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Physicochemical characterization of chitosan nanoparticles and nanocapsules incorporated with lime essential oil and their antibacterial activity against food-borne pathogens

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ABSTRACT

Lime oil has recognized fungicidal and antibacterial properties. Nanoparticles and nanocapsules are structures that have been developed to overcome the high volatility of essential oils. Chitosan nanoparticles and chitosan nanocapsules incorporated with lime essential oil were synthesized by nanoprecipitation and nanoencapsulation methods, respectively. The samples were characterized by transmission electron microscopy (TEM) and Fourier Transform Infrared Spectroscopy (FTIR), and Z potential was measured. Also, particle size distribution was analyzed by dynamic light scattering (DLS) and the antibacterial activity was studied. According to TEM, the average size of nanocapsules was higher than for nanoparticles. When lime essential oil was incorporated, the particle size increased. Lime essential oil incorporation was evidenced by FTIR. Chitosan nanocapsules showed higher Z potential value compared to chitosan nanoparticles. The antibacterial activity was tested against four food-borne pathogens, being higher for nanoparticles than for nanocapsules. The highest antibacterial activity was observed for chitosan nanoparticles incorporated with lime essential oil applied against *Shigella dysenteriae*, attaining an inhibition halo (IH) value of 3.5 cm for 40 μL of minimum inhibitory volume (MIV). The novelty of incorporating lime essential oil into chitosan nanoparticles and nanocapsules and the study of their enhancing effect on antibacterial activity are shown in this paper.

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1. Introduction

Nowadays, the use of synthetic pesticides and fertilizers poses a problem for human health and the environment. Therefore, the use of new natural bioactive substances from plants is an alternative in order to overcome this problem (Luiz de Oliveira, Ramos, Bakshi, Abhilash, & Fernandes, 2014).

It has been found that the main antibacterial, antifungal, antiviral, insecticidal and antioxidant properties of essential oils are due to their bioactive components (Bey-Ould et al., 2016; Rivera, Crandall, ÓBryan, & Ricke, 2015). Lime is one of the most important citrus fruits for essential oil extraction (Kosakowska et al., 2015; Sánchez, Andrade-Ochoa, Aguilar, Contreras-Esquivel, & Nerváez-Moorillón, 2015). The main bioactive compounds of lime oil reported in the literature are limonene and other terpenes (Souza et al., 2014). Its antibacterial properties have been proved against *P. aeruginosa* (Thomas et al., 2014), *E. coli*, *S. typhimurium*, and *S. aureus* (Matan, Nisoa, & Matan, 2014). Due to the high volatility of essential oils, their encapsulation allows a progressive release of the bioactive compound (Souza et al., 2014). In order to encapsulate essential oils, many polymers such as liposomes (Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015), sodium alginate (Liakos et al., 2014) and chitosan (Khalili et al., 2015) have been used.

Chitosan has been used for encapsulation of different compounds because it is a biocompatible and biodegradable polymer (Mendes et al., 2016; Zhaveh et al., 2015). Chitosan nanoparticles have been loaded with cinnamon (Hu, Wang, Xiao, & Bi, 2015), *Eucalyptus staigeriana* (Correia et al., 2013), oregano (Hosseini,

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Zandi, Rezaei, & Farahmandghavi, 2013), and limonene (Souza et al., 2014) essential oils, among others. Moreover, the antibacterial activity of chitosan has been demonstrated in a wide variety of bacteria: *S. pyogenes*, *E. coli*, *S. aureus*, *S. dysenteriae and B. fragilis* (Benhabiles et al., 2012; Chang, Lin, Wu, & Tsai, 2015; Zhu et al., 2016).

The aim of this work was to characterize and study the antibacterial activity of chitosan nanoparticles and nanocapsules incorporated with lime essential oil for use in coatings or packaging in the food industry to prevent food-borne pathogen contamination in food. This is the first time that incorporation of lime essential oil into chitosan nanoparticles and nanocapsules and their combined antibacterial activity has been reported in the literature.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan (deacetylation degree 75–85%) was purchased from Sigma-Aldrich. Distilled Mexican lime essential oil (FDA) was supplied by Essential Oils-essencefleur. Glacial acetic acid and methanol were acquired from Fermont Chemicals Inc. Lecithin (Epikuron 145 V) was obtained from Cargill. Acetone and ethanol were acquired from J.T. Baker.

2.2. Nanoprecipitation method

Nanoprecipitation particles (NPs) were prepared according to the methodology proposed by Luque-Alcaraz et al., 2012. The solvent or aqueous phase was prepared by dissolving chitosan (0.05% w/v) in acetic acid. Then 2.5 mL of the solvent phase was added to the non-solvent phase composed of 20% lime and methanol (40 mL) under moderate magnetic stirring by using a peristaltic pump (Bio-Rad, EP-1 Econo Pump). The obtained solution was placed in a rotary evaporator at 40 °C with a speed of 50 rpm (Rotary Evaporator RE 300, BM 500 Water Bath, Yamato CF 300).

2.3. Nanoencapsulation method

The methodology followed for nanocapsule (NC) elaboration was as follows (Lozano et al., 2008): 30 mg of lecithin were dissolved in 0.5 mL of ethanol. Afterwards, 0.125 mL of lime essential oil and 9.5 mL of acetone were added. The organic phase was incorporated into 20 mL of chitosan (0.5% w/v) under constant agitation for 10 min. The obtained solution was placed in a rotary evaporator (Büchi R-210, Switzerland) at 40 °C with a speed of 50 rpm.

2.4. Transmission electron microscopy (TEM) and dynamic light scattering (DLS)

For TEM observation, a drop of the sample suspension was deposited on a copper grid. The morphology of the nanoparticles and nanocapsules was observed using a Transmission Electron Microscope (JEOL-JEM 2010), with an acceleration voltage operating at 200 kV. Average particle size was calculated from TEM images using the ImageJ program. To determine the Z potential and particle size by Dynamic Light Scattering (DLS), a Zetasizer Nano-ZS90 (Malvern Instruments) was used, and 3 mL of each sample were placed in a quartz cell and analyzed.

2.5. Fourier transform infrared spectroscopy (FTIR)

The Fourier Transform Infrared spectra of the nanoparticles and nanocapsules were collected using a Nicolet 6700 spectrometer with 1 nm resolution and 200 scan/sample in the range of 400–4000 cm^{-1}.

2.6. Antibacterial activity

Four strains of bacteria, *Staphylococcus aureus*, *Listeria monocytogenes*, *Shigella dysenteriae*, and *Escherichia coli,* were used as test microorganisms. Bacterial strains were obtained by clinical isolation from the CINVESTAV-IPN and UPIBI-IPN research institutes. The concentration of culture suspensions was adjusted to 10^8 CFU/mL according to the McFarland turbidity standard no. 0.5 (Keawchaoon & Yoksan, 2011).

Nutrient Agar (10 mL) was allowed to solidify and then a 20 μL bacterial suspension previously added to the semisolid agar (10 mL) was incorporated into Petri dishes. Once solidified, cavities were made on the agar plate (5 mm in diameter). Subsequently, 20 μL of the solution with the bioactive compound to be tested were placed in each small well. Finally, the Petri dishes were sealed and incubated for 24 h at 37 °C. After that, the inhibition zones (lighter areas around the well) that indicated death or bacterial growth inhibition were measured using a Vernier Caliper (Colome, Kubinske, Cano, & Grady, 1998).

Based on the minimum inhibitory concentration (MIC) that inhibited the growth of the bacterial strain (chitosan 0.05%, 0.5% w/v., lime 20%, 2.5% v/v, for nanoparticles and nanocapsules, respectively), formulations of the nanoparticles and nanocapsules were made. The MIC was determined for six replicates. For the *in vitro* evaluation, different volumes (2.5–20 μL) were tested in order to establish the MIV.

2.7. Statistical analysis

A completely randomized design was used for statistical analysis. One-way analysis of variance (ANOVA) with a significance level of $P < 0.05$ was applied. Similarly, when significant differences were found, a comparison of means was performed using Tukey's multiple comparison test. A confidence interval of 95% was employed. The analysis was performed using a SigmaStat 3.5 program.

3. Results and discussion

Chitosan nanoparticles (CSNPs) and chitosan nanoparticles incorporated with lime essential oil (CSNPs-EO) were prepared by nanoprecipitation. On the other hand, chitosan nanocapsules (CSNCs) and chitosan nanocapsules incorporated with lime essential oil (CSNCs-EO) were synthetized using the nanoencapsulation method. The characterization and results of the nanostructures are presented below.

3.1. Transmission electron microscopy (TEM) and dynamic light scattering (DLS)

In Fig. 1, average particle size and TEM micrographs of CSNPs and CSNPs-EO are shown. In brief, by using ImageJ software, scale was set and the threshold of the images was found to stablish the contrast between particles and background. Then, the particle size (diameter) was calculated automatically assuming round particles. Fig. 1a corresponds to average particle size, being 4.7 ± 1.2 nm and 6.1 ± 0.4 nm for CSNPs and CSNPs-EO, respectively. Spherical and equally distributed nanoparticles for CSNPs (Fig. 1b) and CSNPs-EO (Fig. 1c) are also observed. TEM micrographs for CSNCs and CSNCs-EO are seen in Fig. 2. In Fig. 2a the average particle size is shown. It was 5.8 ± 1.6 nm for the CSNCs and 6.1 ± 0.6 nm CSNCs-EO. Spherical nanocapsules of different sizes are observed for both

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