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ORIGINAL ARTICLE

Antibacterial effects of *Myristica fragrans*, *Zataria multiflora* Boiss, *Syzygium aromaticum*, and *Zingiber officinale* Rosci essential oils, alone and in combination with nisin on *Listeria monocytogenes*

Mohammad Rahnama · Mohsen Najimi · Shahraki Ali

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Abstract Plant essential oils and their components are perceived to exhibit antimicrobial activities. In this study the antibacterial effects of essential oil extracted from *Myristica fragrans*, *Zataria multiflora* Boiss, *Syzygium aromaticum*, and *Zingiber officinale* Rosci, alone and in combination with nisin, were evaluated against *Listeria monocytogenes* in brain heart infusion broth. Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) with essential oils and pH values (5, 6, and 7), alone and in combination with nicin (5 μg ml^{-1}), were determined. The individual essential oils all showed antibacterial effects against this bacterium. Combination of essential oils and nisin showed synergetic effects such that when the essential oils were utilized in combination with nisin, MIC and MBC decreased. By decreasing pH, these antibacterial effects were increased.

Keywords *Myristica fragrans* · *Zataria multiflora* Boiss · *Syzygium aromaticum* · *Zingiber officinale* Rosci · Nicin · *Listeria monocytogenes*

M. Rahnama
Department of Food Hygiene, School of Veterinary Medicine,
University of Zabol,
Zabol, Iran

M. Najimi (✉)
Department of Pathobiology, School of Veterinary Medicine,
University of Zabol,
Zabol, Iran
e-mail: moh_najimi@yahoo.com

S. Ali
Department of Biology, Faculty of Science,
University of Sistan and Baloochestan,
Zahedan, Iran

Introduction

Although, significant advances have occurred in health and safety of food processing and food production techniques, food safety is still one of the most important issues in public health. It has been estimated that 30% of people in developed countries suffer from food-borne diseases. In 2000 approximately 2 million people died from diseases which resulted in diarrhoea (Burt 2004). Therefore, we need to develop new methods for removing or decreasing food pathogens by combining new approaches with the present methods. Since industrial chemicals have many side effects and most are carcinogenic substances, there is a growing interest in using natural preservatives (Schuenzed and Harrison 2002). It seems that focusing on new methods which provide the possibility of using essential oils, spices, herbs, and natural antibiotics is growing (Burt 2004). One natural food preservative is nisin, an amphipathic polypeptide bacteriocin which consists of 34 amino acids and is produced by a particular species of *Lactococcus lactis*. It has been used extensively as a food preservative due to its antimicrobial properties and low toxicity in humans. Nisin can prevent gram-positive bacterial growth such as *Clostridium* spp., *Bacillus* spores, *Listeria monocytogenes*, and *Staphylococus aureus* (Kuwano et al. 2005; Thomas and Wimpenny 1996). Nisin is the only bacteriocin approved by WHO and is widely used in food industries (Delves-Brougthon and Gasson 1994). Nisin acts on the cell membrane phospholipids of gram-positive bacteria; it alters the plasma membrane morphology and induces cell membrane pore formation, resulting in bacterial destruction (Breukink et al. 1999). Nisin has no effect on gram-negative bacteria, because their plasma membrane consists of an external lipopolysacharide layer and internal glycerophospholipid layer. It is believed that nisin molecules are too large to pass through the plasma membrane of gram-negative bacteria (Helander and Mattila-Sandholm 2000).

Antimicrobial activities have also been reported from several plant extracts including onion and garlic (Holley and Patel 2005; Kumar and Berwal 1998). Essential oils are aromatic oils which are prepared from different parts of the plant. Phenolic compounds of the extracts made in the phospholipids of the plant's cell wall and higher phenolic compound in the essential oils result in higher antimicrobial activities (Moreira et al. 2005; Singh et al. 2002). The combination of nisin and essential oils has synergetic effects on reducing intracellular ATP in microorganisms (Bagamboula et al. 2003).

L. monocytogenes is small gram-positive, nonspore-forming, non-capsulated bacilli. This intracellular bacterium is a facultative anaerobe and able to grow at a wide range of temperatures and is relatively resistant to extreme growth conditions, e.g., from pH 4.5–9 and in 10% NaCl. *L. monocytogenes* infects human through the ingestion of contaminated food (Buchrieser 2007).

In order to replace chemical preservatives in food with natural preservatives such as herbal essential oils, it is necessary to evaluate their antimicrobial and preservation properties in laboratory models and in food substances. In this study we investigated the antibacterial effects on *L. monocytogenes* of four essential oils, *Myristica fragrans*, *Zataria multiflora* Boiss, *Syzygium aromaticum*, and *Zingiber officinale* Rosci alone and in combination with nisin under different conditions.

Material and methods

The following herbs were obtained commercially and identified by an expert botanist at Tehran Institute of Medicinal Plants: *M. fragrans*, *Z. multiflora* Boiss, *S. aromaticum*, *Z. officinale* Rosci.

Extracting essential oils Aerial parts of the collected plants were dried in the shade and were ground into a fine powder. The essential oil was obtained from 100 g powder with Clevenger-type apparatus. The essential oil obtained was 1% of the dry weight and was dried over anhydrous sodium sulfate and kept refrigerated in a dark container until tested.

Identification of the essential oils composition

The essential oils were analyzed by gas chromatography (GC) mass spectrometry. GC was Hewlett Packard 6890N equipped with a DB5 capillary column with 30 m length, 0.25 mm diameter, and 0.25 nm thickness with operating conditions as follows: temperature program was performed 50°C (6 min) to 240°C at a gradient rate of 3°C/min. The carrier gas was helium at a rate of 0.8 ml/min. GC was coupled by MS Hewlett Packard 5973N operating at 70 electron volts. Injector and MS transfer line temperature were set at 220°C and 290°C. Identification of the components was performed by comparison of their retention indices and mass spectra with those reported in library database, using Wiley software (Adams 1995).

Nisin

Nisin stock solutions were prepared by dissolving 5 g of nisin (Sigma Aldrich) containing 2.5% active nisin in acidic distilled water (pH 1.6 using 0.02 mol l^{-1} HCl) and sterilized by filtering through a disposable and non-pyrogenic syringe filter.

Test microorganism

Bacterial strain *L. monocytogenes* ATCC1911 was obtained from the Department of Microbiology, School of Veterinary Medicine, Tehran University. Initially, lyophilized cultures were transferred to brain heart infusion (BHI) broth (Merck KGaA, Darmasteadt, Germany) and incubated at 35°C for 18 h at two consecutive times. This was followed by culturing on BHI agar (Merck) and incubated at 35°C for 18 h. The plate was stored at 4°C to be used as a culture and subcultured media.

Preparation of bacterial suspension

A colony of bacterium was added to the BHI broth and incubated at 35°C for 18 h. Second bacterial culture was performed from the primary culture by transferring of 0.01 ml to new BHI broth and incubated at 35°C for 18 h. The sterile tubes containing 5 ml BHI broth were prepared, and different amounts of the second culture were transferred to these tubes and incubated at 35°C for 24 h. Optical absorbance of the BHI broth culture was measured at 600 nm using spectrophotometry (Pharmacia LKB–Nova spa cell England). Simultaneously, colony counting was also performed by pure plate method and tubes with 1×10^7 bacteria of determined preparation of BHI broth.

BHI powder (9.25 g) was added to 235 ml distilled water in a 500-ml flask in a mild heating environment. A method described previously (Misaghi and Basti 2007) was used for stabilizing the oil and water emulsion in the broth. Five percent dimethylsulfoxide (Merck) as an emulsifier and agar 5% (Merck, KGaA) were added as a stabilizer to the culture media, and pH was measured. The solution was sterilized by autoclave at 121°C for 15 min. Five milliliters of the solution was added to sterile screw capped tube.

Determination of MICs and MBCs

The inoculated tubes were incubated at 35°C for 24 h. In each serial dilution, the lowest concentration of essential oil which inhibited the visible growth of bacteria (no turbidity) was identified as the minimum inhibitory concentration (MIC). Subcultures on BHI agar were prepared from all nonvisible growth tubes and plates with CFU less than 0.1% and determined as minimum bacterial concentration (MBC, Baron and Finegold 1990).

Results

M. fragrans contained mainly sabinene (16.56%), α-pinene (10.48%), and α-thujene (7.87%). Three major components of *Z. officinale* were β-phelanderne (13.6%), curcumeme (8.92%), and camphene (7.94%). The composition of the essential oil *S. aromaticum* consisted mainly of eugenol (52.11%), cyperen (23.76%), and eugenol acetate (6.77%). The active components of *Z. multiflora* Boiss were carvacrol (26%) and thymol (23%).

According to the results (Table 1), synergetic effects were observed between *Z. multiflora* Boiss and nisin on *L. monocytogenes*. Their combined effect caused a decrease in MICs and MBCs on *L. monocytogenes*. By decreasing pH, these effects were increased. Synergetic effects between *S. aromaticum* and nisin were revealed on *L. monocytogenes*, and also such a synergistic effect was seen between nisin and *M. fragrans*. *Z. officinale* Rosci also showed synergistic effect with nisin against the microorganism *L. monocytogenes* (Table 1).

Discussion

Essential oils are one of the most useful antimicrobial agents that might be exploited as pathogen controlling agents in food (Bagamboula et al. 2004). Many different studies have been undertaken on the inhibitory effects of natural antibiotics and essential oils to replace chemical preservatives with safer natural preservatives (Ettayebi et al. 2000; Yamazaki et al. 2004).

In order to validate the usefulness of natural antimicrobial agents, they must be examined alone and in combination with other available preservatives to elucidate whether there are any synergetic effects. Therefore, we evaluated the efficacy of different essential oils alone and in combination with nisin on *L. monocytogenes.* Most bacteriocins only inhibit closely related species of the bacterium, that produces it, but nisin is a broad-spectrum bacteriocin that is effective against many gram-positive bacteria. In the present study we evaluated the effects of nisin on gram-positive bacterium *L. monocytogenes* in the presence of certain essential oils including *M. fragrans*, *Z. multiflora* Boiss, *S. aromaticum*, *Z. officinale* Rosci. The amount of essential oils in the plants is affected by many factors such as: geographical origins, harvesting age, choice of plant organ, extracting method, and solvent used for extraction. Shaffiee and Javidnia (1997) reported that 24.61% carvacrol and 25.18% thymol existed in the *Z. multiflora* Boiss essential oil. While, in a study by Misaghi and Basti (2007), it was reported that *Z. multiflora* Boiss essential oil contained 72.12% carvacrol without any thymol. In the present study, the major components of this oil were carvacrol (22.96%) and thymol (17.73%).

A study by Sharififar et al. (2007) showed that *Z. multiflora* Boiss essential oil could be used as a natural food preservative and antimicrobial agent against *S. aureus*, *Escherichia coli*, *Klebsiella* spp., *Pneumonia* spp., *Staphylococcus epidermidis*, and *Salmonella typhimorium*.

In a study by Misaghi and Basti (2007) the authors showed that *Z. multiflora* Boiss essential oil has a significant inhibitory effect on *Bacillus cereus* and in combination with nisin, the antibacterial effect increased. Previously it has been demonstrated that *S. aromaticum* has an important antibacterial effect against *L. monocytogenes*, *S. typhimorium*, *S. aureus*, *Campylobacter jejuni*, and *Clostridium perfringens* (Nevas et al. 2004). The antibac-

Table 1 MICs and MBCs of studied essential oils (PPM) alone (−) and with nisin (5 μg/ml) (+) at pH 5, 6, and 7 on *L. monocytogenes* ATCC1911

pH	5				6				7			
Nisin (5 μg ml^{-1})	−		+		−		+		−		+	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Z. multiflora	9.5	19	1.2	2.4	19	38	2.4	4.8	38	75	9.5	19
S. aromaticum	19	75	4.7	19	38	75	9.5	19	75	150	19	38
M. fragrans	38	75	9.5	19	75	150	19	38	300	300	38	150
Z. officinale	150	300	19	38	300	300	75	150	600	–	150	300

terial affects of *S. aromaticum* essential oil are also present against *L. monocytogenes* in cheese and meat, suggesting that it can be used as a suitable natural preservative in these foods (Vrinda Menon and Garg 2001). Our results also showed that this essential oil has a significant antibacterial effect against *L. monocytogenes*. Hefnavy et al. (1993) investigated *M. fragrans* and showed that it has a slight effect against *L. monocytogenes*. However our study showed that this essential oil in combination with nisin has moderate antibacterial effects against this bacterium. The essential oil from *Z. officinale* in comparison with the other studied essential oils showed lesser antibacterial effect against *L. monocytogenes*.

Statistical analysis showed strong correlation between essential oils, nisin, and pH on *L. monocytogenes*. The data presented in Table 1 demonstrated a higher inhibitory effect of the essential oils and nisin on this bacterium when used in combination rather than applied alone.

The results represent higher inhibitory effects of essential oils and nisin with a synergetic effect on *L. monocytogenes* when the incubated temperature changed from 35°C to 20°C and 10°C (data not shown). As the results show (Table 1), the synergetic effect of essential oils and nisin was elevated at a lower pH which could be explained by improved entry of essential oil and nisin through the membrane pores of the bacterium. The results of this study suggest the use of these essential oils as antibacterial compounds with other preservative methods such as reduced pH and temperature or other natural preservatives such as nisin against the bacterial pathogen *L. monocytogenes*.

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