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*Research Article*

# The Combined Effect of *Mentha spicata* Essential Oil and Nisin Against *Listeria monocytogenes*

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**ABSTRACT**

***Background:*** *Listeria monocytogenes* is one of the major causes of infections in developing countries. The aim of the present study was to investigate chemical composition and the combined effect of *Mentha spicata* essential oil with nisin against *L. monocytogenes* at different temperatures (4, 9 and 14°C), pHs (5, 6 and 7) and NaCl concentrations (1, 2 and 4g/100ml) in *in vitro* condition. ***Methods:*** Chemical composition of the essential oil was evaluated by GC-MS analysis. Minimum inhibitory concentration (MIC) values of the essential oil and nisin were determined by using broth micro-dilution test. The Differences in Population (DP) assay and Fractional Inhibitory Concentration (FIC) index were applied to investigate their synergistic effects. ***Results:*** The main components of the essential oil were carvone (78.76%) and limonene (11.50%). MIC values of the essential oil and nisin against *L. monocytogenes* were 320IU/ml and 160µl/ml, respectively. Concentration of 80µl/ml essential oil in combination with 160IU/ml nisin significantly (P<0.001) inhibited the bacterium at all pHs. Also, concentration of 40µl/ml and 80 essential oil in combination with 80 and 160IU/ml nisin significantly (P<0.001) inhibited the bacterium at all temperatures. 2g/100ml and 4g/100 ml NaCl concentration enhanced the sensitivity of *L. monocytogenes* toward four combinations. Reduction in the pH and incubation temperature and increasing of salt content led to enhance the anti-listerial effects of the essential oil and nisin. ***Conclusion:*** nisin and *M. spicata* essential oil could be considered as potential strong antimicrobials that can be used for the growth inhibition of *L. monocytogenes* in food products.

## Introduction

*Listeria monocytogenes*, is gram-positive, facultative anaerobe, catalase-positive, oxidase-negative and a non-spore forming bacterium, is one of the most food-borne pathogens that has been found in different environments including soil, water, and raw and processed foods (especially refrigerated foods such as ready-to-eat food, meat, cheese and milk).[1-3] This bacterium seems to be a major concern for at-risk consumers, especially pregnant women, newborns and adults with weakened immune system, due to severe diseases caused by the bacterium such as stillbirth and premature delivery, meningitis and septicemia in perinatal cases.[4-6] One of the most important characteristics of this bacterium is its ability to grow at under relatively extreme physicochemical conditions such as 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.[3,7]

Safe chemical antimicrobials have been widely used for the preservation of food products.[8] Nisin is only the Generally Recognized as Safe (GRAS) bacteriocin by Food and Drug Administration (FDA) and World Health Organisation (WHO) that produced by certain strains *Lactococcus lactis* or *Streptococcus uberis*.[9] This peptide shows strong antimicrobial activity against gram-positive and rarely gram-negative bacteria.[10,11] It has been applied as a food preservative additive since the 1940s and currently approved as food additive in over 50 countries.[4] It has been reported that nisin is not able to inhibit some gram-positive bacteria such as *L. monocytogenes* and spore forming bacteria.[2] To enhance the effect of nisin on some gram-positive bacteria such as *L. monocytogenes* and gram-negative bacteria, combination of this antimicrobial agent with other antimicrobials such as essential oil, chelating agents (ethylenediamine tetraacetic acid (EDTA)), food

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grade acids (acetic acid), as well as sodium fluoride or chlorhexidine, and heat treatment should be used.[12,13]

In recent years, besides the use of chemicals, food preservation by natural products and preservatives has been considered as a new and safe approach for inhibiting the growth of food borne pathogens and spoilage bacteria.[14,15] Essential oils are considered as natural components for food industries.[16] These materials usually exhibit different characteristics such as antimicrobial and flavoring effects.[16] *Mentha spicata* (spearmint) belonging to the Lamiaceae family grows in throughout the world and this plant is widely employed as a flavoring agent in several foods, also cosmetic, confectionary and pharmaceutical industries.[17,18] Historically, the genus of *M. spicata* has been applied to treat gastro-intestinal disorders.[18] Carvone and limonene the main components of the essential oil of *M. spicata*, has been reported to have antibacterial, antioxidant, antiseptic and antifungal properties.[17-19]

The antibacterial effect of the essential oil of *M. spicata* and nisin has previously been reported against some of bacteria.[10,11,17,18,20] However, based on the knowledge of the authors, no report is available on their combination effect against gram-positive or gram-negative bacteria. The objective of this study is to evaluate the effect of nisin and *M. spicata* oil in combination against *L. monocytogenes* at different temperatures (4, 9 and 14°C), pHs (5, 6 and 7) and NaCl concentrations (1, 2 and 4g/100 ml) in laboratory medium.

## Material and Methods

### *Antimicrobials and chemicals*

Nisin (Sigma-Aldrich, UK) stock solution was prepared by dissolving 20mg in 0.02M HCL to yield $10^4$ IU/ml.[10] The solution was heated at 80°C for 7 min, and maintained at -20°C until use.[21] The fresh aerial parts of *Mentha* plant were gathered from Tabriz, East Azarbaijan at the full flowering stage at July 2011. Authentication of the botanical of the plant was conducted by Faculty of Agriculture, University of Tabriz, Tabriz, Iran. The specimen of the collected plant materials were recognized as *M. spicata*. The Stock solution of the oil (2560µl/ml) was prepared in 10ml of Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) containing 5% v/v dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and 0.05% w/v agar-agar (Merck, Darmstadt, Germany). Culture media used in the present study was BHI agar (Merck, Darmstadt, Germany) and BHI broth, which adjusted to pH 5, 6 and 7 using citric acid and NaOH.

### *Analysis of the essential oil*

The analysis of *M. spicata* essential oil was performed using an Agilent Agilent 7890/5975C chromatographer that was equipped with a DB-624 capillary column (30.00 length × 0.25mm ID; 0.25mm film thickness). Carrier gas was helium with a flow rate of 1.2ml/min. The column temperature was initially 60°C, and then gradually increased to 220°C at a 5°C/min rate, held for 1 min, and finally increased to 220°C. The procedure was operated at 70eV. Identification of the major constituents of the essential oil was accompanied based on comparison between their retention indices (RIs), Standard Mass Spectral fragmentation pattern (Wiley/NBS) and the NIST (National Institute of Standards and Technology). The percentage of each essential oil compositions was calculated from GC peak areas.

### *Test microorganism*

The strain used in the current study was *L. monocytogenes* ATCC 19115. Lyophilized cultures of the organisms were obtained from the Iranian Research Organisation for Science & Technology (IROST), Tehran, Iran.

### *Preparation of nisin and M. spicata essential oil*

Serial dilutions of nisin were prepared in BHI broth from the stock solution of nisin ($10^4$IU/ml) to obtain different concentrations ranged from 2.5 to 2560 IU/ml. A similar procedure was used to prepare the serial dilutions of the essential oil (20 to 2560µl/ml).

### *Determination of Minimum inhibitory concentration (MIC) of nisin and M. spicata essential oil*

Minimum inhibitory concentration (MIC) value of nisin was determined using broth micro-dilution test according Azizkhani *et al*., and Rohani *et al*. with some modification.[5,15] For this purpose, sterile 96 well micro-plates (Extragene, USA) were used. Briefly, 160µl of BHI broth, 20µl of different concentrations of nisin (2.5 to 2560 IU/ml) and 20µl of inoculum diluted in BHI broth with $10^5$CFU/ml of *L. monocytogenes* were located into each well. The last well containing 180µl BHI broth, 20µl of inoculum without nisin was designed as the positive control sample. For negative control, un-inoculated BHI broth was used in order to determine sterility. Contents of each well were mixed using a plate shaker at 300 rpm for 30 seconds and afterwards, microplates were incubated at 35°C for 24h. The MIC was defined as the lowest concentration of the antimicrobial that prevented the growth of *L. monocytogenes* completely. To determine MIC values of *M. spicata* essential oil, the procedure presented above was used, but the concentrations of the oil were adjusted at different levels (20 to 2560µl/ml).

### *Mentha spicata essential oil and nisin combination procedure*

Three temperatures (4, 9 and 14°C), three pHs (5, 6 and 7), three NaCl concentrations (1, 2 and 4 g/100ml) along with two dilution from each nisin (80 and 160 IU/ml) and essential oil (EO) (40 and 80 µl/ml) preparation were used to define combination. Before each experiment in this study, the BHI broth containing different NaCl concentrations (1, 2 and 4 g/100ml) and

various pHs (5, 6 and 7) were prepared by using citric acid and NaCl (Merck, Darmstadt, Germany), respectively and then autoclaved.

Four combination tests, two dilutions lower than the MIC of the essential oil and nisin were considered: 1) 40µl/ml EO + 80IU/ml nisin; 2) 40µl/ml EO + 160IU/ml nisin; 3) 80µl/ml EO + 80IU/ml nisin; 4) 80µl/ml EO + 160IU/ml nisin. Then, the micro-plates incubated at three temperatures (4, 9 and 14°C) for 24h. After incubation, samples were enumerated 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).[5,22]

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

Where $N$ and $N_0$ are the bacterial population (CFU/ml) at times $t$ and zero, respectively.

The broth dilution checkerboard method, which was frequently used to assess interactive inhibition *in vitro*, was used to determine the antimicrobial effects of EO and nisin combinations obtained in antimicrobial activity testing. For this purpose, Fractional Inhibitory Concentration (FIC) index was calculated. The FICI was calculated as FICA+FICB,[23] where $FIC_A$= ($MIC_A$ of the combination/$MIC_A$ alone) and $FIC_B$= ($MIC_B$ of the combination /$MIC_B$ alone). The results were interpreted as synergy (FICI<0.5), addition (0.5≤FICI≤1), indifference (1<FICI≤4) or antagonism (FICI>4)

## Statistical Analysis

Analysis of variance and Turkey's test were considered to all data sets by using SPSS software. P< 0.05 was considered as significant differences.

## Results

### *Chemical composition of M. spicata essential oil*

Essential oil composition of *M. spicata* is presented in Table 1. According to GC/MS analysis, 13 components were identified representing 96.8% of the total oil. The main components were phenolic monoterpene carvone (78.76%), followed by 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).

### *Antimicrobial effect of essential oil and nisin in different conditions*

Antibacterial effect of the essential oil and nisin are presented in different conditions in Table 1, 2 and 3. MIC values of the essential oil and nisin against *L. monocytogenes* were 320IU/ml and 160µl/ml, respectively. As it is shown in Table 3, by lowering the pH level to 5, the antibacterial activities of the essential oil and nisin were significantly (P<0.001) increased. pHs 5 and 6 significantly decreased the number of *L. monocytogenes*.

**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 |

Concentration of 80µl/ml essential oil in combination with 160IU/ml nisin significantly (P<0.001) inhibited the bacterium at all pHs. Increasing in incubation temperature led to a decrease concentration of the essential oil and nisin. Briefly, concentration of 40µl/ml and 80 essential oil in combination with 80 and 160IU/ml nisin significantly (P<0.001) inhibited the bacterium at all incubation temperature (Table 2). As well as, based on our results, 2g/100ml and 4g/100 ml NaCl concentration enhanced the sensitivity of *L. monocytogenes* toward four combinations (Table 4). According to the results presented in this study, reduction in the pH and incubation temperature and increasing of salt content led to an increase in the anti-listerial effects of the essential oil and nisin.

**Table 2.** Effect of *M. spicata* and nisin against *L. monocytogenes* at different temperatures (4, 9 and 14°C).

| Temperature | 4°C | | | 9°C | | | 14°C | | |
|---|---|---|---|---|---|---|---|---|---|
| Combination | FIC | log DP | p value | FIC | log DP | p value | FIC | log DP | p value |
| 40µl/ml EO + 80IU/ml N | III | 0.53 | ns | II | -0.31 | p<0.05 | II | -0.37 | p<0.05 |
| 80µl/ml EO + 80IU/ml N | III | 0.14 | ns | II | -0.62 | p<0.05 | I | -1.02 | p<0.001 |
| 40µl/ml EO + 160IU/ml N | I | -0.47 | p<0.001 | I | -1.47 | p<0.001 | I | -1.73 | p<0.001 |
| 80µl/ml EO + 160IU/ml N | I | -1 | p<0.001 | I | -2.32 | p<0.001 | I | -2.86 | p<0.001 |

* ns: non-significant

* The results were interpreted as synergy (I: FIC<0.5), addition (II: 0.5≤FIC≤1), indifference (III: 1<FIC≤4) or antagonism (IV: FIC>4).

**Table 3.** Effect of *M. spicata* and nisin against *L. monocytogenes* at different pHs (5, 6 and 7).

| pH | 5 | | | 6 | | | 7 | | |
|---|---|---|---|---|---|---|---|---|---|
| Combination | FIC | log DP | p value | FIC | log DP | p value | FIC | log DP | p value |
| 40µl/ml EO + 80IU/ml N | I | -0.42 | p<0.001 | П | -0.13 | P<0.05 | Ш | 1 | ns |
| 80µl/ml EO + 80IU/ml N | I | -0.73 | p<0.001 | П | -0.38 | P<0.05 | Ш | 0.98 | ns |
| 40µl/ml EO + 160IU/ml N | I | -2.11 | p<0.001 | I | -1.07 | P<0.001 | Ш | 0.5 | ns |
| 80µl/ml EO + 160IU/ml N | I | -2.31 | p<0.001 | I | -1.98 | P<0.001 | П | -0.2 | P<0.05 |

* ns: non-significant
* The results were interpreted as synergy (I: FIC<0.5), addition (П: 0.5≤FIC≤1), indifference (Ш: 1<FIC≤4) or antagonism (IV: FIC>4).

**Table 4.** Effect of *M. spicata* and nisin against *L. monocytogenes* at different NaCl concentration (1, 2 and 4g/100ml).

| Salt (g/100ml) | 1 | | | 2 | | | 4 | | |
|---|---|---|---|---|---|---|---|---|---|
| Combination | FIC | log DP | p value | FIC | log DP | p value | FIC | log DP | p value |
| 40µl/ml EO + 80IU/ml N | Ш | 0.82 | ns | I | -0.33 | p<0.001 | I | -1 | p<0.001 |
| 80µl/ml EO + 80IU/ml N | Ш | 0.58 | ns | I | -0.68 | p<0.001 | I | -1.14 | p<0.001 |
| 40µl/ml EO + 160IU/ml N | П | -0.14 | p<0.05 | I | -0.81 | p<0.001 | I | -1.38 | p<0.001 |
| 80µl/ml EO + 160IU/ml N | П | -0.41 | p<0.05 | I | -1.48 | p<0.001 | I | -1.99 | p<0.001 |

* ns: non-significant
* The results were interpreted as synergy (I: FIC<0.5), addition (П: 0.5≤FIC≤1), indifference (Ш: 1<FIC≤4) or antagonism (IV: FIC>4).

## Discussion

Various studies have been conducted on the chemical composition and antimicrobial effect of essential oil of plants belonging to Lamiaceae family, particularly the essential oil of *M. spicata* and in most of them, carvone and limonene are reported as the main components of *M. spicata* essential oil. Telci *et al*. reported carvone as the main compounds of *Mentha spicata* essential oil obtained from Turkey.[17,19,24] According to other studies, carvone, limonene and 1, 8-cinole were determined as the major compounds.[20,24,25] The results obtained in this study showed that the *M. spicata* essential oil exhibited strong antibacterial activity against *L. monocytogenes*. The antimicrobial activity of the *M. spicata* essential oil could be associated to the presence of carvone and limonene. It has been reported that carvone is one of the most efficient antimicrobial agents of various plants.[26] The mechanism of action of carvone is related to the destabilization of the phospholipid bilayer structure, interaction with membrane enzymes and proteins and its act as a proton exchanger reducing the pH gradient across the membrane.[27-29] As well as, the variability and diversity of the reports regarding to chemical composition of *M. spicata* essential oil can be attributed to different geographical conditions, climate and seasonal variations and the stage of the plant growth.[30-32]

According to the results obtained here, nisin also showed significant anti-listerial activity. MIC value of nisin was detected as 160 IU/ml against *L. monocytogenes.* Rohani *et al.* and Murdock *et al.* reported an MIC of nisin for *L. monocytogenes* 12.5 IU/ml and 25 IU/ml, respectively.[5,33] Elevated MIC in our study may be due to use different bacterial species and media.[5,16]

Antibacterial effect of the *M. spicata* essential oil and nisin was evaluated by differences in population (DP) determination. As mentioned in results section, in general, antibacterial effect of these components increased when the pH value was reduced, also incubation temperature and NaCl concentration were increased. The results of the present study about susceptibility of the bacterium to reduction of pH and increasing of incubation temperature and NaCl concentration are agreement with other studies.[5,25,34-36] Based on our results, the pHs 5 and 6 significantly decreased the number of *L. monocytogenes* in all concentrations. Boziaris *et al*. reported the most reduction growth of *L. monocytogenes* was occurred at pH 4.81.[34] As well as, Bouttefroy *et al.* and Rohani *et al.* found that 2.5g/100 ml and 4.5g/100 ml NaCl concentrations and increasing incubation temperatures (20°C and 30°C) were enhanced the anti-listerial activities of nisin and various essential oils.[5,35]

In the present study, essential oil and nisin alone at concentrations shown to be non-inhibitory (two concentrations lower than the MIC), were combined with each other (four combination) as described in Tables 2, 3 and 4. Previous study of authors indicated that *M. spicata* essential oil or nisin alone had a slight effect of the growth of *L .monocytogenes* in the laboratory medium.[37] In the current research, it was found that *M. spicata* oil, along with various combinations of nisin, had an enormous influence in reducing the growth of *L. monocytogenes.* 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. However, several researchers reported that the combined use of nisin and various essential oil may be affected by factors such as pH, NaCl concentration and incubation temperature.[16,25,38,39]

The current study indicated that *M. spicata* essential oil and nisin exhibit strong antibacterial effect against *L.*

*monocytogenes* and the combination of these components was revealed to be a more potent inhibitor against this bacterium. In conclusion, nisin and *M. spicata* essential oil could be considered as potential strong antimicrobials that can be used for the growth inhibition of *L. monocytogenes* in food products.

## Conflict of interest

There is no conflict of interest in this study.

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