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Antimicrobial activity of nanoemulsions containing essential oils and high methoxyl pectin during long-term storage

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ABSTRACT

The antimicrobial activity against *Escherichia coli* and *Listeria innocua* of nanoemulsions containing oregano, thyme, lemongrass or mandarin essential oils and high methoxyl pectin was assessed during a long-term storage period (56 days). On one hand, a higher antimicrobial activity was detected against *E. coli* compared to *L. innocua* regardless the EO type. Transmission Electron Microscopy (TEM) images showed a significant damage in the *E. coli* cells for both the cytoplasm and cytoplasmic membrane, led to cell death. The antimicrobial activity of the nanoemulsions was found to be strongly related to the EO type rather than to their droplet size. The lemongrass-pectin nanoemulsion had the smallest droplet size (11 ± 1 nm) and higher antimicrobial activity reaching 5.9 log reductions of the *E. coli* population. Nevertheless, the freshly made oregano, thyme and mandarin EO-pectin nanoemulsion led to 2.2, 2.1 or 1.9 *E. coli* log-reductions, respectively. However, the antimicrobial activity decreased significantly during storage regardless the EO type, which was related to the loss of volatile compounds over time according to our results. The current work provides valuable information in order to make progress in the use of nanoemulsions containing EOs as decontaminating agents in food products.

1. Introduction

Essential oils (EOs) are volatile substances obtained from aromatic plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (Noorizadeh, Farmany, & Noorizadeh, 2011; Bakkali et al., 2008), usually extracted by steam vaporization and cold-press techniques (Saad, Muller, & Lobstein, 2013). EOs are commonly used as antioxidants, flavorings or colorants in a wide range of food products (Edris, 2007; Santin, Oliveira, Cristina, Ferreira, & Ueda-nakamura, 2009). Moreover, EOs have been described as strong natural antimicrobial agents for food preservation purposes (Muriel-Galet et al., 2012). The antimicrobial properties of EOs are mainly due to their volatile components, including terpenoids and phenolic compounds (Cosentino et al., 1999). The mechanism of EOs to inactivate food-borne microorganisms relies on their interaction with the microbial membrane. EOs phenolic compounds are known to penetrate through the microbial membrane and cause the leakage of ions and cytoplasmatic content thus leading to cellular breakdown (Burt, 2004; Bajpai, Baek, & Kang, 2012). Several studies have shown that EOs are effective antibacterial agents against a wide spectrum of pathogenic bacterial strains including *L. monocytogenes*, *L. innocua* (Solomakos, Govaris, Koidis, & Botsoglou, 2008), *E. coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhimurium* (Saad et al., 2013). However, antimicrobial EOs are rarely used directly in food products as bulk oils since they present limitations such as intense aroma and low water solubility (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014).

Nanotechnology is a tool used to modify nano-scale material characteristics, in this case, to improve the EOs properties, which can be incorporated as nano-sized delivery systems in order to overcome their limitations (Huang, Yu, & Ru, 2010). A wide variety of delivery systems have been developed to encapsulate active ingredients, including colloidal dispersions, biopolymer matrices or emulsions (Weiss, Takhistov, & McClements, 2006). Emulsions containing very small oil droplet size are desirable for certain applications since they present advantages over systems containing larger particles. Nanoemulsions are defined as conventional emulsions that contain tiny particles (diameter between 100 and

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300 nm) that make them kinetically stable to the particle aggregation and gravitational separation (Qian & McClements, 2011; Tadros, Izquierdo, Esquena, & Solans, 2004). Moreover, due to the reduced droplet sized and therefore weak light scattering, they may be incorporated in optically transparent beverages or certain food products without altering their optical properties (Qian & McClements, 2011). Additionally, nanoemulsions have a much higher surface area of the active ingredients compared to conventional emulsions and therefore present an enhanced functionality when interacting with biological systems (Salvia-trujillo, Qian, Martín-belloso, & McClements, 2013). Microfluidization is a device widely used for the production of food-grade nanoemulsions (Guerra-Rosas, Morales-Castro, Ochoa-Martínez, Salvia-Trujillo, & Martín-Belloso, 2016; Lovelyn & Attama, 2011; Salvia-Trujillo et al., 2014). Salvia-Trujillo et al. (2014) reported an enhanced antimicrobial activity of nanoemulsions produced by microfluidization compared to conventional emulsions with a larger droplet size, which was attributed to the higher penetration of nano-sized droplets through the microbial membrane. Nonetheless, the fate of nanoemulsion functionality after being incorporated in food formulations has been hardly described. On the other hand, the stability of the antimicrobial activity of nanoemulsions during longer periods of time also needs to be evaluated. Therefore, the aim of the present work was to study and compare the antimicrobial activity of nanoemulsions containing EOs (oregano, thyme, mandarin and lemongrass) as oil phase and high-methoxyl pectin solution (1% w/v) as the aqueous phase against *E. coli* and *L. innocua* during storage. For that purpose, EO-pectin nanoemulsions were stored at room temperature for 56 days and the antimicrobial activity of such nanoemulsion was assessed at several time intervals in terms of *E. coli* and *L. innocua* population reductions. Moreover, changes in the volatile fraction of EOs during storage was studied and related to the antimicrobial capacity of nanoemulsions. Also changes in the microbial cell structure were assessed by Transmission Electron Microscopy (TEM), a useful technique in biological science for the observation of cellular structures.

2. Materials and methods

2.1. Chemical compounds

Chemical compounds studied in this article: High methoxyl pectin (PubChem CID: 441476); carvacrol (PubChem CID: 10364); thymol (PubChem CID: 6989); citral (PubChem CID: 638011); limonene (PubChem CID: 440917); Tween 80 (PubChem CID: 443315).

2.2. Materials

Food-grade high methoxyl pectin (Unipectine QC100 from citrus source) was kindly donated by Cargill Inc. (Spain). Oregano (*Origanum compactum*) and thyme (*Thymus vulgare*) EOs were purchased from Dietetica Intersa, S.A. (Spain), whereas lemongrass (*Cymbopogon citratus*) was purchased from Laboratories Dicana, S. L. (Spain), and mandarin (*Citrus reticulata*) was kindly provided by Indulleida, S.A. (Spain). The main volatile active compounds present in each EO used in this study based on bibliographic references is described in Table 1. Tween 80 (Poly oxyethylene sorbitan Monoesterate) (Lab Scharlab, S. L., Spain) was used as non-ionic food-grade surfactant. Ultrapure water obtained from Millipore water system (Millipore S. A., Molsheim, France) (0.22 μm) was used for the formulation and analysis of all nanoemulsions.

2.3. Essential oil-pectin nanoemulsions preparation

High methoxyl pectin powder (1% w/v) was dissolved in hot water at 80–85 °C, with continuous stirring until being fully solubilized and it was cooled down to 25 °C. Coarse oil-in-water emulsions were made by mixing pectin solutions as aqueous phase with oregano, thyme, lemongrass or mandarin EOs (2% v/v) and Tween 80 (5% v/v) emulsified with a high sheer laboratory mixer Ultraturrax T-25 (IKA, Staufen, Germany) for 2 min at 9500 rpm. The final volume was 1000 mL. Afterwards, the coarse emulsion was passed through a microfluidizer device (Microfluidics, Massachusetts, USA) at 150 MPa for 5 cycles. For the stability studies, 15 mL of each nanoemulsion were kept in capped plastic test tubes and stored in the dark at room temperature (~25 ± 2 °C).

Physical, chemical and antimicrobial assays were performed in duplicate immediately after nanoemulsions preparation and every 7 days up to 56 days. Every sampling day, new tubes were used in order to avoid the oxidation or volatilization of the volatile compounds of EOs after opening the plastic tubes.

2.4. Droplet size and droplet size distribution

The average droplet size of nanoemulsions was determined by dynamic-light-scattering (DLS), using a Zetasizer Nano-ZS laser diffractometer (Malvern Instruments Ltd., Worcestershire, UK), working at 633 nm, equipped with a backscatter detector (173°). The DLS measures particle diffusion moving under Brownian-motion. Nanoemulsions were diluted 100 times with milli-Q water to avoid multiple-scattering effect and stirred to ensure sample homogeneity. The refractive indexes (RI) of the oil phases were measured with a manual refractometer (model J357, Rudolph research, New Jersey, USA) being 1.501, 1.497, 1.484 and 1.475 for the oregano, thyme, lemongrass and mandarin EOs, respectively. The absorbance of the EOs at 633 nm was measured with a spectrophotometer Cecil CE 1021 (Cambridge, England) being 0.002, 0.002, 0.024 and 0.004 for the oregano, thyme, lemongrass and mandarin EOs, respectively. Measurements were made and the droplet size was reported as the volume weighted average diameter (nm).

2.5. Antimicrobial properties of nanoemulsions

In order to evaluate the changes on the antimicrobial activity of nanoemulsions during storage, their bactericidal capacity was assessed against *Escherichia coli* 1.107 and *Listeria innocua* 1.17, as Gram-negative and Gram-positive bacteria, respectively. For that purpose, bacterial cultures were prepared to grow at their stationary phase and then put in contact with nanoemulsions

Table 1
Main volatile components and percentages present of EOs used in the present work according to the bibliographic references.

Essential oil	Main volatile compounds	References
Oregano (*Origanum vulgare*)	Carvacrol (7.8–80) and thymol (3.24–72%)	(Daferera et al., 2000; Giatrakou et al., 2008; Martino et al., 2009)
Thyme (*Thymus vulgaris*)	Thymol (10–74.8%) and carvacrol (2.2–11%)	(Daferera et al., 2000; Lee et al., 2005; Salehi et al., 2014)
Lemongrass (*Cymbopogon citratus*)	Citral (70–85%)	(Desai et al., 2014)
Mandarin (*Citratus reticulata*)	Limonene (52.2–96.2%)	(Fisher & Phillips, 2008)