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Antimicrobial Effects of *Mentha pulegium* Essential Oil on *Listeria monocytogenes* in Iranian White Cheese

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Abstract

Background: *Listeria monocytogenes* is an important Gram-positive disease-causing bacterium existing in milk and dairy products. Inhibitory effects of the *Mentha pulegium* essential oil at concentrations of 0.03, 0.015, and 0.0075% on growth of *L. monocytogenes* were studied in Iranian white cheese during 60 days of storage.

Methods: Essential oil of *M. pulegium* plant, collected from north of Iran, was extracted by Clevenger apparatus and analyzed by gas chromatography mass spectrometry (GC-MS). The antibacterial effects of the oil were evaluated by growth of the microorganism in control and treatment cheese samples. Also, sensory properties of the cheese samples containing different concentration of *M. pulegium* were determined. Statistical analyses were performed by ANOVA and Fisher's Least Significant Difference (LSD) procedure using SPSS 16.0 software.

Results: GC-MS analysis showed that the major compounds of *M. pulegium* essential oil were pulegone (36.68%), piperitenone (16.88%) and 1,8 cineole (14.58%). In control group, *L. monocytogenes* grew in 7 days and then their growth decreased gradually during 60 days. But, in all treated samples there is a log reduction in bacterium count while the maximum growth lasted for 14 days with a significant difference ($p<0.05$) compared with control samples. Although 0.03% concentration of mentha oil had the most strong antibacterial effects, but samples with 0.015% essential oil had significantly higher organoleptic properties score comparing the other samples ($p<0.05$).

Conclusion: *M. pulegium* essential oil not only can improve organoleptic properties of cheese but also can reduce and postpone the growth of *L. monocytogenes* in this product.

Introduction

Although there is a sharp improvement in food safety of issues, food-borne diseases still have their own victims all over the world. Thus, good hygiene practice and using food additives to promote food safety are important goals (Burt, 2004). Among food additives, essential oils attract much attentions because of their natural origin (Fazlara et al., 2012). They are used not only for their antimicrobial,

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antiparasitic, or antioxidant properties, but also for their pleasant aromatic properties (Sadeghi et al., 2015; Smith-Palmer et al., 2001). These components influence microbial structure and distract cell membranes eventually lead to death of microorganisms (Hafedh et al., 2010).

Mentha pulegium L. is an edible vegetable belongs to *Mentha* species commonly known as pennyroyal (Goodarzi and Nanekarani, 2014). Diversity of *Mentha* species and their different morphological, cytological, and chemical features have been determined previously. The types of the genus *Mentha* L. (Lamiaceae family) contain more than 20 species. The main habitats for this kind of plant are Eurasia, Australia, South Africa, and Asia where weather is humid or wet. It is wildly used in herbal medicine and particularly valuable in empowering the immune system (Gulluce et al., 2007). The chemical composition of plants is known to be influenced by several external factors, including climate, thus, different kind of compounds may present in regarding to where the plant grows. It is known that the antimicrobial activity of the *M. pulegium* essential oils is attributed to presence of components such as menthol, menthone, limonene, and carvone that have been determined by the disk diffusion method (Hussain et al., 2010). Remarkable plant diversity is seen in the west of Iran especially in Kermanshah and Hamadan provinces. Accordingly, antimicrobial activity of some Iranian plants have been studied during past years (Bahraminejad et al., 2013; Misaghi and Basti, 2007; Moradi et al., 2014; Sadeghi et al., 2013).

Listeria monocytogenes is a Gram-positive, rod shaped, non spore forming, and motile microorganism commonly exists in air, soil, water, and food (Arslan and Ozdemir, 2008; Jamali et al., 2013; Sandasi et al., 2008). This pathogenic bacterium probably spreads through the mammary glands, feces, and other secretions of infected animals. Additionally, animals without clinical symptoms, human resources and contaminated instruments cause to contaminate milk (Tehrani and Sadeghi, 2015). Consumption of raw milk and dairy product can easily transmit the microorganism (Jakobsen et al., 2011; Rahimi et al., 2010; Sharma et al., 2012; Zarei et al., 2015). *L. monocytogenes* is an important pathogen that may cause some serious disease such as abortion and encephalitis in sheep, cattle, other mammals, birds, and fish (Kalorey et al., 2008; Schoder et al., 2011). It is able to grow or survive at low temperature that causes it as an important hazard in food safety. Listeriosis is a serious infection manifested by fever, diarrhea, headache, and myalgia. In some severe cases, septicemia, meningitis, and abortion may be occurred (Aygun and Pehlivanlar, 2006).

Effects of some plant oils on *L. monocytogenes* have been studied in the past. Findings show reduced growth and survival of the microorganism when the essential oils were added to model foods (Burt, 2004). As elimination of *L. monocytogenes* in dairy products is so important, applying effective preservatives like essential oils that their inhibitory effects on microorganisms have been approved appears to be fundamental. On the other hand, *M. pulegium* is a member of the essential oil group having pleasant aromatic properties; thus, the aim of this study was to find the relationship between adding *M. pulegium* essential oil and *L. monocytogenes* growth behaviors in Iranian white cheese.

Materials and methods

Preparation of herb and essential oil

M. pulegium was collected from the mountains of Sari, Mazandaran province in north of Iran, in spring and identified by herbarium of Medicinal Plants Research Center, Iranian Institute of Medicinal Plants, Karaj, Iran. The leaves of *M. pulegium* were soak in water and exposed to hydro distillation for 2 h, in a Clevenger-type apparatus. The obtained essential oil of *M. pulegium* was dried with anhydrous sodium sulfate and kept in dark glass bottles in refrigerator at 4 °C. The essential oil was analyzed by gas chromatography mass spectrometry (GC-MS; Agilent 6890, Wilmington, PA) with HP-5MS capillary column (30×0.25 mm ID×0.25 mm film thickness).

Essential oil of *M. pulegium* was analyzed in following conditions:
Initial temperature: (50 °C)
Program rate: (15 °C/min)
Final temperature 300 °C (holding for 20 min)
Injector temperature: 290 °C
Carrier gas: helium (0.8 ml/min)
Electron ionization mode: (70 eV)

Bacterial strains

The *L. monocytogenes* (ATCC7644) were prepared from Food Science and Quality Control Laboratory, Faculty of Public Health, Kermanshah University of Medical Sciences, Kermanshah, Iran.

Experimental design

In this study the effect of different levels of *M. pulegium* essential oil (0, 7.5, 15 and 30 µl/100 ml) were investigated on growth of *L. monocytogenes* during the manufacturing process of Iranian white brined cheese up to 60 days. The experiments were totally carried out in triplicate.

Bacterial inoculation

L. Monocytogenes was inoculated in tryptic soy broth (TSB, Merck®, Germany) culture for 18-20 h (overnight)

at 37 °C. Afterwards, cultures were diluted with sterile glycerin and stored in micro tubes at -20 °C for our research. To obtain fresh bacteria, it was cultured in TSB at 37 °C for 20 h and was inoculated again, kept in the same condition. Then, each sterile cuvette with 5 ml TSB was mixed with fresh bacteria, put in spectrophotometer to read turbidity in order to show amount of bacteria at 600 nm (Moosavy et al., 2015).

Manufacturing of Iranian white cheese

According to the following procedure, Iranian white brined cheese was produced. Pasteurized cow's milk (75 °C/16 s with 2.5% fat) was put in a stainless steel container and fixed in a larger pilot-plant-sized steam-jacketed cheese vat. Milk was warmed up to 35 °C and was inoculated with *L. monocytogenes* (1×10^3 cfu/ml). As starter cultures, *L. bulgaricus* and *S. thermophilus* 0.5% (w/v) were added to the treatment groups. Two ml of fungal origin rennet (DK-2970, CHR HANSEN) and $CaCl_2$ (0.2 mg/ml) were added to milk (pH=5.6). Then, it was supplemented with 0, 7.5, 15 and 30 µl/100 ml essential oil of *M. pulegium*. Coagulum was made about 1 h after adding rennet, then it was cut and transferred into rectangular metal hoops ($28\times12\times12$ cm^3) and drained 6 h at room temperature (22 °C). The cheese was cut and put into 20% sterile salt brine for 8 h at 22 °C. After that, brine was removed and each cheese piece was put into other sterile container and covered with 8% sterile salt brine. Cheese was ripened for 15 days at 14 °C, then kept and stored for 75 days at 4 °C. *L. monocytogenes* were enumerated in cheese samples in these steps and times including pasteurized milk, 0 h inoculated milk, day 7 (168 h), day 15 (360 h), day 30 (720 h), day 45 (1080 h) and day 60 (1440 h) after cheese ripening. All of the procedures were also carried out for preparation of uninoculated (*L. monocytogenes*) cheese which is used for sensory evaluation (Tehrani and Sadeghi, 2015).

Bacterial enumeration

Ten g of each sample along with 90 ml of sterile 0.1% (w/v) peptone water was blended in a stomacher apparatus (Interscience, France) for about 3 min. Then, bacterial enumeration was carried out according to Fazlara et al. (2012) using some culture media ecpeically Palcam listeria agar purchased totally from Merck®, Germany. Plates contained spherical shape, small, gray to green with dark color margins colonies were counted and the number of *Listeria* per gram of cheese were studied. Each inoculation was done tree times and mean of counted number of colonies was reported.

Sensory evaluation

Sensory effects of adding different concentration of essential oil of *M. pulegium* (0, 7.5, 15 and 30 µl/100 ml milk) to Iranian white cheese were evaluated by an acceptance test. The samples were evaluated by 10 trained panelists from staff who had received training and postgraduate students of Department of Food Sciences and Technology, Faculty of Public Health, Kermanshah University of Medical Sciences, Kermanshah, Iran. A 5-point hedonic scale for aroma, taste and overall acceptability was carried out, indicating 5 for extremely good and 0 to unacceptable (Tehrani and Sadeghi, 2015).

Statistical analysis

Statistical analyses were performed using SPSS Inc., Chicago, IL, USA (version 16.0). The effects of essential oil of *M. pulegium* on *L. monocytogenes* counts were evaluated by analysis of variance (ANOVA). Also, the variability of sensorial acceptance of the samples was assessed by ANOVA and Fisher's least significant difference procedure (LSD). Results were considered statistically significant at $p<0.05$.

Results

Table 1 shows GC-MS analysis of the *M. pulegium* essential oil used in our experiments. Accordingly, the essential oil contains roughly 15 different compounds (99.07%). The major compounds were pulegone (36.68%), piperitenone (16.88%), and 1,8 cineole (14.58%).

Statistical results revealed no significant differences among control and treated samples in the first day of experiments ($p>0.05$), while there were significant differences between mean of bacterial enumeration and various essential oil concentrations in the other storage days ($p<0.05$). As seen in Table 2, in control group, *L. monocytogenes* grew in 7 days and then their growth decreased gradually during 60 days. But, in all treated samples there was a log reduction in bacterium count while the maximum growth lasted for 14 days with a significant difference ($p<0.05$) compared with control samples. Also, it was found that 0.03% concentration of *M. pulegium* oil had the strongest ($p<0.05$) antibacterial effects compared to the other groups (Table 2).

Organoleptic tests showed that 0.015 and 0.03% concentrations of *M. pulegium* essential oil, had the highest and lowest score, respectively, showing significant difference ($p<0.05$).

Discussion

Our present study revealed considerable antimicrobial effects of *M. pulegium* essential oil on the growth of *L. monocytogenes* in Iranian white cheese. In fact, adding

Table 1: GC-MS analysis of the essential oil of *M. pulegium* collected from northern Iran

No.	Compounds	%	Retention Index*
1	Pulegone	36.68	1254
2	Piperitenone	16.88	1367
3	1,8 Cineole	14.58	1095
4	Alpha Terpineol	5.98	1255
5	Menthone	4.72	1165
6	Cis Salvene	3.56	1398
7	Piperitenone oxide	3.27	1309
8	Delta Terpineol	3.19	1231
9	Endo Borneol	3.04	1278
10	β-Caryophyllene	1.79	1476
11	Caryophyllene oxide	1.57	1649
12	Carvacrol	1.34	1463
13	Limonene	1.26	1009
14	β-Pinene	0.78	987
15	α-Pinene	0.43	921
Total		99.07	

Table 2: Enumeration of *L. monocytogenes* (CFU/g) with various concentrations of *M. pulegium* essential oil in different storage days

Essential oil concentration (%)	Storage time (day)					
	0	7	14	30	45	60
0	$1\times10^{3}\pm101$	$8.7\times10^{6}\pm1000$	$5\times10^{6}\pm996$	$5\times10^{5}\pm601$	$5\times10^{5}\pm472$	$4\times10^{5}\pm753$
0.0075	$1\times10^{3}\pm54.8$	$6\times10^{5}\pm521.4$	$4\times10^{6}\pm1051$	$1.3\times10^{5}\pm462$	$7.2\times10^{4}\pm295.5$	$7\times10^{4}\pm413$
0.015	$1\times10^{3}\pm50.8$	$2\times10^{5}\pm231$	$1.2\times10^{6}\pm970.5$	$1\times10^{5}\pm310.4$	$4\times10^{4}\pm500.5$	$3\times10^{4}\pm342.6$
0.03	$1\times10^{3}\pm30.3$	$1.5\times10^{5}\pm325.5$	$8\times10^{5}\pm520.8$	$1\times10^{5}\pm200.2$	$1.5\times10^{4}\pm340.1$	$2\times10^{4}\pm250.6$

oil in different concentrations during several days showed reduction in microbial population of *L. monocytogenes*. Use of essential oils in different food matrices has an obvious upward trend last years. Having both antibiotic and organoleptic properties caused to attract food producers' attention (Sandasi et al., 2008), owing to this fact, there are a lot of researches studying antimicrobial and antioxidant activities of different essential oil in foods. For example, antimicrobial and antioxidant activities of essential oil and methanol extract from *M. longifolia* L. ssp. *longifolia* were been studied by Gulluce et al. (2007). They reported that the essential oil had strong antimicrobial activity against all tested microorganisms whereas the methanol extract almost had no effect (Gulluce et al., 2007). Moosavy et al. (2015) showed high antimicrobial combined effect of *M. spicata* essential oil and nisin against *L. Monocytogenes* which is in accordance with our results (Moosavy et al., 2015). Some other researchers investigated the effects of various essential oils on some pathogenic microorganisms. For example, it has been proved that *M. piperita* L. and *Myrtus communis* L. essential oils had active compounds that may have antibacterial, antifungal and antioxidants impacts in food (Yadegarinia et al., 2006). Also, Fu et al. (2007) reported high antimicrobial effect of two essential oils (clove and rosemary) against *Escherichia coli*. In a similar study, Ehsani and Mahmoudi (2013) assessed the effects of *M. longifolia L.* essential oil on the growth of *Staphylococcus aureus* and *L. monocytogenes* during the manufacturing, ripening and storage of Iranian white-brined cheese. They found that this essential oil had inhibitory effects on the both pathogenic bacteria that are in agreement with our findings. According to another work, a group of researchers evaluated antibacterial effects of *Cuminum cyminum* essential oil on *L. monocytogenes* in Iranian white cheese and their results found to be consistent with ours. They reported that presence of the essential oil caused to eliminate the microorganisms after several days, however the bacteria survived in control sample during the period of study (Fazlara et al., 2012). Additionally, Darderafshi et al. (2014) revealed that 0.03 and 0.015 concentration of *Ferulago angulata* essential oil had antibacterial effects of *S. aureus* added to Iranian white cheese. Most of the previous researches showed effectiveness of plant essential oils as natural antibacterial additives in food models in which with increasing the concentrations of essential oil, the bacterial count decreased significantly which is similar to our data. On the other hand, acceptable sensory score seen in this study determined a practical concentration of *M. pulegium* essential oil that could be used as preservative in Iranian cheese without any side effect from viewpoint of organoleptic characteristics.

Conclusion

It is concluded that *M. pulegium* essential oil not only can improve organoleptic properties of Iranian white

cheese but also can reduce and postpone the growth of *L. monocytogenes* in this product.

Conflicts of interest

There is no conflict of interest in this work.

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