10,11]. Although many studies have been conducted on the antibacterial effect of various EOs in laboratory media, few studies exist investigating this antimicrobial effect in milk. Therefore, in this study, we decided to study the impact of peppermint and pennyroyal essential oils in milk.

Result

GC/MS analysis

The chemical constituents of peppermint and pennyroyal EO are given in Tables 1 and 2. Eight constituents were determined in peppermint, which represented 98.57% of the total oils. Carvone (63.02%) and limonene (24.48%) were found as

Table 1.

Chemical composition (relative % of peak area) of essential oil of peppermint determined by GC-MS analyses.

No.	Components	Retention time (min)	Area sum%
1	(-)- β -Pinene	4.931	0.91
2	D-Limonene	6.007	24.48
3	p-Menth-8-en-2-one, trans-	10.177	2.88
4	(\pm)-Pulegone	11.23	2.13
5	(-)-Carvone	11.438	63.02
6	Carvyl acetate E	14.357	1.08
7	(-)- β -Bourbonene	15.039	0.88
8	Caryophyllene	15.939	3.19

Table 2.

Phytochemical components (relative % of peak area) of pennyroyal essential oil assessed by GC-MS analyses.

No.	Components	Retention time (min)	Area sum%
1	3-Carene	4.105	1.58
2	Sabinen	4.817	1.43
3	β -Pinene	4.936	2.12
4	β -Myrcene	5.103	0.76
5	Cymene	5.892	0.8
6	D-Limonene	6.003	2.37
7	Eucalyptol	6.08	14.57
8	1-Cyclohexene-1-methanol, $\alpha,\alpha,4$ -trimethyl-	8.939	2.61
9	Menthone	9.092	0.79
10	.p-Menthan-3-one	9.323	0.78
11	endo-Borneol	9.516	0.96
12	Isopulegon	9.592	0.92
13	L-.alpha.-Terpineol	10.112	0.95
14	Pulegone	11.285	48.16
15	Carvone	11.381	1.55
16	Piperitone	11.64	1.45
17	Thymol	12.601	1.02
18	Piperitenone	13.837	10.09
19	Terpinyl acetate	14.058	1.01
20	3-Methoxy-5-propylphenol	14.395	4.17
21	Caryophyllene	15.942	1.42

the major components. GC/MS analysis of the pennyroyal EO showed 21 various constituents (99.51% of its chemical composition). Three major compounds were: pulegone (48.16%), eucalyptol (14.57%), and piperitenone (10.09%).

MIC and MBC

The effects of peppermint, pennyroyal EOs, and positive control (lincospectinomycin) on bacteria are declared in Table 3. The antibacterial effect was shown on the microorganisms. The MIC and MBC were 0.62% and 1.25% for pennyroyal, 0.31-1.25% and 0.62-2.5% for pep-

Table 3.

MIC and MBC of peppermint and pennyroyal essential oils against bacteria compared with a positive standard antibiotic (lincospectinomycin) in milk

	Bacterium	MIC (%V/V)	MBC (%V/V)
Peppermint	Escherichia coli	1.25	2.5
	Staphylococcus aureus	0.62	1.25
	Streptococcus agalactiae	0.31	0.62
Pennyroyal	Escherichia coli	0.62	1.25
	Staphylococcus aureus	0.62	1.25
	Streptococcus agalactiae	0.62	1.25
Peppermint+pennyroyal	Escherichia coli	0.62	1.25
	Staphylococcus aureus	1.25	2.5
	Streptococcus agalactiae	0.31	0.62
Lincospectinomycin	Escherichia coli	0.078	0.156
	Staphylococcus aureus	0.039	0.078
	Streptococcus agalactiae	0.039	0.078

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

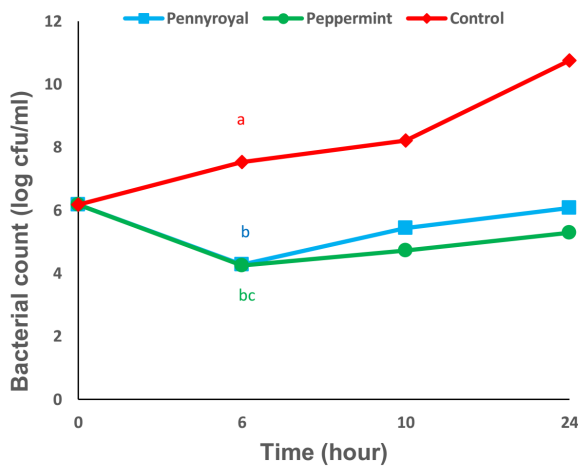


Figure 1. Time-kill curve for *E. coli* exposed to 0% (♦, control) and sub-MIC of peppermint (●) and pennyroyal (■) EOs in milk. ^{a-c} Values with different letters are significantly ($p < 0.05$) different within the same time.

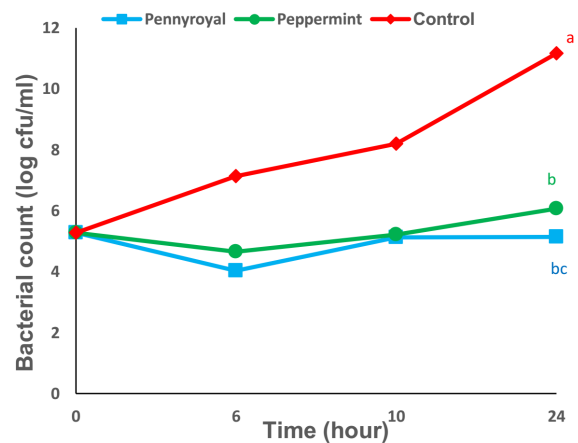


Figure 2. Time-kill curve for *S. aureus* exposed to 0% (♦, control) and sub-MIC of peppermint (●) and pennyroyal (■) EOs in milk. ^{a-c} Values with different letters are significantly ($p < 0.05$) different within the same time.

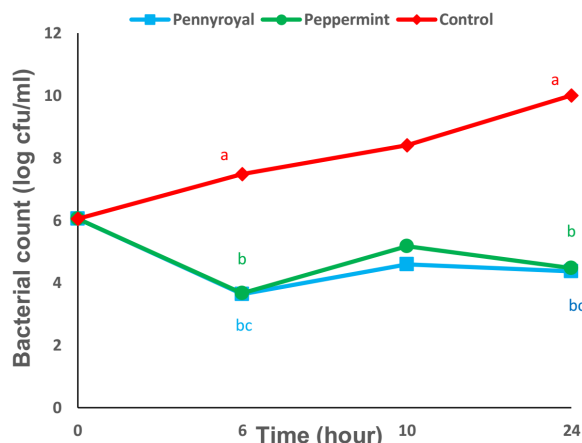


Figure 3. Time-kill curve for *S. agalactiae* exposed to 0% (♦, control) and sub-MIC of peppermint (●) and pennyroyal (■) EOs in milk. ^{a-c} Values with different letters are significantly ($p < 0.05$) different within the same time.

permint, 0.31-0.62% and 0.62-2.5% for peppermint and pennyroyal, respectively.

Time kill assay

The influence of peppermint and pennyroyal on the bacteria is depicted in Figures 1, 2, and 3. The bacterial counts of the treatment and control groups were initially about 6.0 log₁₀cfu/ml for all three bacteria. At 6-h, the bacterial reduction of treatments compared to the control group was significant for *E. coli* ($p = 0.00$) and *S. agalactiae* ($p = 0.01$) bacteria. The *S. agalactiae* ($p = 0.00$) and *S. aureus* ($p = 0.01$) counts significantly decreased in peppermint and pennyroyal group at 24-h.

Discussion

In our study, the main components of peppermint were carvone (63.02%) and limonene (24.48%). The *Mentha piperita* EO extracted from Poland included menthyl acetate (19.2%), menthone (22.7%), and menthol (29.0%) [12]. In another study from Saudi Arabia, the main constituents of peppermint were menthofuran (6.88%), menthyl acetate (8.95%), menthone (24.56%), and menthol (36.02%) [5]. Major chemical compositions of peppermint EO from Brazil were terpin-4-ol (8.00%), 3-octanol (10.1%), carvone (23.42%), and linalool (51.0%) [13]. In several studies from Iran, major components of peppermint were reported as 1,8-cineole (2.15%), menthyl acetate (4.61%), menthofuran (6.49%), and menthol (25.16%) [14]; menthofuran (11.18%), menthyl acetate (15.1%) and menthol (53.28%) [6]; isomenthone (10.3%), trans-carveol (14.5%), pipertitinone oxide (19.3%), and α -terpene (19.7%) [15]. The major components of peppermint in our study were similar to a previous study from Iran [15] but different from other Iranian studies [6, 14].

Carvone is an oxygenated monoterpene and limonene is a monoterpene hydrocarbon. Most of the antimicrobial activity in the oils has been attributed to the oxygenated monoterpenes [16]. The mechanism of monoterpene's antimicrobial action is mainly associated with the lipophilic nature of their aglycones, which allows them to rapidly cross biological membranes and then interact with a plethora of biomolecules [17].

Major constituents of pennyroyal in the present study were pulegone (48.16%), eucalyptol (14.57%), and piperitenone (10.09%). These results are similar to EO from Iran [18] that report (E)-p-mentha-2-en-1-ol (12.157%), 3,3'-dimenthol (18.859%), and

cis-pulegone oxide (45.676%) as major components and essential oil from Portugal [19] represents pulegone (86.64%), isomenthone (4.60%), and piperitenone (2.58%) as main components. Also, another study from Portugal reported the main components of pennyroyal as neo-menthol (9.2%), pulegone (23.2%), and menthone (35.9%) [20]. The difference in principle components may be the result of climate, the effect of sunlight, and geographical conditions.

The strong antibacterial activity of pulegone have been demonstrated against a set of bacteria, including *S. typhimurim* and *E. coli*. Cytotoxicity of this essential oil appears to include a bacterial membrane damage that occurs when the essential oil passes through the cell wall and cytoplasmic membrane, and disrupts the structure of their different layers of polysaccharides, fatty acids and phospholipids [20].

To the best of our knowledge, there is no study about the antibacterial effects of peppermint and pennyroyal oil against these three bacteria in milk. Nevertheless, various MIC and MBC values have been declared against *S. aureus* and *E. coli* in laboratory mediums. In one study, the MIC value ranged from 0.39 to 3.12 mg/ml, and the MBC value ranged from 0.78 to 12.48 mg/ml for peppermint against multidrug-resistant strains of *S. aureus* [12]. In other studies, MIC values of peppermint were reported against reference strains of *S. aureus* 0.5 mg/ml [21], 0.75 ± 0.03 μ g/ml [5], 0.10% [22] and against *E. coli* 0.20 ± 0.09 μ g/ml [5] and 0.25% [22]. The MIC and MBC of pennyroyal oil reported by Luis and Domingues (2021) against *E. coli* and *S. aureus* were %8. In another study, MIC and MBC of pennyroyal against *S. aureus* and *E. coli* were reported 0.004% and 0.0005%, respectively [23]. In our study, the MIC of peppermint against *E. coli* and *S. aureus* was 1.25% and 0.62%, respectively and the MIC of pennyroyal on *S. aureus* and *E. coli* was 0.62% which was higher than most of other studies in the laboratory mediums.

Based on findings of the present study, the antibacterial activity of peppermint and pennyroyal was lower than lincospectin in milk, but in a previous study, the antibacterial effect of peppermint was the same (*Streptococcus agalactiae*) or higher (*S. aureus* and *E. coli*) than lincospectin and pennyroyal effect on *Streptococcus agalactiae* was higher than lincospectin in laboratory synthetic media [24].

According to the MIC and MBC results of the present study, *E. coli* (Gram-negative bacteria) was more resistant than *S. aureus* and *S. agalactiae* (Gram-positive bacteria) to peppermint EO. The resistance of the three bacteria to pennyroyal EO was the same. The resistance of Gram-negative bacteria is due to the outer membrane surrounding the cell wall which restricts the diffusion of hydrophobic

compounds through the lipopolysaccharide. In addition, the periplasmic space contains enzymes, which are able to break down foreign molecules introduced from outside [25].

Significant reductions in bacterial population were observed at 6 and 24-hour in the time-kill assay. The antibacterial activity of peppermint and pennyroyal oil appears promising based on their activity at sub-MIC concentrations. To the best of our knowledge, we did not find any other studies comparing the impact of these essential oils on the bacteria in milk.

Though much information exists about the antibacterial activity of EOs, many studies have been done in model broth systems. The essential oil concentrations for antibacterial effect are higher in complex foodstuffs such as vegetables, dairy products, fish, and meat than in laboratory media [26]. For the application of essential oils in the treatment of bovine mastitis, oils must have miscibility in milk and obtain in vivo antibacterial effect in milk. In this study, we investigated the antibacterial effect of peppermint and pennyroyal EOs using the MIC&MBC method and time-kill assay in milk. According to MIC and MBC results, the antibacterial effects of our EOs were lower in milk than those effects in laboratory mediums in other studies. In a few studies, the activity of essential oils in milk and laboratory medium has been compared. Zhue et al. (2016) studied the antibacterial effect of Cinnamon cassia on major bacterial bovine mastitis in culture media and its miscibility in milk [9]. They showed that 4 MBC of oil of *C. cassia* was eliminated and had similar activity against *S. aureus* and *E. coli* 29 in milk within 8 hours [9]. In another study, milk composition, especially fat content, decreased the antibacterial efficacy of eugenol in milk [27].

In conclusion, peppermint and pennyroyal essential oils exhibited antibacterial properties against the three bacteria tested, indicating potential as a supplement or alternative to antibiotics in treating bovine mastitis.

Materials and Methods

Essential oils Analysis

EOs of peppermint and pennyroyal were obtained from Dorrin Golab Company, Kashan, Iran. Chemical composition determination was conducted with gas chromatography coupled to GC/MS (Model 5977A, Agilent Technologies, USA) using an HP-5MS capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 μm . The temperature of the injection port was set to 260 °C, while the oven temperature was programmed to increase from 50°C

to 250°C at a rate of 4°C per minute. Helium was used as the carrier gas, with an injection volume of 1 μl and a flow rate of 1 mL/min.

Cultures and Medias

The antibacterial effects were assessed against three major mastitis reference bacteria purchased from Persian Type Culture Collection, Tehran, Iran (PTCC) as a lyophilized culture: one gram-negative bacteria (*Escherichia coli* ATCC 25922) and two gram-positive bacteria (*Streptococcus agalactiae* ATCC 13813 and *Staphylococcus aureus* ATCC 9144). Cultures were prepared with twice the growth of the lyophilized cultures in tryptic soy broth (TSB) (Biolife, Milano, Italy) for 18 - 20 h at 37 °C. Sterile glycerin (1:5) was used for dilution of the cultures, and these cultures were kept at - 20 °C. Stock cultures were obtained by twice culture in TSB and streaking on tryptic soy agar (TSA) (Biolife, Milano, Italy) slant. After incubation at similar conditions, these cultures were kept at 4 °C and subcultured once a month [28].

Inoculum Preparation

Bacterial inoculum was obtained by transferring cells from working cultures to TSB tubes, and after incubation for 18 h at 35 °C, the subcultures were performed. The optical density (OD) (absorbance) of the bacterial broth cultures was adjusted to an optical density at 600 nm of 0.1 with a spectrophotometer (Libra S12, Biochrom Ltd., Cambridge, London). The cell concentration of these cultures was 1.64×10^{11} cfu/ml of *S. agalactiae*, 3.4×10^{10} cfu/ml of *S. aureus*, and 2.4×10^{11} cfu/ml of *E. coli* [28].

Preparation of Milk

Free antibiotic residue milk was obtained and sterilized with autoclavation (121 °C, 15 min) [4].

Determination of MIC and MBC

DMSO (Sigma, Germany) was used to increase the solubility of EOs in milk with the preparation of a 1:1 dilution of the oils. Antibacterial analysis was done using this dilution. Autoclaved milk was applied as the growth medium rather than the laboratory medium. MIC was determined using double serial dilutions (10, 5, 2.5, 1.25, 0.62, 0.31, 0.15, and 0.07%) of the oils in milk. These tubes containing 1 mL of liquid were vortexed for 90 s. 100 μl of 1:400 dilution of bacterial inoculums were inoculated in tubes, and after 15 s vortex, the cultures were incubated at 37 °C for 24 h. After the vortex, eight dilutions (10-fold) were made with 0.1 mL of culture and normal saline. Eighths of a TSA plate were inoculated with dilutions and incubated at 37 °C for 24 h. Then, bacterial counts were

determined. The lowest concentration that did not result in the growth of bacteria after subculture on TSA was regarded as MBC. MIC was considered the Ante-MBC concentration [9].

For assessment of synergism/antagonism in antibacterial action between the EOs of peppermint and pennyroyal, the essential oils were mixed volume to volume (1:1). Some controls were employed in experiments. The negative control was milk to assess autoclavation. Positive control was milk containing the bacteria to evaluate the growth of bacteria in the milk. Also, bacterial culture and DMSO without the EOs were other positive controls. Another control was the antibiotic Linco-Spectin (Linco-Spectin 5% + spectinomycin 10%) (Lincoject, Rooyan Darou, Semnan, Iran) to compare the antibacterial activity of essential oils. Each treatment was done in duplicate. Each trial was repeated at two separate times.

Time-kill assay

Sub-MIC of EOs and each bacteria were inoculated to milk. Inoculated media lacking in EO were regarded as control. After 0, 6, 10, and 24 h of incubation at 37 °C nine 10-fold dilutions using sterile normal saline were created. TSA plates were used to plate on dilutions, and after incubation for 24 h at 37 °C, a count of bacteria in dilutions was carried out. Each trial was repeated at two separate times. Plotting log₁₀ cfu/ml versus time (hour) was used to construct curves [9,28].

Statistical analysis

The data were analyzed utilizing one-way ANOVA along with Tukey's test employing the SPSS (18) software (IBM Corp., Armonk, NY, USA) at $p < 0.05$.

Authors' Contributions

R.R.: study design and preparation of manuscript. S.N.: conduct the study and data collection. J.B.K.: analysis and interpretation of the data.

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Competing Interests

The authors declare that there is no conflict of interest regarding to publication of this paper.

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