Research Article

Antibacterial Effect of *Mentha piperita* Essential Oil Against Foodborne Pathogens in Minced Meat During Storage at Abuse Refrigeration Temperature

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Abstract | With expansion of food trade in today's world, preserving food products and extending their shelf lives is a necessity. In this regard, using natural and safe preservatives such as essential oils has a particular importance. The aim of the present study is to determine the chemical composition of *Mentha piperita* essential oil (MEO) using Gas chromatography–mass spectrometry,evaluate the *in vitro* antibacterial capacity of MEO against *L.monocytogenes* and *S.typhimurium* using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, and evaluate the effects of MEO on fate of inoculated *L.monocytogenes* and *S.typhimurium* in minced beef during 9 days storage at 7°C. The essential oil was composed of18 various compounds (96.16% of total oil). Among all components menthol was the most abundant compound (43.12%). The MIC and MBC of MEO against *L.monocytogenes* and *S.typhimurium* were 1250 and 2500 µg/mL, as well as 2500 and 5000 µg/mL, respectively. The addition of 2% MEO caused a reduction about 2 and 3 $\log_{10}$ CFU/g against inoculated *L.monocytogenes* and *S.typhimurium* ($P< 0.05$) in minced meat compared with the control group, respectively. The results showed that MEO as a naturalpreservative caninhibit the growth of *L.monocytogenes* and *S.typhimurium* in meat and maintain this pathogens at acceptable levels in order to prevent the risk of food infections for consumers.

Keywords | *Mentha piperita*, Foodborne pathogen, Ground meat, *Listeria monocytogenes*, *Salmonella typhimurium*

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INTRODUCTION

Consumer preference to use fresh-like and minimally processed food products has increased dramatically during recent years; on the other hand, the globalization of food trade and the transportation of food over long distances pose major challenges for food safety and quality. Food-borne diseases (FBDs) considered asone of the most costly and important public health concerns worldwide (Abdollahzadeh et al., 2014). Meat and meat products are of the most perishable foods and that is because of high content of essential nutrients in these products. Regarding these products, several pathogenic microorganisms including *Listeria monocytogenes* and *Salmonella typhimurium* can result in foodborne illnesses such as listeriosis and salmonellosis in consumers if the products are not preserved and handled properly. *Listeria monocytogenes* is a Gram-positive pathogen which can grow at low temperatures because of its psychrotrophic nature. *L.monocytogenes* contaminating unprocessed foods like raw meat, fish and milk and also

some processed foods such as cheese, ice cream, and processed meat may cause listeriosis (Andritsos et al., 2013, de Noordhout et al., 2014, Ehsani et al., 2016) which has a high mortality rate of 20-30% (D'Ostuni et al., 2016, Moon et al., 2017). *Salmonella typhimurium* is a Gram-negative rod-shaped bacterium available in the gastrointestinal tract of animals, considered as one of the most important food-borne pathogens and exists in animal food sources like raw meat (D'Ostuni et al., 2016). In United States of America about 1.4 million cases develop salmonellosis, which results in nearly 600 deaths and 17000 hospitalizations annually (Leekitcharoenphon et al., 2016).

The major preservation technique currently employed to prevent or delay spoilage is reduction in temperature. Temperature control in refrigeration units of retail outlets and homes, however, is not always an efficient way to control foodborne pathogens since several studies have shown that a significant number of home and grocery store refrigerators operate at above the optimum temperature (Likar and Jevšnik, 2006, Lundén et al., 2014a, Lundén et al., 2014b, Morelli et al., 2012).

For many years, synthetic preservatives have been used in the food industry because of their anti-bacterial properties. Synthetic additives can reduce food spoilage however they have been accused for some allergies, intoxications, cancer and other serious diseases. Thus many consumers are desired to consume healthier products containing natural preservatives and additives instead of synthetic ones. Plant essential oils (EOs) and extracts are natural and safe antibacterial agents which have been applied in traditional medicine and as food preservatives for centuries. EOs are volatile and complicated mixture of compounds which defined by a strong odor made by aromatic plants as secondary metabolites (Aminzare et al., 2016). Commercial EOs have been categorized as Generally Recognized as Safe (GRAS) at low concentrations for food use. Recently study of the EOs and extracts of many herb species have become popular, and several studies have been investigated their antifungal and antibacterial activities on different microorganisms (Djenane et al., 2011, Hsouna et al., 2011, Dashipour et al., 2015). Based on the results of these studies application of EOs in meat products could reduce the growth of pathogens and thus the risk of foodborne outbreaks in consumers (Moon et al., 2017).

Peppermint (*Mentha piperita* L.) belongs to the *Lamiaceae* family and aerial parts of this plant in the flowering season have been traditionally applied for their antiseptic properties. The essential oils and extracts obtained from *Mentha* spp. are recently applied in the production of food stuffs. Since this essential oil has antioxidant and antimicrobial properties, it can be probably used as a natural alternative to chemical-based antibacterial and flavoring agents in foods and beverages (de Sousa Guedes et al., 2016, Djenane et al., 2012). The aim of the present study is to evaluate (1) the chemical composition of *Mentha piperita* essential oil (MEO) using Gas chromatography–mass spectrometry, (2) the *in vitro* antibacterial capacity of MEO against *L. monocytogenes* and *S.typhimurium* using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, and (3) the effects of different concentrations of MEO on fate of inoculated *L.monocytogenes* and *S.typhimurium* in minced beef during 9 days storage at 7°C.

MATERIALS AND METHODS

Essential Oil Preparation

The plant of *Mentha piperita* was collected from local markets of Urmia city, Iran. Briefly, 100 g of dried plant was grounded using mixer grinder (Pars Khazar, Tehran, Iran) and placed with distilled water (900 ml) in distillation flask of clevenger apparatus (Electro mental, Iran). The MEO was extracted by hydrodistillation method and the extraction was performed for 3 h with the temperature maintained at 100°C. This procedure repeated several times to obtain enough MEO for further experiments. Then essential oil was sterilized using 0.45 µmmilipore syringe filters, dehydrate with sodium sulfate and kept in a dark place at 4°C for more analysis (Raeisi et al., 2016).

Chemical Analysis

Chemical composition of MEO was analyzed with a gas chromatograph (Hewlett-Packard, Santa Clara, CA; 6890N) including a column HP-5MS (30 m length × 0.25 mm i.d., film thickness 0.25 mm) and connected to a mass spectrometer (Hewlett-Packard 5973N). The gas chromatograph program was as follows: helium flow rate was 1.5 mL/min and temperature increased from 40 to 240°C with a gradient of 3C/min. The initial and final temperature was hold for 6 min followed by an increase to 300°C for 15C/min holding for 3 min. Injector port and detector temperature were 290°C and 250°C, respectively. Identification of the spectra was carried out using the Willey-229 mass database, retention time, calculating the Kovats' index, the mass spectrum analysis of compounds and comparison with standard mass spectra and valid sources such as national institute of standards and technology (NIST) (Aminzare et al., 2015).

Preparation of Bacteria

L. monocytogenes (PTCC 1163) and *S. typhimurium* (ATCC 13311) cultural were gathered from the Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. The bacterial strains prepared in 15 ml of the Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 h (Ojagh et

al., 2010). The bacterial cells were centrifuged and washed with a physiological solution two times. The optical density (OD) method was used in order to prepare and calculate the number of bacterial inoculations. Different dilutions were prepared from bacterial cultures and their absorbance was read at 600 nm using the spectrophotometer (Biotek Instrument Inc., Winooski, VT, USA) in order to adjust to 0.5 McFarland standard turbidity (10^8 CFU/mL) and to dilute to the desired bacterial density (10^6 CFU/mL). Eventually, bacterial counting was performed using the spread plate count method in BHI agar medium (Laboratorios Conda S.A) at 37°C for 24 h in order to confirm the results.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of MEO

The lowest concentration of the MEO, preventing the growth of *S. typhimurium* and *L. monocytogenes* were determined according to the method described by Raeisi et al. (2016). (Raeisi et al., 2016). *S. typhimurium* and *L. monocytogenes* were moved to a 96-well microplate containing serial double dilutionof the MEO (The final concentration of each bacterial suspensions and EO were approximately 10^5 CFU/ml and 5,000 to 156.25 µg/mL, respectively). After incubation for 24 h at 37 °C, the absorbance was determined at 600 nm by a spectrophotometer (Biotek Instrument Inc., Winooski, VT). In this experiment, positive control contained inoculated broth without any antibacterial agent (MEO) while the negative control encompassed un-inoculated broth with MEO.

The MBC values were determined by serial sub-culturing of wells without any visible growth upon BHI agar plates and further incubation for 24 h at 37 °C. The lowest concentrations with no visible growth on BHI agar plates were defined as MBC values.

Inoculation of Ground Meat with MEO and Bacteria

Beef meat was minced in a meat grinder (Pars Khazar, Iran) and homogenized with various concentrations of MEO (0, 0.3, 0.5, 1 and 2 % v/w) in aStomacher (Seward Stomacher 400 Circulator, London, UK) under sterile condition. Half of the meat samples containing different concentrations of MEO were inoculated with 10^5 CFU/g of *S. typhimurium* and *L. monocytogenes* separately. After another homogenization step, meats were stored at 7 °C for subsequent analysis after 3, 5, 7 and 9 days of storage. In regards to the control group, water was added in the samples instead of MEO.

Bacterial Enumeration

For enumeration of *S. typhimurium*, 25 gram of samples were weighed and put into a plastic bag including 225 ml of 0.1% peptone water. Then homogenized for 1 min (Seward Stomacher 400 Circulator, London, UK) and pre enriching of samples was done at 35°C for 24 h. For enrichment in selective liquid medium, 1 ml of last step sample was added into 9 ml tubes with Tetrathionate broth (TT-Merck, Darmstadt, Germany) and Selenite Cystine broth (SC-Merck, Darmstadt, Germany) separately and were incubated at 35°C for 24 h. Bismuth Sulphite agar (BS-Merck, Darmstadt, Germany) and Xylose Lysine Decarboxylase agar (XLD-Merck, Darmstadt, Germany) were used for chosen plating and incubated at 35°C for 24–48 h. For confirmation of suspected colonies were inoculated into Lysine Iron Agar (LIA-Merck, Darmstadt, Germany) and Triple Sugar Iron agar (TSI-Merck, Darmstadt, Germany) and incubated at 35°C for 24 h. The Methyl Red-Voges Proskauer (MR-VP, Sigma-Aldrich chemical Co. St. Louis, USA) tests were carried out which *Salmonella* genus was MR positive and VP negative by this test.

For enumeration of *L. monocytogenes* 5 g of beef meat was homogenized with 45 ml of peptone water (0.1%). Serial dilutions were provided and 0.1 ml of each serial dilutions was spread on Listeria CHROM agar (CHRO Magar Microbiology, France) incubating at 37° C for 24 h. Blue colonies with white halos were considered as *Listeria monocytogenes* (Hitchins et al., 2011).

Statistical Analysis

Statistical analysis was carry out using SPSS version 18.0 and all experiments were done in three times. For comparison of results *among* experimental groups *analysis* of *variance* (one-way ANOVA) was performed. Turkey's post-hoc test was also performed to compare the differences among mean values during the storage. $P<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Chemical Compositions of MEO

GC-MS analysis of MEO identified eighteen various compounds representing 96.16% of total oil. Menthol was the most abundant compound among all constituents (43.12%). Other important evaluated compounds were mentone (17.12%), 1.8-Cineole (7.12%), viridiflorol (5.24%), menthyl acetate (4.55%) and germacrene-d (4.11%). Other compounds identified in the essential oil were less than 3%. The phytochemicals, with their relative percentages are shown in Table 1. de Sousa Guedes et al. (2016) and Djamel Djenane et al. (2012) also reported menthol as the main compound of MEO with the percentage of 59.73% and 33.28%, respectively, which were in accordance with results of the present study (de Sousa Guedes et al., 2016, Djenane et al., 2012). Menthol is the

main bioactive component available in MEO and other terpenes have the antimicrobial activity by synergistic effects (İşcan et al., 2002). The differences in percentage of menthol between present study and former studies can be due to various factors including climate change, geographical origin, extraction methods, changes in standardized or applied hydro-distillation, the part used of plant, cultivation conditions and genetic background such as cultivar and maturity of the plants (Najari et al., 2014).

Table 1: Chemical composition of *M. piperita* essential oilby GC-MS.

No	Component	Retention Time (Min)	%
1	Alpha-pinene	5.43	0.93
2	Sabinene	6.32	2.49
3	β pinene	6.41	2.21
4	Alpha-terpinene	7.31	1.12
5	Limonene	7.63	1.88
6	1,8-Cineole	7.72	7.12
7	Gamma terpinene	8.29	0.21
8	Terpinolene	8.99	0.82
9	Linalool	9.33	0.36
10	Mentone	10.58	17.12
11	Menthol	11.13	43.12
12	Isomenthol	11.26	2.1
13	Piperitone	12.70	0.25
14	Menthyl acetate	13.43	4.55
15	Beta-bourbonene	15.19	1.21
16	caryophyllene	15.85	1.32
17	Germacrene-d	16.69	4.11
18	Viridiflorol	18.95	5.24
	Total		96.16

MIC and MBC Values

Results of MIC and MBC of MEO against pathogenic bacteria are shown in Table 2. *L.monocytogenes* and *S. typhimurium* growth were inhibited at the concentration of 1250 and 2500µg/mL while bactericidal effects were observed at 2500 and 5000µg/mL, respectively. In a study by McKay and Blumberg, it was reported that MEO has a significant inhibitory effect on *L. monocytogenes* by 0.16–63 mg/mL which is in line with our findings (McKay and Blumberg, 2006). In the current study the antibacterial activity of MEO was measured at different concentrations against *S. typhimurium* and *L. monocytogenes*. Results indicated that MEO has inhibitory effect on the growth of *L. monocytogenes* in lower concentrations compared with *S. typhimurium* and *L. monocytogenes* was more sensitive than *S. typhimurium*. This may depend on the nature of the Gram-negative cells and their external peptidoglycan membrane (Tyagi and Malik, 2011). The bactericidal effect of EOs can be attributed to their impact on cell membrane. As our results have indicated, the dominant compounds of MEO are menthol and mentone which have lipophilic nature. These compounds can destroy membrane of the cells which may lead to the discharge of cell contents and subsequently their death (Dashipour et al., 2015, Saei-Dehkordi et al., 2010). In line with our findings, Soković M. et al. (2010) found significant effect of MEO on *L. monocytogenes* and *S. typhimurium* (Soković et al., 2010).

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *M. piperita* essential oil against *L.monocytogenes* and *S. typhimurium*.

	Bacteria	
	L.monocytogenes	***S. typhimurium***
MIC (µg/mL)	1250	2500
MBC (µg/mL)	2500	5000

The Effects of Meo Against Inoculated *L. Monocytogenes* and *S. Typhimurium* in Minced Meat

Antibacterial effect of *M.piperitta* essential oil on *L. monocytogene* and *S. thyphimurium* count in minced beef during storage time were presented in Table 3 and 4, respectively. In general, the rate of *S. typhimurium* and *L. monocytogenes* growth were higher in the control group compared with the samples treated with MEO (P<0.05). According to Table 3, the count of *L. monocytogenes* enhanced during the storage time in the control group. The number of bacteria in the minced beef which was treated with 0.3% of MEO was increased during first 3 days of storage and then decreased. In the samples which were treated with 0.5%, 1% and 2% of MEO, the number of *L. monocytogenes* had a decreasing pattern until the end of storage (P<0.05). All treatments had significant difference compared with the control group at the end day of storage (P<0.05). The addition of 2% of MEO, showed a greater reduction compared to other treatments during the storage period (P<0.05). In agreement with our results Djenane et al. (2012) reported that essential oil of *M. piperita* significantly inhibited the growth of inoculated *S. aureus* (as a Gram-positive bacterium) in minced beef during 9 days storage at abuse temperature (Djenane et al., 2012). Tassou et al. (1995) investigated the antimicrobial effect of *M. piperita* essential oil against *L. monocytogenes* in three food systems with different compositions; the food system containing beef required higher concentrations of this essential oil for bacterial inhibition, due to the higher concentration of protein and fat (Tassou et al., 1995).

As shown in Table 4, the population of *S. typhimurium* in the control group increased during the storage time (P<0.05). This increment was observed in other groups ex

Table 3: Antibacterial effect of *M. piperitta* essential oil on *L. monocytogenes* count in minced beef at 7°C for 9 days.

Treatments	Bacterial Count (log_{10} CFU/g)				
	Storage Time (Day)				
	0	3	5	7	9
Control	5.00 ± 0^{Aa}	5.23 ± 0.25^{Ba}	5.50 ± 0.10^{Ba}	5.70 ± 0.20^{Ca}	5.87 ± 0.45^{Ca}
0.3%	5.00 ± 0^{Aa}	5.20 ± 0.30^{Aa}	4.90 ± 0.30^{Ab}	4.60 ± 0.10^{Bb}	4.53 ± 0.45^{Bb}
0.5%	5.00 ± 0^{Aa}	4.90 ± 0.40^{Ab}	4.77 ± 0.15^{Bc}	4.37 ± 0.25^{Cc}	4.30 ± 0.20^{Db}
1%	5.00 ± 0^{Aa}	4.60 ± 0.20^{Bb}	4.23 ± 0.15^{Cd}	3.90 ± 0.40^{Dd}	3.70 ± 0.20^{Dc}
2%	5.00 ± 0^{Aa}	4.10 ± 0.30^{Bc}	3.83 ± 0.35^{Be}	3.60 ± 0.10^{Ce}	3.10 ± 0.30^{Dd}

Different capital letters in each row and different small letters in each column indicate significant difference ($P< 0.05$).

Table 4: Antibacterial effect of *M. piperitta* essential oil on *S. thyphimurium* count in minced beef at 7°C for 9 days.

Treatments	Bacterial Count (log_{10} CFU/g)				
	Storage Time (Day)				
	0	3	5	7	9
Control	5.00 ± 0^{Aa}	5.80 ± 0.30^{Ba}	6.33 ± 0.55^{Ba}	6.60 ± 0.10^{Ca}	6.70 ± 0.20^{Da}
0.3%	5.00 ± 0^{Aa}	5.70 ± 0.20^{Ba}	5.90 ± 0.30^{Bb}	5.80 ± 0.10^{Bb}	5.63 ± 0.35^{Bb}
0.5%	5.00 ± 0^{Aa}	5.63 ± 0.15^{Ba}	5.43 ± 0.25^{Bc}	5.33 ± 0.45^{Cc}	5.13 ± 0.45^{Cc}
1%	5.00 ± 0^{Aa}	5.10 ± 0.30^{Ab}	4.83 ± 0.35^{Ad}	4.63 ± 0.25^{Bd}	4.47 ± 0.35^{Cd}
2%	5.00 ± 0^{Aa}	4.80 ± 0.30^{Bc}	4.37 ± 0.35^{Ce}	4.07 ± 0.35^{De}	3.90 ± 0.40^{Ee}

Different capital letters in each row and different small letters in each column indicate significant difference ($P< 0.05$).

cept for 2% MEO in the initial days of storage period. At the end day of the storage period, the bacterial reduction was observed only in 1% and 2% of MEO compared to the initial inoculation (10^5 CFU/g). Comparing the antimicrobial results of MEO against *L. monocytogenes* and *S. typhimurium* indicates that the MEO has a weaker effect on *S. typhimurium* as a Gram-negative bacterium. The peptidogly can layer of Gram-positive bacteria like *L. monocytogenes* take parts as a main permeability barrier whilst the outer membrane of Gram-negative bacteria such as *S. typhimurium*, plays key role as a main permeability barrier (Arqués et al., 2008, Burt, 2004). Our results indicated that MEO had a lower repressive effect on the growth of *S. typhimurium* that is maybe due to the hydrophilic outer membrane of this Gram-negative bacterium (Soković et al., 2010).

CONCLUSION

According to results of the present study the growth of *L. monocytogenes* and *S. typhimurium* was significantly affected by higher concentrations of *Mentha piperita* essential oil. Our findings showed that the essential oil of this plant has bacteriostatic and bactericidal effects on *L. monocytogenes* and *S. typhimurium*. Moreover, MEO improved the microbiological safety (inoculated *L. monocytogenes* and *S. typhimurium*) of the minced beef during 9 days storage at abuse refrigeration temperature. Therefore, this additive can be applied as a natural preservative in order to prevent the growth of food-borne pathogens and increase meat shelf-life.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS CONTRIBUTION

Mojtaba Raeisi designed and monitored the study. Mohammad Hashemi carried out the experiments. Elham Ansarian and Shahrzad Daneshamooz drafted the manuscript. Hassan Hassanzad Azar, Jalal Hejazi and Behrooz Jannat revised the manuscript. Majid Aminzare checked and submitted the manuscript and revised it according to reviewer's comments. All authors read and approved the final manuscript.

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