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Antibacterial mechanisms of *Zataria multiflora* Boiss. essential oil against *Lactobacillus curvatus*

Esmaeil Ziaee, Maryam Razmjooei, Ehsan Shad, Mohammad Hadi Eskandari*

Department of Food Science and Technology, College of Agriculture, Shiraz University, Shiraz, Iran

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Sodium Sulphate (PubChem CID: 24436)
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Potassium dihydrogen phosphate (PubChem CID: 516951)
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Glutaraldehyde (PubChem CID: 3485)
Osmic acid (PubChem CID: 30318)
Ethanol (PubChem CID: 702)

ABSTRACT

The chemical composition of ZMEO was analyzed by GC-FID, and consequently 14 components were identified. Carvacrol (39.29%) and thymol (25.24%) were found to be the major components. The diameter of inhibition zone (2.68 cm), minimum inhibitory concentration (6.6 μl/mL), and minimum bactericidal concentration (9.10 μL/ml) of ZMEO against *L. curvatus* was evaluated. To determine antibacterial mechanisms of ZMEO against *L. curvatus* the cell permeability, membrane integrity and membrane potential changes and cell microstructure were determined. When ZMEO was added at 2 × MIC levels, the number of cells decreased significantly from 5.45 to 1.34 $\log_{10}$ CFU/mL over 24 h of incubation. The REC of the treated samples increased after the addition of ZMEO at MIC and 2 × MIC. In addition, the ZMEO revealed its mode of action on membrane integrity as confirmed by loss of 260-nm absorbing materials. After 0, 30, 60 and 120 min of exposure time, concentration of cell constituents in suspensions treated with MIC essential oil increased by 1.44, 4.16,11.94 and 16.11 times, while they increased by 2.11, 11.72, 18.77 and 20.77 times when treated at 2 × MIC. The TEM analysis showed the inhibitory effect of ZMEO as confirmed by considerable morphological alterations on the cell wall *L. curvatus*.

1. Introduction

Lactic acid bacteria (LAB) are known to be major lactic acid producers in food products and have been characterized as the main spoiling flora of many cooked meat products (Björkroth, Ristiniemi, Vandamme, & Korkeala, 2005; Samelis, Kakouri, & Rementzis, 2000; Vasilopoulos et al., 2008). In raw or precooked chicken fillets, LAB was identified as the main bacteria present after treatment (Patsias, Badeka, Savvaidis, & Kontominas, 2008; Patsias, Chouliara, Badeka, Savvaidis, & Kontominas, 2006). Dykes, Cloete, and Von Holy (1995) have been reported that the majority isolated organisms from spoiled cooked cured sausages are *Lactobacillus sake*, *Lactobacillus curvatus* and *Leuconostoc mesenteroides*. LABs are able to grow under a variety of conditions which prevent the growth of Gram-negative microorganism. LABs do not require oxygen for growth, are resistant to inhibition by CO_2, nitrite and smoke, able to grow at relatively high salt concentrations and they tolerate lower pH values than the Gram-negative bacteria commonly found on meats, especially under anaerobic conditions. Therefore conditions favorable for the growth of these organisms occur in meat and meat products packaged in vacuum films of low gas permeability. The use of essential oils in food formulation has gained significant attention by consumers and the food industry. This is mainly due to increasing consumers' awareness of the potential adverse effects of chemical preservatives on health versus the benefits of natural additives. *Zataria multiflora* Boiss., is a thyme-like plant which grows in some parts of Iran, Pakistan and

* Corresponding author. Tel.: +98 71 32286110; fax: +98 71 32286110.
E-mail address: Eskandar@shirazu.ac.ir (M.H. Eskandari).

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Afghanistan (Fazeli et al., 2007; Gahruie, Ziaee, Eskandari, & Hosseini, 2017; Gandomi et al., 2009). It is traditionally used in folk remedies for its antiseptic, analgesic and carminative properties (Akrami et al., 2015). *Z. multiflora* essential oil (ZMEO) shows strong antimicrobial and antioxidant activities because of having large quantities of phenolic oxygenated monoterpenes (Moradi et al., 2012; Saei-Dehkordi, Tajik, Moradi, & Khalighi-Sigaroodi, 2010). However, to our knowledge, there are little data available explaining the mechanism of antibacterial action of ZMEO against LAB in detail. Our study aimed to investigate the antibacterial properties of ZMEO by adopting the mechanistic approach to provide fundamental understanding on the mode of antibacterial action.

2. Materials and methods

2.1. Microorganisms and chemicals

Lactobacillus curvatus was previously isolated from vacuum packed spoiled cooked cured sausages marketed in Fars province, Iran. The bacterium was identified using 16S rDNA sequencing and deposited in the culture collection of Shiraz University (SUCC 1365). This bacterium was grown in MRS agar (Merck, Darmstadt, Germany) for 48 h at 30 °C. Dimethyl sulfoxide (DMSO), n-alkanes C8-C22 were purchased from Sigma (USA). Other chemicals used were all of analytical grade.

2.2. Essential oil extraction

The *Zataria Multiflora* Boiss. was collected from the Shiraz suburbs (30°.06′ N, 52°.56′ E) and their dried aerial parts were subjected to hydro-distillation for approximately 2 h in a Clevenger-type apparatus according to the method outlined by Moosavi-Nasab et al. (2016). The distilled EO was dried over anhydrous sodium sulphate and stored in closed dark vials at −18 °C.

2.3. GC-FID analysis

GC analysis was performed using an Agilent gas chromatograph series 7890A equipped with a flame ionization detector (FID). The analysis was carried out on fused silica capillary HP-5 column (30 m × 0.32 mm i.d.; film thickness 0.25 μm). The injector and detector temperatures were kept at 250 °C and 280 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 1 mL/min; oven temperature program was 60–210 °C at the rate of 4 °C/minutes, which was then programmed to 240 °C at the rate of 20 °C/minutes, and finally, held isothermally for 8.5 min. The split ratio was 1:50.

2.4. Antibacterial assays

2.4.1. Agar diffusion

The antibacterial activity of ZMEO was described using agar diffusion method according to Goni et al. (2009).100 μl of bacterial suspension (1×10^7 cfu/ml) was spread onto the Mueller-Hinton agar plates. The sterile filter paper disc (6.4 mm) containing 10 μl of ZMEO was placed in the surface of plate. Afterwards, the plate was incubated at 37 °C for 24 h. The antibacterial activity of ZMEO was expressed by measuring the diameter of inhibition zone (DIZ).

2.4.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC were determined according to the method used by Moosavi-Nasab et al. (2016). Briefly, broth subcultures were prepared by inoculating a single colony to an Erlenmeyer containing MRS broth and then incubating at 37 °C for 24 h at 160 rpm. Then concentration of bacterial solution adjusted to a final density of 1.5×10^6 CFU/mL, and used as inoculum. The ZMEO was completely dissolved in ratio of water: DMSO (95:5) to reach a final concentration of 20 μL/mL. Serial dilutions were made in a concentration ranging from 4 to 10 μl/mL. The micro plates were prepared in triplicate and incubated at 37 °C for 24 h. After incubation, bacterial growth was evaluated by the presence of turbidity and a pellet on the well bottom. To evaluate MBC, 100 μL of each well in which microbial growth was not observed was spread plated on MRS agar and plates were incubated for 24–48 h at 37 °C.

2.5. Mode of antibacterial action

2.5.1. Antibacterial dynamics of the antimicrobials

The antibacterial dynamics were determined using a micro dilution assay as described previously in section 2.3. The L. *curvatus* was cultured at 37 °C with an oscillating speed of 150 rpm, and the absorbance was measured at 600 nm every 1 h using an automatic micro plate reader (CYTATION 3, BioTek, Winooski, Vermont, USA). Each treatment was repeated in triplicate. The area under the curve (AUC) was calculated as antibacterial activity using the following formula:

$$\mathrm{AUC} = \left(\sum_{i=1}^{n-1} \frac{\left(m_{(i+1)} + m_i\right)}{2} \right) - \left(m_i \sum_{i=1}^{n-1} t_i \right)$$

where, m_i is absorbance at t_i and $m_{(i+1)}$ is absorbance at $t_{(i+1)}$.

2.5.2. Cell membrane permeability

The cell membrane permeability of *L. curvatus* was studied according to the method described by Diao, Hu, Zhang, and Xu (2014). To determine changes in cell membrane permeability the Relative electric conductivity (REC) was examined. The EC was measured with an electrical conductivity meter (Mi 180 bench meter, Martini, Romania). First of all, the *L. curvatus* cells were separated after incubation for 24 h at 37 °C by centrifugation from MRS broth at 5000×*g* at 4 °C for 5 min. The pellets were washed with 5% glucose until the EC of the cells were near to that of the 5% glucose. These washed cells were isotonic bacteria. Then, 5% glucose was added to isotonic bacterial solution with different concentrations of ZMEO (control, MIC and 2 × MIC) and measured their EC (L_1). Then, the samples were incubated at 37 °C for 12 h and their conductivities were measured after two hours (L_2). The conductivity of *L. curvatus* in 5% glucose treated in boiling water for 5 min was used as control and marked as L_0. The permeability of cell membrane is calculated using the following equation:

$$\mathrm{REC} = 100 \times \frac{L_2 - L_1}{L_0}$$

2.5.3. Cell membrane integrity

The integrity of cell membrane was evaluated by measuring the release of cell nucleic acids into cell suspension. According to Lv, Liang, Yuan, and Li (2011), *L. curvatus* cells were collected by centrifugation from cell suspension (100 mL) at 5000×*g* at 4 °C for 5 min. The supernatant was discarded and the pellets were washed three times and resuspended in phosphate buffer solution (PBS) (0.1 M, pH 7.4). The 100 mL washed suspension were shaken and incubated at 37 °C for 2 h in the presence of variable concentrations of ZMEO (control, MIC and 2 × MIC). Then, the suspensions were centrifuged at 6000×*g* at 4 °C for 5 min. After that, the supernatants were collected, diluted with PBS, and the absorption at 260 nm was measured by using an UV–vis spectrophotometer (SHANGHAI PI, China). Correction was carried out for the absorption of the