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Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce

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A B S T R A C T

Although antimicrobial activities of plant essential oils are well documented, challenges remain as to their application in fresh produce due to the hydrophobic nature of essential oils. Oregano oil nanoemulsions were formulated with a food-grade emulsifier and evaluated for their efficacy in inactivating the growth of foodborne bacteria on fresh lettuce. Lettuce was artificially inoculated with *Listeria monocytogenes*, *Salmonella Typhimurium* and *Escherichia coli* O157:H7, followed by a one-minute dipping in oregano oil nanoemulsions (0.05% or 0.1%). Samples were stored at 4 °C and enumerated for bacteria at fixed intervals (0 h, 3 h, 24 h, and 72 h). Compared to control, 0.05% nanoemulsion showed an up to 3.44, 2.31, and 3.05 log CFU/g reductions in *L. monocytogenes*, *S. Typhimurium*, and *E. coli* O157:H7, respectively. Up to 3.57, 3.26, and 3.35 log CFU/g reductions were observed on the same bacteria by the 0.1% treatment. Scanning Electron Microscopy (SEM) demonstrated disrupted bacterial membranes due to the oregano oil treatment. The data suggest that applying oregano oil nanoemulsions to fresh produce may be an effective antimicrobial control strategy.

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1. Introduction

Nearly half (46%) of foodborne illnesses from 1998 through 2008 in the US have been attributed to fresh produce (Painter et al., 2013). *Listeria monocytogenes*, *Salmonella Typhimurium*, and *Escherichia coli* O157:H7 are among the major foodborne bacteria implicated in produce outbreaks (Beuchat, 1996; Harris et al., 2003). Prevention and control of bacterial contamination on fresh produce is thus an imperative task to ensure food safety. The current microbial control strategy remains industrial washing of the product in water containing chlorine. However, due to sanitizer ineffectiveness leading to pathogen reductions of only 1–2 log CFU/g (Zhang et al., 2009; Kim et al., 2011) and the potential health concerns associated with chlorine by-products (Kim et al., 2011), there is an urgent need to identify alternative antimicrobials, particularly those of natural origin, for the produce industry.

Plant essential oils are secondary metabolites of plants that are generally recognized as safe (GRAS) as flavoring agents for consumption by animals and humans in the US (Hyldgaard et al., 2012; Shah et al., 2012). They have been shown to be promising alternatives to chemical sanitizers against foodborne bacteria. Essential oils from clove, basil, lemongrass, and thyme have demonstrated antimicrobial effects on fresh produce (Singh et al., 2002; Gunduz et al., 2009; Gunduz et al., 2010; Kim et al., 2011; Moore-Neibel et al., 2011). A less than 2.0 log-reduction in *S. Typhimurium* was observed on lettuce treated by myrtle oil (Gunduz et al., 2009) and oregano oil (Gunduz et al., 2010). Yossa et al. (2013) reported that 1000 ppm of Sporan (a proprietary antimicrobial containing clove, rosemary, and thyme oil) alone caused less than 2.7 log-reduction in *E. coli* and *Salmonella* on iceberg lettuce and about 1 log reduction in the same pathogens on romaine lettuce. Although an up to 4.0 log-reduction was achieved by lemongrass oil against *Salmonella* Newport on organic lettuce, the concentration of essential oil was as high as 0.5% (5000 ppm) (Moore-Neibel et al., 2011). Another study (Kim et al., 2011) also used 5–10% of clove extract to inhibit *E. coli* O157:H7 and *S. Typhimurium* by up to 4.0 log. However, an obvious caveat of using high concentrations of essential oils or extracts is the potential negative impact on the sensory attributes of products.

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The majority of work on the antimicrobial efficacy of essential oils has been conducted using products made by mixing immiscible oils in water or phosphate buffered saline (PBS). Due to the hydrophobic nature of essential oils, organic compounds from produce may interfere with essential oils to reduce their sanitizing effect and/or shorten the duration of effectiveness (Perumalla and Hettiarachchy, 2011). Formulation of essential oils in emulsions provides an alternative strategy to improve their efficacy in food systems by stabilizing the compounds and enhancing their release and coverage of the leafy produce. A peppermint oil emulsion showed the high stability over at least 30 days of storage at room temperature and prolonged antibacterial activities against *L. monocytogenes* Scott A and *Staphylococcus aureus* ATCC25923 using pure bacteria culture (Liang et al., 2012). A basil oil emulsion demonstrated antibacterial activity against pure *E. coli* culture (Ghosh et al., 2013). Spoilage microorganisms were inhibited by a terpenes nanoemulsion in fruit juices (Donsì et al., 2011). A thymol dispersion showed pH- and food matrix-dependent inhibition on *E. coli* O157:H7 and *L. monocytogenes* in apple cider and 2% reduced-fat milk (Shah et al., 2012). Regardless of these formulations and applications, research is lacking on the antimicrobial efficacy of essential oil nanoemulsions using fresh produce models.

Oregano oil is one of the most effective essential oils for antimicrobial control (Baranauskiene et al., 2006). The present study was aimed to formulate, characterize, and evaluate oregano oil nanoemulsions as a potential microbial control method applied to fresh lettuce.

2. Materials and methods

2.1. Preparation and characterization of oregano oil nanoemulsions

An oil-in-water nanoemulsion of oregano oil was prepared by a high energy ultrasound approach (Ghosh et al., 2013). Oregano oil (LorAnn Oils, Lansing, MI) and Tween 80 (Sigma–Aldrich, St Louis, MO), a food-grade non-ionic surfactant, were mixed at a 2:1 mass ratio and added to deionized water. The mixture was subjected to the continuous sonication for 10 min using an ultrasonicator (Model 300, Fisher Scientific Sonic Dismembrator) at 750 W at room temperature.

Light Scattering (LS, in a Malvern ZetaSizer Nano ZS) was used to evaluate the size and Zeta Potential (ζ) of the nanoemulsion. Samples of three independent batches were diluted 1:10 in water and measurements were performed at 25 °C using refractive index, viscosity, and dielectric constant of deionized water (Mishra et al., 2012). Zeta potential was calculated using the Smoluchowski Model (Sze et al., 2003).

2.2. Determination of MIC of oregano oil nanoemulsions

Minimum Inhibitory Concentrations (MIC) of oregano oil nanoemulsions against *L. monocytogenes* ATCC 19115, *S. Typhimurium* ATCC 19585 and *E. coli* O157:H7 ATCC 700927 were determined by a broth microdilution method according to Clinical Laboratories Standards Institute (CLSI) guidelines (CLSI, 2012). Nanoemulsions (20,000 μl/L) were dissolved in Mueller–Hinton Broth (MHB, Difco, Detroit, MI) and serially diluted in a 96-well plate. Bacteria inoculum in 50 μl broth (MHB for *S. Typhimurium* and *E. coli* O157:H7, and MHB + 2–3 % Lysed Horse Blood (LHB) for *L. monocytogenes*) was added to the wells to obtain final concentrations of oregano oils at 0, 19, 39, 78, 156, 312, 625, 1,250, 2500, 5000 and 10,000 μl/L. The final concentration of bacteria was 5×10^5 CFU/ml. Plates were incubated at 37 °C for 24 h and growth was measured after 24 h using a BioAssay Reader (HTS7000, Perkin Elmer). The MIC was determined as the lowest concentration of the nanoemulsion that inhibited microbial growth with an absorbance value <0.05 at 595 nm (Ravichandran et al., 2011). To determine minimum bactericidal concentrations (MBC), 100 μl aliquots from wells where no growth was observed were plated on Triptic Soy Agar (TSA, Difco). The plates were incubated at 37 °C for 24 h. MBC was defined as ≥99.9% decrease in viable cells (Moreira et al., 2005). Nutrient broth only and broth containing Tween 80 only and bacteria only served as control.

2.3. Inoculation of lettuce and application of oregano oil nanoemulsions

Stock cultures of *L. monocytogenes* ATCC 19115, *S. Typhimurium* ATCC 19585 and *E. coli* O157:H7 ATCC 700927 were grown in Tryptic Soy Broth (TSB, Difco) at 37 °C for 18 h, followed by washing in PBS for 2 times before inoculation. Bagged romaine and iceberg lettuce were purchased from a local supermarket and only lettuce not carrying any of the three pathogens was used in the study. Lettuce leaves were cut into approximately 6 × 2 cm pieces using a sterile knife to represent ready to eat packages of salad lettuce. Romaine and iceberg lettuce were mixed in equal proportions. One milliliter of individual bacterial suspension in PBS (10^9 CFU/ml) was added to 100 g of lettuce, followed by mixing and drying for 15 min in a Biosafety cabinet. The lettuce was then dipped in 200 ml nanoemulsions with 0.05% or 0.1% v/v of oregano oil for 1 min, dried for 30 min, and then stored in double-zipper Ziploc bags at 4 °C. Sterile distilled water was used as control. Nanoemulsion concentrations of 0.05% (500 μl/L) and 0.1% (1000 μl/L) were selected based on the MIC results.

2.4. Microbiological studies

Samples were taken for microbiological determinations at 3 h, 24 h, and 72 h after the nanoemulsion treatment. Ten grams of lettuce leaves were macerated in 90 ml of 1% PBS in a stomacher (400 Circulator, Seward) for 2 min. Samples were then diluted and plated onto PALCAM (Difco), Xylose Lysine Deoxycholate (XLD, Difco), and MacConkey (Difco) agars for the enumeration of *L. monocytogenes*, *S. Typhimurium*, and *E. coli* O157: H7, respectively.

2.5. Scanning Electron Microscopy (SEM) studies

Overnight bacterial cultures was adjusted to 10^7 CFU/ml and washed 3 times with 1× PBS before mixing with 0.05% of the nanoemulsion for 10 min at room temperature. The morphology of bacteria was examined by SEM (Bozzola, 2007). Untreated and treated cells were deposited on a Millipore filter that was then dipped in 2.5% glutaraldehyde (Sigma–Aldrich) in PBS for 45 min for fixing. Re-fixing was performed in 1% OsO_4 (osmium tetroxide, Sigma–Aldrich) in PBS for 1 h. The filter was then dehydrated by successive 10 min incubations in 30% ethanol, 50% ethanol, 70% ethanol, and 100% ethanol. The filter was attached to an SEM stub with a double-sided tape and coated with gold/palladium after critical point drying. Samples were visualized with SEM (Hitachi S-2400).

2.6. Statistical analysis

All experiments were performed in triplicate. Microbial survival in different treatment groups was compared by one way ANOVA using SPSS version 21 (IBM Corp, Armonk, NY). Tukey's test was used to determine the significant differences of mean values. A p value of less than 0.05 was considered statistically significant.

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