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Antilisterial Effects of *Ziziphora clinopodioides* Essential Oil and Nisin in Milk

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The aim of the present study was to evaluate the antibacterial effect of *Ziziphora clinopodioides* essential oil (0.1 and 0.2%) separately and in combination with nisin (250 and 500 IU/ml) on survival of *Listeria monocytogenes* inoculated in pasteurized milk sample during storage under refrigerated temperature for 9 days. Chemical composition of the essential oil was analyzed by gas chromatography coupled with mass spectrometer detector (GC-MS). Carvacrol (64.22%), followed by thymol (19.22%) and *p*-cymene (4.86%) were the most abundant components of the essential oil. Based on our findings, there was no significant difference ($p>0.05$) between samples treated with the essential oil at 0.1 and 0.2% and also nisin at 250 and 500 IU/ml. Our samples treated with the essential oil in combination with nisin showed the populations of pathogen significantly ($p<0.05$) lower than the untreated samples. Moreover, samples treated with the combination of the essential oil at 0.2% and nisin at 500 IU/ml, showed populations of *L. monocytogenes* significantly ($p<0.05$) lower than those of samples treated with other groups. According to the results of this work, *Z. clinopodioides* essential oil and nisin could be applied in food industries as alternative food preservatives and to control the growth of *L. monocytogenes.*

Key words: Milk, nisin, *Listeria monocytogenes*, *Ziziphora clinopodioides* essential oil.

Listeria monocytogenes is considered as one of the most important food-borne pathogen concerns to the dairy industries[1]. In recent years, there are several reports of sporadic cases and outbreaks of listeriosis due to consumption of contaminated pasteurized milk and dairy products such as cheese and yoghurt[2-4]. It has been established that this pathogen can survive at minimum pasteurization treatment and is more thermotolerant than most non-spore-forming bacterial pathogens[5,6]. Moreover, it capable of growing at wide range temperatures (-0.4°C-45°C), various pHs(4.3-9.6), high salt concentrations (up to 10%), anaerobic conditions and conditions with low levels of oxygen[4,7]. Numbers of different chemical and synthetic compounds have been used as antimicrobials in order to control growth of *L. monocytogenes* in milk and dairy products[1,3,5,6]. However, recently, the exploration of naturally occurring antimicrobials as alternative preservatives for inhibiting the growth of *L. monocytogenes* has been increased[8,9].

Various plant essential oils, individually or synergistically with other chemicals or natural antibacterial agents, have been reported that had strong antibacterial effects on the growth of *L. monocytogenes* in milk and dairy products[5,6,10-12]. *Ziziphora clinopodioides*, belonging to the family of *Lamiaceae*, is propagated and distributed throughout worldwide especially Iran and Turkey[13]. In Iranian folk medicine, the fresh leaves and stem frequently were applied as appetitive, carminative, antiseptic, wound healing material, sedative, stomach tonic, expectorant and antiseptic[14]. Pulegone, 1,8-cineole, thymol,

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carvacrol, *p*-cymene and limonene as major compounds of the essential oil of *Z. clinopodioides* have been found to have antibacterial, anthelmintic and antifungal properties[14,15].

Nisin is an antimicrobial peptide that produced by certain strains of *Lactococcus lactis* or *Streptococcus uberis* and its importance is due to its widespread of inhibitory effect against Gram-negative and positive bacterial pathogens[16]. The use of nisin as a natural antimicrobial agent has been extensively examined in a large variety of foods especially milk and dairy products such as cheese and yoghurt[1, 5-7]. It has been reported that nisin is not able to inhibit completely some Gram-positive bacteria such as *L. monocytogenes* and spore forming bacteria[3,6]. Hence, the combination of nisin and other natural compounds such as essential oils is receiving increasing attention[10,17]. However, there is no report available in the literature on the antibacterial effect of *Z. clinopodioides* essential oil separately and in combination with nisin in milk. Hence, the aims of the present study were to evaluate chemical composition of *Z. clinopodioides* collected hydro-distilled essential oils from Zagros Mountain ranges, Kermanshah province, west of Iran by GC–MS and the effect of *Z. clinopodioides* essential oil separately and in combination with nisin on survival of *L. monocytogenes* inoculated in pasteurized milk sample during storage under refrigerated temperature (4±1°C) for 9 days.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Z. clinopodioides* plant were hand collected from the wild, Zagros Mountain ranges (Gilane Gharb city, Kermanshah province, western Iran: latitude 3,776,583 Universal Transverse Mercator (UTM); longitude 585,86 UTM and altitude 833 m above sea level) during the full flowering stage of growth (March-July 2014). Immediately after harvest, the plants were packed and transferred to the laboratory and authenticated by Dr. Seyed Mohammad Masoumi (staff of the Faculty of Agriculture, Razi University, Kermanshah, Iran) and a representative voucher specimen (No. 6816) was deposited in the Herbarium of the Research Center of Natural Resources of Tehran, Iran.

Isolation of essential oil

Prior to the isolation of the essential oil of *Z. clinopodioides* plant, the leaves extensively washed with distilled water and dried indoors. After two weeks, the dried samples (100g) *Z. clinopodioides* of leaves were cut into 5 mm strips and subjected to steam distillation for 3.5h using a Clevenger-type apparatus with 500ml of de-ionised water. The essential oil floating on the top of the condensed water was obtained and dried over anhydrous sodium sulfate (Na_2SO_4) (Merck, Darmstadt, Germany), stored in darkness in an amber vial and was kept at low temperature (4±1°C).

Gas chromatography–mass spectrometry (GC–MS) analysis of essential oil

The essential oil, isolated as described above, was analyzed by gas chromatography coupled with mass spectrometer detector (GC-MS) to identify their components. GC-MS was conducted using a Thermo Quest Finningan gas chromatograph coupled with Thermo Quest Finningan mass spectrometer detector. The column used was HP-5MS 5% phenyl methylsiloxane capillary column (30.00m length×0.25mm ID, 0.25µm film thickness). GC-MS operating conditions followed those described by Adams,1995[18]: Injector and detector temperatures 290ºC and 300ºC, respectively; oven temperature, from 50°C to 265ºC at 2.5°C/min; carrier gas, Helium with a constant flow rate 1.2ml/min; electron (EI) ionization energy, 70eV with scan range of 30–550 amu; sample injection volume, 1 µl. The essential oil sample was also analyzed by gas chromatography (Thermo Quest Finningan, UK) using the similar capillary column and analytical conditions as described above.

Identification of chemical compounds

The most volatile chemical compounds of the essential oil were identified by comparison between their retention indices (*RIs*), *retention indices* of published data, Standard Mass Spectral fragmentation pattern (Wiley/NBS) and the NIST (National Institute of Standards and Technology). The GC peak area normalization of the three injections was expressed as mean percentage of individual essential oil composition.

Preparation of nisin

Nisin with a label activity of 10^4 International Unit (IU/g) was purchased from Sigma-Aldrich Company, UK. Stock solution was

prepared by dissolving of nisin in 0.02M HCl, centrifuged at 1500×g for 20min, filtered by 0.22µm pore filter (Sigma-Aldrich, UK) and stored at -20°C until use[19]. Before each experiment, the stock solution was thawed at 25°C and diluted in sterile water to a concentration of 250 and 500 IU/ml nisin.

Test microorganism

In the present study, lyophilized culture of *Listeria monocytogenes* (ATCC19118) was purchased from the culture collection of the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The strain was kept frozen at -20°C in Brain Heart Infusion broth (BHI; Merck, Darmstadt, Germany) medium containing 17% glycerol. Before experiment, strain of bacterium was activated by two consecutive sub-culturing in BHI broth at 37°C for 24h. Cell counts were determined by serial dilution and subsequent enumeration on Brain Heart Infusion agar (BHI; Merck, Darmstadt, Germany). Moreover, the density of bacterial culture needed for the inoculation of milk samples examined by using a spectrophotometer at 600nm. An inoculum with a population of pathogen cells of 5 log CFU/ml was used for the inoculation of milk sample.

Experimental design

Pasteurized milk samples were purchased from a market of Kermanshah city, west of Iran. For each experiment, nine batches (Batch A-Batch H) were designed as follows: control(no essential oil or nisin added), A (0.1% *Z. clinopodioides* essential oil), B (0.2% *Z. clinopodioides* essential oil), C (250 IU/ml nisin), D (500 IU/ml nisin), E (0.1% *Z. clinopodioides* essential oil + 250 IU/ml nisin), F (0.1% *Z. clinopodioides* essential oil + 500 IU/ml nisin), G (0.2% *Z. clinopodioides* essential oil + 250 IU/ml nisin) and H (0.2% *Z. clinopodioides* essential oil + 500 IU/ml nisin). Control was inoculated with tested microorganism without the essential oil or nisin. After homogenization for 30s, all samples were kept at refrigerated temperature (4±1°C) until measurements were made. The microbial analysis of milk samples for populations of *L. monocytogenes* was done at 2-day intervals up to the 9th day of refrigerated storage (0, 1, 3, 5, 7 and 9 days).

Microbiological analysis

At each sampling time, 10ml of milk samples in the stomacher bags were aseptically filled with 90 ml of 0.1% peptone water. The content was homogenized in the stomacher for 2 min at room temperature. Then, ten-fold serial dilution (1:10) in 0.1% sterile peptone water for enumeration was prepared. For *L. monocytogenes*, sample dilutions (0.1 ml) of milk were spread plated on selective Palcam Listeria selective agar (Merck, Darmstadt, Germany) in duplicate. After incubation of the plates at 30°C for 48h, populations of the pathogen were determined and expressed as log CFU/ml. All experiments were conducted in independent triplicate.

Statistical analysis

SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) software package was used for data analyses. Mean and standard deviations of each experiment were calculated and then were subjected to analysis of variance. Tukey's test at 95% confidence interval was used to determine mean differences among the treatments.

RESULTS AND DISCUSSION

Chemical composition of *Z. clinopodioides* essential oil

Results obtained the GC and GC-MS chemical composition of *Z. clinopodioides* essential oil along with retention indices and percentage composition is presented in Table 1. Based on our results, in total, twenty four compounds accounting for 99.65% of the whole essential oil were identified. Among these, the oxygenated monoterpenes afforded a main portion of the oil (86.1%) with carvacrol (64.22%) and thymol (19.22%) as the major abundant constituents. Monoterpene hydrocarbons the second major class of compounds constituted (11.97%) of the oil with *c*-terpinene (4.63%) and *p*-cymene (4.86%) as the main components, whereas, sesquiterpene hydrocarbons and oxygenated sesquiterpenes comprised 1.07% and 0.43%, respectively. In contrast with our findings, Ozturk and Ercisli *et al*.,[13], who investigated the chemical composition of *Z. clinopodioides* essential oil gathered from the Erzurum–Palandoken mountain of Turkey, reported that pulegone (31.86%), 1,8-cineole (12.21%), limonene (10.48%), menthol (9.13%), *β*–pinene (6.88%), menthone (6.73%), piperitenone (5.30%) and piperitone (4.18%) as the major constituents the essential oil. Moreover, Behravan *et al*.,[14] showed that pulegone (44.5 %),

terpineol (14.5%), methyl acetate (10.9%), iso-neomenthol (7.1%) and 1, 8-cineole (4.1%) were the most abundant components of the essential oil obtained from Mashhad, Khorasan Razavi province (North East of Iran). In agreement with our results, Aghajani *et al*.,[15] and Schulz *et al*.,[20] reported that carvacrol and thymol were the major compounds of the essential oil of *Z. clinopodioides* plant harvested from Iran and Turkey, respectively. In general, The diversity and variety of chemical composition of the essential oils of plants could be due to several factors such as genetic and growth stage, age of the plant, part of the plant, seasonal and environmental condition, geographical location, the method used to extraction of the essential oil and other factors[21-23]. Previous study reported that the main components related to the antibacterial activity of *Z. clinopodioides* essential oil were carvacrol and thymol[14]. The mechanism of action of carvacrol is due to the destabilization of the phospholipid bilayer structure and interaction with membrane enzymes[24,25].

Control of *L. monocytogenes* in milk during storage at 4°C

Based on our results, it was found that *Z. clinopodioides* (at concentrations of 0.1 and 0.2%) had high antibacterial effect against *L. monocytogenes* compared to the control group in milk as shown in Fig 1. In detail, the initial inoculum population of pathogen was 5 log CFU/ml that was reached at 7.29 log CFU after 9 days in control samples. In treated group with essential oil at 0.2%, the population of pathogen was kept below 1 log

Table 1. Essential oil composition of *Z. Clinopodioides* identified by GC-MS

No.	Compound name	Composition%	Retention time (min.)	KI[a]
1	*á*-Thujene	0.26	11.33	927
2	*á*-Pinene	0.27	11.71	934
3	Camphene	0.13	12.61	952
4	*â*-Pinene	0.06	14.06	981
5	1-Octen-3-ol	0.08	14.32	986
6	Myrcene	0.51	14.62	992
7	*á*-Phellandrene	0.13	15.58	1010
8	*á*-Terpinene	0.79	16.11	1021
9	*p*-Cymene	4.86	16.62	1030
10	Limonene	0.1	16.77	1033
11	*â*-Phellandrene	0.11	16.89	1036
12	*c*-Terpinene	4.63	18.31	1063
13	*cis*-Sabinene hydrate	0.07	19.02	1077
14	Terpinolene	0.08	19.69	1089
15	Linalool	0.13	20.5	1105
16	Borneol	0.61	24.36	1183
17	Terpinene-4-ol	0.48	24.7	1190
18	*á*-Terpineol	0.08	25.49	1206
19	Carvacrol, methyl ether	0.04	27.38	1246
20	Thymol	19.51	29.61	1293
21	Carvacrol	65.22	30.57	1315
22	*E*-Caryophyllene	1.07	35.47	1427
23	Spathulenol	0.12	42.10	1590
24	Caryophyllene oxide	0.31	42.30	1595
	Other	0.08		
	Total	99.65%		

*Expressed as percentage of the total peak area
** The dominant compounds are indicated in bold
[a] Kovats index

CFU/ml on day 9 of storage. Based on our results, there was no significant difference (p>0.05) between samples treated with the combination of the essential oil at 0.1% and 0.2%. With respect to treated groups with nisin, concentration of 500 IU/ml nisin completely inhibited the growth of *L. monocytogenes* at 9 day and no cell count formation was observed (Fig 2). Based on our results, a strong antibacterial effect was observed when milk sample was treated with 0.1 and 0.2% essential oil in combination with 250 and 500 IU/ml nisin (Fig 3). Our samples treated with the essential oil in combination with nisin showed the populations of the pathogen significantly (p<0.05) lower than the untreated samples. Moreover, samples treated with the combination of the essential oil at 0.2% and nisin at 500 IU/ml, showed populations of *L. monocytogenes* significantly (p<0.05) lower than those of samples treated with other groups. The present study showed certainly for the first time, the antibacterial activity of *Z. clinopodioides* essential oil separately and in combination with nisin in milk. Previous studies reported that the combination of essential oils and nisin had a greater effect than the essential oil or nisin separately against *L. monocytogenes* in milk samples. Yoon *et al*[5] who investigated the antibacterial activity of nisin and cone essential oil of *Metasequoia glyptostroboides* Miki ex Hu against *L. monocytogenes* in milk, reported that the essential oil at 1 and 2 % and nisin at 62.5, 125, 250 and 500 IU/ml, separately and in combination, had strong antilisterial activity in whole (8%), low (1%) and skim (no fat content) milks. Their results related to antibacterial activity of nisin are agreement with our findings.Moreover, Kim *et al*[1], reported that nisin (62.5, 125, 250 and 500 IU/ml) and garlic shoot juice (2.5 and 5%) had synergistic effects against *L. monocytogenes* in milk. In addition, our results are accordance with Bajpai *et al*.,[6] that reported nisin at concentration 250 and 500 IU/ml had antilisterial effect in pasteurized milk. Henning *et al*.,[26] stated the antlisterial activity of nisin is due to its amino acid residues that react with the fatty acids of the membrane phospholipids of the pathogen. With regard to the synergistic effects of nisin and the essential oil, Singh *et al*.,[11] revealed that the use of combination of nisin and garlic extract lead to secure both microbial stability and safety to maintain the sensory, nutritive and economic properties of the foods. The mechanism of combination effects of nisin and various essential oils is not fully understood. It seems that essential oil enhance the effect of nisin by increasing the number of pores in the phospholipid bilayer membrane structure by nisin and also by increasing the size of the pores formed[27,28]. Hence, due to synergistic combinations of

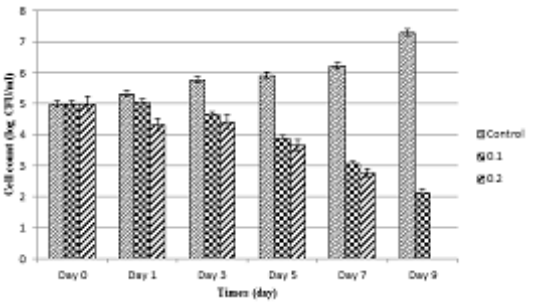

Fig. 1. Antibacterial effect of *Z. clinopodioides* essential oil (0.1 and 0.2%) against *Listeria monocytogenes* in milk sample for 9 days

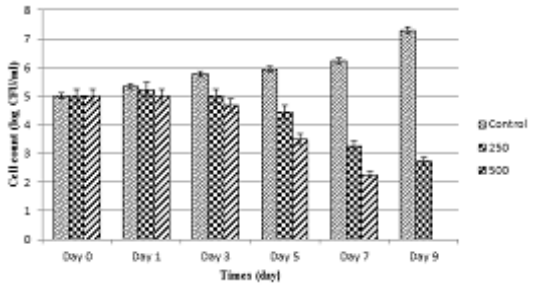

Fig. 2. Antibacterial effect of nisin (250 and 500 IU/ml) against *Listeria monocytogenes* in milk sample for 9 days

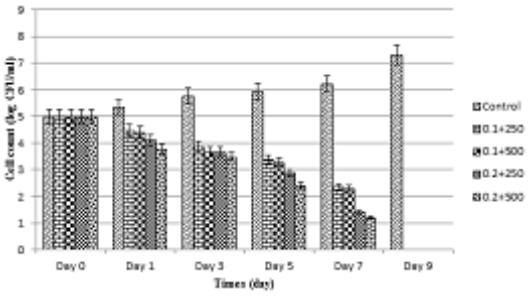

Fig. 3. Antibacterial synergistic effect of various combinations of *Z. clinopodioides* essential oil (0.1 and 0.2%) and nisin (250 and 500 IU/ml) on *Listeria monocytogenes* in milk for 9 days

Z. clinopodioides essential oil and nisin in milk could apply as the alternative antimicrobial agents in foods and food products.

CONCLUSION

In conclusion, our finding showed that thymol, *p*-cymene and c-terpinene the main compounds of *Z. clinopodioides* essential oil collected from Zagros Mountain ranges, west of Iran. Based on our results, the essential oil separately and in combination with nisin had strong antilisterial activity in milk after 9 days of storage under refrigerated temperature. Hence, this essential oil separately and in combination with nisin could be applied in food industries as alternative food preservatives and to control the growth of food microbial pathogens such as *L. monocytogenes.*

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