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Determination of Antimicrobial Effects of Nisin and *Mentha spicata* Essential Oil against *Escherichia coli* O157:H7 Under Various Conditions (pH, Temperature and NaCl Concentration)

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ABSTRACT

Background: Plant essential oils and nisin have been known as antimicrobial agents that could be used to control food-borne pathogenic bacteria such as *Escherichia coli* O157:H7. The aim of this study was to evaluate the antimicrobial efficacies of nisin and *Mentha spicata* essential oil (EO) both separately and in combination, against *Escherichia coli* O157:H7 at different temperatures (4, 9 and 14°C), pH (5, 6 and 7) and NaCl concentrations (0, 1, 2 and 4%). ***Methods:*** The chemical components of EO were analysed by GC-MS. The minimum inhibitory concentrations (MIC) of nisin and EO were assessed using a broth micro-dilution method. For combinations of the antimicrobials, the Differences in Population assay were used to determinate their effects. ***Results:*** The dominant active components of EO were carvone (78.76%) and limonene (11.50%). The EO MIC value was 40µl/ml, but nisin did not inhibit the growth of *E.coli* O157:H7. The susceptibility of *E.coli* O157:H7 to nisin and EO was found to enhance with increasing incubation temperature, pH and NaCl concentration. ***Conclusion:*** Our findings demonstrate that a combination of nisin and *Mentha spicata* essential oil might be a potential source of preservative for the control of *E.coli* O157:H7 in the food industry.

Introduction

Many strains of verocytotoxigenic *Escherichia coli* (VTEC) are recognised as zoonotic agents which cause a potential human illness.[1] VTEC serotype O157:H7 is deemed as an important food-borne pathogen.[2,3] Numerous food-borne illness outbreaks of *Escherichia coli* O157:H7 have been reported worldwide with a clinical spectrum which includes diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome (HUS), which is the leading cause of acute renal failure in children.[4] Consumption of products with different pH, such as undercooked ground beef, unpasteurized milk, and juices, has been deemed the most common major potential sources for this microorganism.[5] Control of this bacterium has a significant impact for the food industries.[6]

Researchers are currently investigating several approaches for eliminating *E. coli* O157:H7 from livestock, including antibiotics, antimicrobials, probiotics, vaccines and bacteriophage.[3] Although chemical and artificial preservatives, such as antibiotics have been used for years in food and pharmaceutical industries,[7] increasing bacterial resistance to these constituents,[8] in addition to increasing concerns about the significant drawbacks of synthetic additives, has lead to wider public acceptance of natural preservatives with their lower levels of toxicity and fewer negative environmental effects.[3]

Many essential oils have demonstrated antimicrobial activity against food-borne pathogens. *Mentha spicata* is an aromatic plant belonging to the Lamiaceae family that grows in Africa, Temperate Asia and Europe. The fresh and dried plants and their essential oils are widely used medicinally for nausea, vomiting and gastro-intestinal disorders, as well as being used as biopreservatives in the food and pharmaceutical industries.[9] The main constituents of the essential oil of this plant are phenolic compounds such as carvone and limonene.[10]

Nisin a well known antibacterial peptide produced by *Lactococcus lactis* or *Streptococcus uberis* strains, is widely used as preservative in foods like cheese.[11] In addition, it is the only bacteriocin that has been approved as safe (GRAS) by the Food and Drug Administration (FDA) and the World Health Organisation (WHO), to be used as an additive in the food industries in over 50 countries. Nisin has been found to inhibit a broad range of Gram-positive organisms such as *Listeria monocytogenes* and *Bacillus cereus.*[12,13] It demonstrates nisin stability under

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refrigerated conditions, heat and acid stability, and is degraded in the digestive system.[2]

The effective suppression of bacteria by nisin depends on a wide range of factors and occurs especially when it is used in combination with other factors.[12] Several studies have shown that the antibacterial activity of nisin can be enhanced by different plant essential oils,[2,14-16] garlic shoot juice,[17] ethanol,[18] sodium fluoride or chlorhexidine,[11] lactoperoxidase[19] and high hydrostatic pressure.[20]

To establish the usefulness of natural antimicrobial preservatives, they must be evaluated alone as well as in combination with other preservation factors such as pH, salt concentration and temperature.[15] Therefore, the objective of this study was to investigate the effect of *Menthe spicata* essential oil and nisin, both separately and in combination, on *E. coli* O157:H7 at different temperatures (4, 9 and 14°C), pH (5, 6 and 7) and NaCl concentrations (0, 1, 2 and 4 g/100 ml) in a laboratory medium.

Materials and Methods

Essential oil

The commercial *Mentha spicata* EO used in this study was purchased from Barij Essence Co. Ltd. (Kashan, Iran). The plant EO was stored at a temperature of 4-6°C prior to analysis.

Gas chromatography and mass spectrometry

The gas chromatography and mass spectrometry (GC-MS) analysis of the essential oil was carried out using an Agilent 7890/5975C GC-MS system, fitted with a DB-624 capillary column (30 m length×0.25 mm; 0.25µm film thickness). The column temperature programme was adjusted as follows: initial temperature of the oven was 60°C, and was held at this temperature for 3 min. The temperature was then increased to 220°C at a rate of 5°C per min. and held for 1 min. The temperature of the injector was 250°C. The amount of injection was 0.2µl. The carrier gas was Helium with a flow rate of 1.2ml/min and a split ratio equal to 1:4. The mass spectrometer was operated in EI ionisation mode at 70eV, and complete scans from 40 to 350amu (atomic mass units) were recorded. The constituents of the oil were identified by the confirmation of the experimental gas chromatographic retention indices (RI) relative to n-alkanes (C8–C24) and mass fragmentation with those of National Institute of Standards and Technology (NIST 08) commercial library, as well as with literature data.[21] All analyses were carried out in triplicate. The details of commercial EO were listed in Table 1.

Nisin preparation

A stock of nisin (10^4) was prepared by dissolving 20mg of pure nisin (Sigma-Aldrich, Dorset, UK) in 0.02M HCL (Merck, Darmstadt, Germany).[15] The solution was heated at 80°C for 7 min, and kept at -20°C until use.[22]

Table 1. Essential oil composition of *Mentha spicata* identified by GC-MS

Compound	Retention index	Percentage
Beta- Myrcene	450	0.25
Limonene	509	11.50
Gamma-Terpinene	553	0.16
Menthone	703	1.01
Menthol	713	1
Terpinen-4-ol	720	0.99
Alpha-Terpinol	737	0.31
Dihydrocarveol	742	0.22
Cis-Dihydrocarveol	746	1.43
Dihydrocarvone	756	0.43
Trans-Carveol	773	0.3
Carvone	819	78.76
Dihydrocarvyl acetate	906	0.57
L-carveol	946	0.32
Beta-Bourbonene	981	1.23
Trans-Caryophyllene	1021	1.04
Gamma-Amorphene	1048	0.21
Alpha-Amorphene	1058	0.16
Others	-	0.11
Sum		100

Bacterial strain

Escherichia coli O157:H7 (ATCC 10536) was obtained from the Iranian Research Organisation for science & Technology. The strain was maintained in the Brain Heart Infusion Broth (BHI; Merck, Darmstadt, Germany) containing 25% v/v glycerol at -80°C. The strain was activated by two consecutive subcultures overnight in BHI at 37°C in 4 replicates. The overnight culture (from the second subculture) was diluted to achieve an initial inoculation level of approximately 10^6 colony forming units per millilitre (CFU/ml).

Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of *Mentha spicata* EO was tested using the micro-broth dilution assay by two-fold serial dilution method.[23] The test sample oil was incorporated in the BHI Broth to obtain a concentration of 2560µl/ml and serially diluted to achieve a concentration range from 2560 to 20µl/ml. A geometric dilution of nisin was prepared with 2.5, 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560IU/ml in sterile test tubes containing BHI broth. The 96 well sterile plates (Extragene, California, USA) were used by dispensing into each well 160µl/ml of BHI Broth and an aliquote (20µl/ml) of EO and nisin at the appropriate concentration was added to the wells. Bacterial culture was diluted to 10^6 CFU/ml in the growth medium and 20µl/ml of the diluted culture was added to each well. A control consisting of the bacterial strain without EO or nisin and two sterility controls containing EO or nisin were run in each plate. The contents of each well were mixed on a plate shaker at 250 rpm for 30s and incubated at 37°C for 24h. The MIC was defined as the lowest concentration of EO or nisin in the last well in which culture growth was not

detected following incubation, and confirmed by plating 20µl from clear wells onto Brain Heart Infusion Agar (BHI; Merck, Darmstadt, Germany) medium.

Mentha spicata essential oil and nisin combination procedure

Three temperatures (4, 9 and 14°C), three pH (5, 6 and 7), four NaCl concentrations (0, 1, 2 and 4 g/100ml), along with two dilutions from each nisin (2.5 and 5IU/ml) and EO (10 and 20µl/ml) preparation were used to define combination.

Four combination systems were studied as follow: 1)10µl/ml EO + 2.5IU/ml nisin; 2) 10µl/ml EO + 5IU/ml nisin; 20µl/ml EO + 2.5IU/ml nisin; 20µl/ml EO + 5IU/ml nisin. The range of concentration tested for these combinations of each agent were two dilutions lower than the MIC of EO and nisin. After incubation at the desired temperatures (4, 9 and 14°C) for 24h, samples were enumerated from each well, by ten-fold serial dilutions with BHI Broth, followed by plating on BHI Agar and incubation for 24h at 37°C. The results were expressed in terms of differences in population (DP) according to the equation (1).[14,24]

$$\text{Log DP} = \log\left(\frac{N}{N0}\right) = \log(N) - \log(N0) \qquad (1)$$

Where N and N0 are the bacterial population (CFU/ml) at times t and zero, respectively.

Statistical analysis

Each set of experimental tests was conducted three times to determine the effect of EO and nisin alone as well as in combination, on survival and growth of the tested bacterial strain. Analysis of variance and comparison of means (Turkey's method) were performed on all data sets using SPSS software (version 19.0; SPSS Inc, USA).

Results

Chemical composition of Mentha spicata essential oil

GC-MS analyses of the oil led to the identification of 18 different compounds, representing 99.89% of the total oil. The identified compounds, together with the retention in indices (RI) of the compounds are shown in Table 1. The main chemical compounds detected in the oil were carvone (78.76%), limonene (11.50%), menthone (1.01%), menthol (1%), cis-dihydrocarveol (1.43%), trans-caryophyllene (1.04%), beta-bourbonene (11.23%) and terpinen-4-ol (0.99) (Table 1).

Effects of Mentha spicata essential oil and nisin on Escherichia coli O157:H7 under various test conditions

For the determination of the antibacterial activities of two antimicrobials, the MIC of *Mentha spicata* EO was 40µl/ml, but nisin did not inhibit the growth of bacteria. Taking these results into account, concentrations of 10 and 20µl/ml for EO and 2.5 and 5IU/ml for nisin were used in the combination systems. The effects of four combinations of EO and nisin on *E.coli* O157:H7 growth in laboratory medium at three temperatures, three pH and four NaCl concentrations are listed in Tables 2, 3 and 4.

Table 2. Effect of *Mentha spicata* and nisin on the difference in population (DP) index of *Escherichia coli* O157:H7 at 14 °C

Treatment		Log DP					
		Mentha Spicata (µl/ml)		*Mentha Spicata* (µl/ml) and Nisin (IU/ml)			
pH	NaCl g/100	10	20	10+2.5	10+5	20+2.5	20+5
5	0	2.07±0.106	-1.55±0.014	0.92±0.325	1.11±0.162	-1.71±0.183	-2.17±0.127
	1	1.32±0.091	-2.04±0.014	0.69±0.558	0.06±0.113	-2.14±0.084	-3.45±0.091
	2	1.11±0.028	-2.87±0.318	0.41±0.756	-0.26±0.325	-2.98±0.084	-3.84±0.219
	4	0.285±0.205	-	-0.315±0.205	-1.26±0.940	-	-
6	0	1.22±0.007	-1.78±0.007	1.07±0.042	0.98±0.134	-1.98±0.063	-2.40±0.077
	1	0.64±0.332	-2.31±0.028	0.695±0.431	-0.51±0.862	-2.46±0.056	-3.60±0.120
	2	-0.21±0.813	-3.49±0.275	-0.41±0.898	-1.14±0.000	-3.84±0.219	-
	4	-0.95±1.29	-	-1.19±0.777	-1.90±0.586	-	-
7	0	0.70±0.226	-2±0.077	0.51±0.042	0.19±0.212	-2.17±0.267	-2.5±0.106
	1	0.55±0.091	-3.60±0.120	-0.59±0.233	-0.67±0.197	-3.84±0.219	-
	2	-1.39±0.685	-	-0.94±0.933	-1.81±0.247	-	-
	4	-1.91±0.113	-	-2.1±0.217	-2.7±0.254	-	-

[a] Logarithm of the difference in population which was estimated from the equation: log DP = log (N/N_0) = (log N)-(log N_0); where N and N_0 are the bacterial populations (CFU/mL) at times t and zero, respectively.

Essential oil and nisin alone, and at combinations shown to be non-inhibitory (two concentration lower than MIC), were combined with each other (four combination systems: 1) 10µl/ml EO+2.5IU/ml nisin; 2) 10µl/ml EO+5IU/ml nisin; 20µl/ml EO+2.5IU/m nisin l; 20µl/ml EO+5IU/ml nisin) as described in Tables 2, 3 and 4 their effectiveness at inhibiting the growth of *E.coli* O157:H7 was measured.

Mentha Spicata essential oil and nisin

Regardless of NaCl concentration, temperature and pH, the number of *E.coli* O157:H7 in culture following

exposure to various concentration of EO (10 and 20µl/ml) significantly varied (P<0.001). In the presence of concentration 20µl/ml EO the number of bacteria were significantly (P<0.001) lower than the control.

For nisin, both concentrations (2.5 and 5IU/ml) significantly (P<0.001) inhibited *E.coli* O157:H7. The numbers of surviving bacteria following exposure to nisin significantly varied (P<0.041) between the two concentrations.

Table 3. Effect of *Mentha spicata* and nisin on the difference in population (DP) index of *Escherichia coli* O157:H7 at 9 °C

Treatment		Log DP					
		Mentha Spicata (µl/ml)		*Mentha Spicata* (µl/ml) and Nisin (IU/ml)			
pH	NaCl g/100	10	20	10+2.5	10+5	20+2.5	20+5
5	0	1.99±0.007	-1.29±0.282	1.46±0.374	1.19±0.021	-1.69±0.049	-2.04±0.007
	1	1.43±0.014	-2.01±0.021	0.86±0.028	0.525±0.205	-2.26±0.084	-2.84±0.275
	2	1.30±0.113	-2.51±0.028	0.215±0.049	-0.215±0.304	-2.92±0.106	-3.60±0.120
	4	0.625±0.544	-4±0.000	-1.33±0.728	-1.14±0.155	-3.84±0.219	-
6	0	1.27±0.021	-1.79±0.021	1.03±0.212	1.31±0.275	-2.07±0.410	-2.47±0.070
	1	1.25±0.007	-2.29±0.056	1.10±0.120	0.53±0.042	-2.47±0.148	-2.69±0.183
	2	0.43±0.042	-3.02±0.028	0.30±0.000	-0.02±0.028	-3±0.120	-3.69±0.000
	4	0.275±0.007	-3.54±0.212	0.06±0.084	-1.62±0.756	-3.60±0.120	-
7	0	1.01±0.155	-2.61±0.077	0.92±0.049	0.43±0.395	-2.94±0.813	-2.88±0.049
	1	0.38±0.120	-3.12±0.042	0.43±0.007	-0.08±0.127	-2.48±0.091	-3.34±0.063
	2	0.04±0.304	-3.50±0.261	0.005±0.275	-0.35±0.233	-3.76±0.339	-
	4	-1.15±0.480	-	-1.33±0.650	-1.19±0.042	-	-

Table 4. Effect of *Mentha spicata* and nisin on the difference in population (DP) index of *Escherichia coli* O157:H7 at 4 °C

Treatment		Log DP					
		Mentha Spicata (µl/ml)		*Mentha Spicata* (µl/ml) and Nisin (IU/ml)			
pH	NaCl g/100	10	20	10+2.5	10+5	20+2.5	20+5
5	0	2.08±0.028	-1.22±0.240	1.90±0.268	1.35±0.035	-1.54±0.000	-1.91±0.155
	1	1.75±0.226	-1.91±0.226	1.59±0.360	0.84±0.141	-1.97±0.247	-2.48±0.431
	2	1.34±0.028	-2.12±0.219	1.18±0.049	0.235±0.148	-2.17±0.035	-3.54±0.212
	4	0.71±0.410	-3.86±1.29	0.85±0.148	0.46±0.735	-2.61±0.551	-
6	0	1.91±0.021	-1.56±0.063	1.73±0.134	1.33±0.452	-1.92±0.254	-2.23±0.289
	1	1.45±0.014	-2.24±0.035	1.35±0.042	1.04±0.431	-2.38±0.148	-2.57±0.608
	2	1.22±0.219	-2.52±0.296	1.18±0.205	0.565±0.586	-2.59±0.318	-3.69±0.000
	4	1.12±0.190	-2.99±0.982	0.55±0.714	0.24±0.537	-3.69±0.000	-
7	0	1.63±0.205	-1.92±0.304	1.51±0.049	0.87±0.148	-2.02±0.325	-2.40±0.127
	1	1.29±0.134	-2.40±0.381	1.16±0.325	0.34±0.098	-2.62±0.141	-3.21±0.671
	2	1±0.077	-3.30±0.304	0.75±0.395	0.16±0.770	-1.80±2	-4±0.000
	4	0.42±0.056	-	0.25±0.247	-1.26±1.23	-	-

Temperature

The numbers of surviving *E.coli* O157:H7 following exposure of antimicrobial compounds differed significantly between incubation temperatures (P<0.002). The numbers of surviving *E.coli* O157:H7 following exposure to *Mentha spicata* EO and nisin decreased significantly with the incubation of all temperatures (4°C (P<0.01), 9°C (P<0.001) and 14°C (P<0.001)). With reference to the effect of temperature on bacterial growth for the EO alone and in combination with nisin, 14°C was more effective than 4°C and 9°C (P<0.05). At 14°C, the numbers of *E.coli* O157:H7 decreased significantly (P<0.001) after exposure to 20µl/ml EO as well as 20µl/ml EO+2.5IU/ml and 20µl/ml EO+5 IU/ml nisin.

pH

As shown in Tables 2, 3 and 4, regardless of NaCl concentration and temperature, the antibacterial activity of EO and nisin were strongly affected by pH, with greater activity being found at a higher pH (P<0.005). In the absence of other factors, all pHs significantly (P<0.001) reduced the numbers of *E.coli* O157:H7. A concentration of 20µl/ml EO in combination with 5IU/ml nisin significantly (P<0.001) inhibited *E.coli* O157:H7 at different pHs. For example, *E.coli*

O157:H7 showed a lower and higher log DP at pH 7 and pH 5, respectively.

Sodium chloride concentrations

The numbers of surviving *E.coli* O157:H7 following exposure to EO and nisin varied (P<0.005) between 4g/100ml NaCl with others NaCl concentrations. We found that at the three temperatures used in this study, the growth of the organism was affected by NaCl concentrations (1 (P<0.046), 2 and 4g/100ml (P<0.001)). According to these results, 4g/100ml NaCl concentration enhanced the sensitivity of *E.coli* O157:H7 towards four combinations, which suggests that NaCl concentration is also an important factor of efficacy of these combination systems.

Synergism or antagonism

Our results demonstrated that among different EO, nisin, NaCl, pH and temperature values, the effect of EO were confounded than the effect other factors (P<0.001).

Discussion

Historically, many aromatic plants oils and their derivatives (e.g., mint, thyme and garlic) have been known to exhibit biological activity and have been used for many purposes, such as food flavouring, medicines and perfumes. At the same time, recent concerns in the food industry and a strong consumers demand for high-quality products that contain fewer preservatives and are less processed, means that quantitative data on plant oils and extracts are required. Essential oils, which are odorous and volatile products of plant secondary metabolism, have a wide application in foodstuffs as culinary ingredients for taste and for preservation industries.[3,8,19]

The synergistic effect of essential oils and nisin on bacteria such as *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* has been reported.[14,15,25] Since essential oil and nisin act on the bacterial cytoplasmic membrane, their antibacterial activity could be enhanced by treatments involving a combination of these compounds, because EO might increase the number of pores created in a layer membrane by nisin, or increase the size of pores formed.[26]

In the present study, it was found that *Mentha spicata* oil, along with various combinations of nisin, had an enormous influence on BHI Broth in reducing the growth of *E. coli* O157:H7.

The essential oil composition of *Mentha spicata* oil was determined by GC-MS and the composition of EO was in general agreement with literature data for *Mentha spicata* EO. Telci et al. (2010), who investigated environmental variation in the new Turkish pulegone-piperitone rich spearmint (*Mentha spicata* L.) chemotype, reported carvone (50-65%) in its essential oils. Other major components of *Mentha spicata* oil are limone and 1, 8-cinole.[10,27] Another study, Sertkaya et al.[28] which evaluated the components of *Mentha spicata* oil, stated 59% carvone, 10% limonene and 7% 1, 8-cinole in GC-MS analysis of its essential oil.

Antimicrobial activity in most experiments has been quantified on two bases, MIC and MBC.[3] The results of this study showed that EO exhibited a MIC of 40µl/ml. Results obtained by researchers working with *E.coli* strains in broth media[1,29] were in contrast to this study. In addition, Mahboubi and Haghi[30] reported that the antimicrobial activity of flowering *Mentha pulegium* L. essential oil against *E.coli*, had a MIC value of 4µl/ml. Different reported MICs in these studies may be the result of using different bacterial species and media.[3,14] Nisin, on the other hand, did not inhibit the growth of *E.coli* O157:H7. The hydrophobic lipopolysaccharide present in the outer layer of Gram-negative bacteria might be responsible for the enhanced resistance to this compound.[31]

In the present study, the antimicrobial activities of EO and nisin against a strain of *E. coli* O157:H7 were assessed under various conditions including pH, temperature and NaCl concentration.

The antibacterial activity of EO and nisin were affected by pH, with greater activity being found at higher pH (P<0.005). Earlier studies have shown that most food-borne pathogens are lethally susceptible low pH.[32] But Tajkarimi et al.[3] reported that acidic a property would not be the key factor influencing the survival of *E. coli* O157:H7. These results are similar to that reported by Han & Linton[33] and Singh et al..[34]

According to our results, the susceptibility of bacteria to the antimicrobial effect of EO and nisin were generally found to enhance, with an increase of incubation temperature. Rivas et al.[1] reported that the antimicrobial activity of thymol and carvacrol against a strain of *E.coli* O157:H7 increased with a decrease of pH. These results are in contrast to this and other studies.[35] On the other hand, Solomakos et al.[2] stated that a combination of thyme EO and nisin showed an additive effect against the pathogen, which was higher at 10°C than at 4°C of storage.

We found the presence of NaCl concentration enhanced EO and nisin effects, which is in agreement with the results of Bouttefroy et al.,[36] Rivas et al.[1] and Razavi Rohani et al..[14]

Conclusion

The antibacterial activity of *Mentha spicata* EO separately and in combination, at different temperature, pH and NaCl concentrations, was assessed by DP method. The combination of high pH, NaCl concentration and temperature have been shown to enhance EO and nisin activities against *E.coli* O157:H7.

In addition, further studies are required to identify the efficacy of these bio-active compounds, to prevent growth of other food-borne pathogens and spoilage microbes and their survival on food products. This

study recommends the application of the *Mentha spicata* EO and nisin as natural antimicrobial compounds in the food and pharmaceutical industries.

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