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Evaluation of *Zataria multiflora Boiss*. essential oil activity against *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* by propidium monoazide quantitative PCR in vegetables

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ABSTRACT

Essential oils (EOs) have long been applied as flavoring agents in foods, and due to their content in antimicrobial compounds, they have potential as natural agents for food preservation. Recently, real-time PCR in combination with PMA has successfully been applied to discriminate between live *Escherichia coli* O157:H7 and dead bacteria killed by cumin, clove, oregano and cinnamon EOs. In this study, initial experiments were performed in order to elucidate the minimum bactericidal concentration of *Zataria multiflora* EOs on *E. coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*. Thereafter PMA-qPCR was applied in order to selectively quantify life cells within a bacterial population treated with *Z. multiflora* EO. Inactivation was obtained at EO concentrations of 0.02, 0.035, 0.045 for *L. monocytogenes*, *E. coli* O157:H7 and *S. enterica*, respectively. *L. monocytogenes* were totally killed in 30 min while it took 1 h 30 min for the gram negative pathogens. As a conclusion *Z. multiflora* EO has potential as natural food additive or biopreservative since it was able to irreversibly inactivate the three pathogens tested, at lower concentrations than other EOs and short exposition times. In addition, the PMA-qPCR approach proved efficient to selectively detect live pathogenic bacteria in vegetables following inactivation with *Z. multiflora* EO.

1. Introduction

Microbial safety of food products is a great concern to consumers and food industries. Many food preservation strategies such as chilling, freezing, fermentation, pasteurization or the use of synthetic antimicrobials have been extensively applied to control spoilage and pathogen microorganisms (Davidson, 2001). Currently, there is increasing consumer demand to replace chemically synthesized antimicrobials with natural alternatives in order to ensure food safety (Wentao et al., 2007). In this context, plant essential oils are attracting interest as natural food preservatives. Besides essential oils (EOs) have long been applied as flavoring agents in foods, they have shown a wide spectrum of antimicrobial activity on foodborne pathogens and spoilage bacteria (Burt, 2004; Ponce, Roura, & Moreira, 2011). Therefore essential oils have potential as natural agents for food preservation (Oussalah, Caillet, Saucier, & Lacroix, 2007).

There are more than 1300 plants with defined antimicrobial compounds but characterization of preservative properties is available for only few EOs (Tajkarimi, Ibrahim, & Cliver, 2010). Some of these EOs such as oregano, clove, cinnamon, garlic, coriander, rosemary, mint, basil, and parsley, or their components have been deeply studied (Angioni et al., 2004; Elizaquivel, Sanchez & Aznar, 2012a; Karagözlü, Ergönül, & Özcan, 2011; Oussalah et al., 2007; Piskernik, Klancnik, Tandrup Riedel, Brondsted, & Smole-Mozina, 2011; Tsigarida, Skandamis, & Nychas, 2000) and demonstrated to exert antimicrobial effects against different foodborne pathogens such as *Campylobacter jejuni* (Piskernik et al., 2011), *Listeria monocytogenes* (Oussalah et al., 2007; Tsigarida et al., 2000), *Salmonella* (Karagözlü et al., 2011; Oussalah et al., 2007), *Staphylococcus aureus* (Bouhdid et al., 2010) or *Escherichia coli* O157:H7 (Karagözlü et al., 2011; Singh, Singh, Bhunia, & Stroshine, 2002).

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Zataria multiflora Boiss (*Z. multiflora*), belonging to the family *Laminacae*, is native from Iran, Pakistan and Afghanistan. This plant is traditionally used in food, especially in yoghurt flavoring, as a stimulant, condiment, carminative and for treatment of pre-mature labor pains and rupture (Ali, Saleem, Ali, & Ahmad, 2000; Hosseinzadeh, Ramezani, & Salmani, 2000). There are also commercial pharmaceuticals with formula based on *Z. multiflora* essential oil. This oil has commonly been used in medicine for the treatment of respiratory tract infections as an antiseptic, antitussive and irritable bowel syndrome treatment (Ali et al., 2000). The main constituents of the *Z. multiflora* EO are phenolic compounds such as carvacrol and thymol (Akhonzadeh Basti, Misaghi, & Khaschabi, 2007). *Z. multiflora* antimicrobial activity has been successfully tested against fungi (Khosravi, Shokri, Sharifrohani, Mousavi, & Moosavi 2012; Mahmoudabadi, Dabbagh, & Fouladi, 2007) and food pathogens such as *S. aureus*, *Salmonella enterica* (Akhonzadeh Basti et al., 2007) or *L. monocytogenes* (Moradi, Tajik, Razavi Rohani, & Oromiehie, 2011), as well as norovirus surrogates (Elizaquivel, Azizkhani, Aznar, & Sánchez, 2012).

EOs activity against pathogen or spoilage bacterial microorganisms has normally been evaluated by traditional culture methods which show some drawbacks such as the relatively long time needed for the growth of bacteria and the lack of sensitivity regarding viable but not culturable cells (VBNC) (Kramer, Obermajer, Bogovic, Rogelj, & Kmetec, 2009). As an alternative, real-time PCR (qPCR) allows rapid, sensitive and specific detection and quantification of pathogens. Moreover, this technique has demonstrated to distinguish between DNA from dead and live cells by including a pretreatment of the sample with a DNA intercalating reagent, e.g. propidium monoazide (PMA) (Rudi et al., 2002). This procedure is based on the integrity of bacterial cells since PMA penetrates only into compromised membrane cells (Nocker & Camper, 2006). Sample pretreatment with PMA combined with qPCR has been successfully tested for the detection of bacterial pathogens such as *L. monocytogenes* (Pan & Breidt 2007), *E. coli* O157:H7 (Elizaquivel, Sanchez, Selma & Aznar, 2012; Nocker, Mazza, Masson, Camper, & Brousseau, 2009), *C. jejuni* (Josefsen et al., 2010). Recently, in a previous study carried out in our group, PMA-qPCR proved to be an efficient tool for the quantification of viable cells after treatment with cinnamon, clove and oregano EOs (Elizaquivel et al., 2012a). The aim of the present study was to evaluate the antimicrobial activity of *Z. multiflora* EO against *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* by using PMA in combination with qPCR.

2. Material and methods

2.1. Bacterial strains, culture conditions and DNA isolation

E. coli O157:H7 CECT 5947 (non-toxigenic), *S. enterica* subsp. *enterica* CECT 4138^T and *L. monocytogenes* CECT 4031 supplied by the Spanish Type Culture Collection (CECT) were used in this study. Bacteria were routinely grown on Tryptic Soy Broth (TSB) at 37 °C for 18 h, and enumerated by plate count on Trypticasein Soy Agar (TSA), at the same incubation conditions. Bacterial DNA was purified using the NucleoSpin Tissue kit (Macherey–Nagel GmbH & Co., Duren, Germany) according to manufacturer's instructions.

2.2. Essential oil

Z. multiflora Boiss. was collected in the Shiraz province of Iran and identified by the Institute of Medicinal Plants, Medical University of Tehran, Iran. To obtain essential oil, air-dried aerial parts of the plant were subjected to steam distillation for 2 h using Clevenger-type apparatus. The obtained essential oil was stored in airtight glass vials covered with aluminum foil at 4 °C. For its application, zataria EO was resuspended 1:10 v/v in 50% ethanol.

2.3. Essential oil analysis

The essential oil was analyzed by gas chromatography (GC) (Thermoquest 2000, UK). The chromatography was equipped with DB5 capillary column (30 × 0.25 mm ID × 0.25 μm film thickness) and data were acquired under the following conditions: initial temperature 50 °C; program rate 2.5 °C, final temperature 265 °C and injector temperature 250 °C. The carrier gas was helium and split ratio was 120. The essential oil was also analyzed by gas chromatography mass spectroscopy (GC/MS) (Thermoquest Finningan, UK) and the same capillary column and analytical conditions indicated above. The MS was run in the electron ionization mode, using ionization energy of 70 eV. The components were identified based on the comparison of their relative retention time (RI) and mass spectra fragmentation with those stored in the Wiley 7n.1 mass computer library and the NIST (National Institute of Standards and Technology) (Adams, 2001), and quantified as the mean value of two injections of EO.

2.4. Determination of the minimum inhibitory concentration (MIC) and the maximal tolerated concentration (MTC)

The MIC and MTC were estimated by a modified broth microdilution method (Klancnik, Piskernik, Jersek, & Mozina, 2010). Briefly, 50 μl of a 2 h culture of bacteria were inoculated in 5 ml of TSB added with different EO concentrations starting with 0.01 and 0.005% and increasing or reducing these concentrations to obtain MIC and MTC values. Cultures were incubated at 37 °C in a shaker incubator and optical density (OD) was measured at 600 nm after 24 h incubation. Four control tubes were prepared as follows: culture medium, bacterial suspensions only, plant extracts only and ethanol in amounts corresponding to the highest quantity present. MIC was determined as the minimum concentration at which optical density was similar to the non-inoculated control containing only the corresponding EO concentration. MTC was determined as the maximum concentration at which the addition of EO did not statistically affect the growth of bacteria with respect to the control without EO.

2.5. Kinetics of inactivation (bactericidal activity)

Bacterial cultures from a 4 h culture in TSB (10^8–10^9 cfu/ml) were diluted to obtain a 5 ml solution of ca. 10^5 cfu/ml and added with *Z. multiflora* at 0.03%, 0.035%, 0.04% for *E. coli* O157:H7, 0.045%. 0.05%, 0.055% for *S. enterica* and 0.02%, 0.025%, 0.03% for *L. monocytogenes* and further incubated at 37 °C in a shaker. Samples were taken at 0, 30 min, 45 min, 1 h, 1 h 30 min, 2 h and 4 h and were spreaded onto TSA plates for plate counts. All experiments were independently repeated three times. Mean log cfu/ml and standard deviations were calculated. EO concentration which resulted in no bacterial growth after 4 h of treatment indicated that all cells had been killed or had entered a VBNC state and was established as the minimum bactericidal concentration (MBC).

2.6. PMA cross-linking

PMA (Biotium, California, US) was dissolved in 20% dimethylsulfoxide (DMSO) to obtain a 20 mM stock solution and stored at −20 °C in the dark. Stock solution was added to 500 μl of either viable or EO-treated cells at a final concentration of 100 μM. Each sample was treated in triplicate to ensure reproducibility of results. After the addition of PMA, samples were incubated for 5 min in the