Antibacterial activity of essential oils encapsulated in chitosan nanoparticles

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Abstract

In this study, cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*) and *Schinus molle* essential oils (at 125, 250 and 500 µg/mL) were encapsulated in chitosan nanoparticles by ionotropic gelation. Their antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Enterococcus* sp., *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* (BLEE +) and *Pseudomona aeruginosa* carbapenemase producer was evaluated. The obtained nanoparticles exhibited a regular distribution and spherical shape with size range of 19.7 to 361 nm as observed by using a laser light scattering equipment. It was found that the studied chitosan nanoparticles with essential oils showed high antimicrobial effect than the chitosan and essential oils alone. *Enterococcus* sp. was the most sensitive bacteria and *S. aureus* the most resistant. Chitosan nanoparticles and chitosan nanoparticles with cinnamon and thyme essential oils were capable to inhibit growth of *Enterococcus* sp. and *K. pneumoniae*. This study demonstrated that chitosan nanoparticles with essential oils were effective against some foodborne pathogens.

Keywords: viability; *Schinus mole*; cinnamon; thyme; essential oils.

Practical Application: Use of essential oils in encapsulated in chitosan to avoid microbial growth.

1 Introduction

Microbial pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and others that can be present in food may cause spoilage and contribute to foodborne disease incidence (Miladi et al., 2016). It has been reported that foodborne illness represents a serious public health threat/risk worldwide, with around 48 million people affected and 3,000 death each year only in the USA (Silva & Domingues, 2016). One of the possible strategies to reduce foodborne infections is the development of effective preservation strategies capable of eradicating microbial contamination of foods. Interest in natural substances has focused attention on plants rich in bioactive compounds some of which are well known for their antimicrobial properties. The composition, structure as well as functional groups of their essential oils (EOs) play an important role in determining their antimicrobial activity. Plant EOs are generally recognized as safe and hence been considered as biodegradable safe natural alternative. Besides antibacterial properties of the EOs and their components, they also have been shown to exhibit antifungal and antiparasitic properties, which are possibly related to the function of these compounds in plants (Elsabee & Abdou, 2013). The antimicrobial activity of several EOs has been attributed to the presence of phenolic compounds such as the thymol, eugenol and carvacrol in thyme, clove and oregano essential oils, respectively (Tsao & Zhou, 2000; Lambert et al., 2001). Encapsulation is an attractive technology for protecting chemical and physical properties of essential oils to avoid unwanted changes and

improving food processing. The encapsulating materials can be formed by one or more compounds such as chitosan, maltodextrin, gum arabic, hydroxypropyl methyl cellulose phthalategelatin, starch, among others, which can be used as a blend or alone (Nale et al., 2018; Prasad et al., 2018; Ahn et al., 2019; Perna et al., 2019). Chitosan has also gained interest due to its applications in food and pharmaceutics. Among others, the antimicrobial and antifungal activities of chitosan are some of its most interesting properties to improve food preservation and to reduce the use of chemical preservatives. The antimicrobial activity of chitosan has been considered in a wide variety of fungi and bacteria (Hernández-Lauzardo et al., 2008; Nazzaro et al., 2013; Feyzioglu & Tornuk, 2016; Liu et al., 2017; Abdeltwab et al., 2019). In addition, the antibacterial activity was enhanced when chitosan was dissolved in acetic, formic or lactic acid No et al. (2002). Furthermore, Liu et al. (2006) showed that chitosan in acetic acid solutions over 200 ppm have a marked biocide response to *Escherichia coli*. This activity of chitosan depends on diverse factors such as the type of chitosan used, its deacetylation degree and molecular weight, the pH of the medium and the temperature, the target organism, etc. Chitosan has been used in combination with essential oils by using nanoencapsulation treatments that may find potential application in the food industries. Due to the fact that EOs are easy to degradation in presence of light, air and high temperatures, nanoencapsulation has been recently developed as an efficient technique to protect them from

evaporation and oxidation (Beyki et al., 2014). It is practical approach to maintain the EOs chemical properties, therefore, the aim of this study was to determine antimicrobial activity of chitosan nanoparticles loaded with essential oils.

2 Material and Methods

2.1 Bacterial strains

Five different human pathogens were tested, two Gram positive bacteria: *Staphylococcus aureus* ATCC 25923 and *Enterococcus* sp. vancomycin resistant, as well as three Gram negative: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* (BLEE +) and *Pseudomonas aeruginosa* carbapenemase producer. They were obtained from the Microbiology Laboratory, Department of the Chemical Biologist Sciences at the University of Sonora (Hermosillo, Sonora, México). Bacteria were cultured on blood agar at 37 °C for 48 h. All cultured were standardized by matching to the McFarland 0.5 turbidity standard using Mueller Hinton broth to produce approx. 1.5 x 10⁸ colony forming units (CFU) per mL (Sánchez-García et al., 2016).

2.2 Raw materials

Chitosan (Cs) from shrimp shells, medium molecular weight (153 kDa) and 78% of degree deacetylation was acquired from Sigma-Aldrich, USA (cat. 448877). Cinnamon (*Cinnamomum zeylanicum*, catalog w-22921-0) and thyme (*Thymus capitatus*, catalog w-28281-2) essential oils (CEO and TEO, respectively) were also acquired from Sigma Aldrich˚ (St. Louis, MO, USA). In the other hand, *Schinus molle* leaves collected near to Hermosillo, Sonora (Mexico) (28° 96' 14" N and 111° 04' 40" W) during the summer of 2019 were shade-dried for two week at 25 °C. To obtain the essential oil (EO) a sample of 100 g of dry leaves were hydro-distillated at 100 °C for 4 h in a Clevenger apparatus.

2.3 Nanoparticles preparation

The nanoparticles were prepared as described by López-Meneses et al. (2018). A solution of chitosan (Cs, 0.2% w/v) was prepared by constant agitation during 12 h in an aqueous lactic acid solution (1%, v/v). Then, it was then centrifuged for 30 min at 6000 rpm in a Hermle Labnet Z326 K centrifuge and the supernatant filtered through 1.6 μm pore filter. Tween 80 (0.45 g) was added into 40 mL of the Cs solution and stirred at 50 °C for 2 h. Aliquots of 10 mL was added individually with 0.04g of each essential oil and a fourth without EOs. After that, 3 mL of sodium tripolyphosphate (TPP, 0.02% w/v) was dropped in a 3 mL aliquot of each of the samples with continuous agitation during 15 min to obtain the chitosan nanoparticles, *S. molle*, thyme, and cinnamon essential oils (NpCs, NpSEO, NpTEO, and NpCEO, respectively. Three concentration of each nanoparticles with EOs were prepared (125, 250 and 500 μg/mL). Finally, they were dialyzed twice with methanol (10% w/v) and twice with deionized water. Particle size or dispersion of size and the zeta potential of nanoparticles was carried out in triplicate using a laser light scattering (MöbiuÇ equipment Wyatt Technology, Santa Barbara, CA, USA) with a laser wavelength of 532 nm and scattering angle of 90° (Luque-Alcaraz et al., 2016).

2.4 Evaluation of the in vitro antimicrobial activity

To evaluate the antibacterial activity of the nanoencapsulated EOs, the cell viability was measured according to Elshikh et al. (2016) using the resarzurin dye assay. For this, a 0.02 mg/mL solution of resazurin sodium salt powder (Sigma-Aldrich, USA cat. R7017) was prepared in distilled water, filtered through 1.2 μm porous paper, sterilized and kept at 4 °C. A volume of 100 μL of each bacteria containing ≈1.5 × 10⁸ CFU/mL in Mueller Hinton broth were deposited into each well of a flat 96-well microplate (Costar Corning, NY, USA) and incubated at 37°C for 4 h. Then, 100 μL of different concentration (125, 250 and 500 μg/mL) of each nanoparticles was added into the wells and incubated during another 14 h. Subsequently, 20 μL of resazurin sodium salt were dropped in each well and incubated for 24 h at 24-25 °C. The plates were read at 570 nm in a plate reader (Multiskan Go Thermo Scientific™). Three controls were used: Gentamicin as positive control, treatments alone, and bacteria + Mueller Hinton broth. The experiments were carried out by triplicate. The inhibition percentage was calculated according to the equation of Lozano & Suárez (2008):

$$\% \text{ Inhibition} = \left[\frac{(ABT - AM) - (ATB - AB)}{ABT - AB} \right] \times 100$$

Where ABT is the total bacteria absorbance, AM is the Mueller Hinton broth absorbance; ATB is the absorbance of the treatments with bacteria and AB is the absorbance of treatments without bacteria.

2.5 Statistical analysis

All experiments were performed in triplicate and data expressed as mean value ± standard deviation. The values were subjected to a variance analysis (ANOVA). To determine the statistical difference among the mean values of treatments with 95% significance level, Tukey's multiple range test (p ≤ 0.05) using the statistical program JMP version 5.0 was used.

3 Results and discussion

3.1 Physicochemical characterization of Cs-EO's nanoparticles

Table 1 shows the average particle size, potential zeta and the polydispersity index of the obtained nanoparticles. The NpCs and NpCsCEO showed irregular shape and had tendency to aggregate. The other nanoparticles showed spherical shape and according to their low polydispersity index (near to zero), they were homogeneous. The potential zeta and average size in the nanoparticles decreased when EOs were added, except for NpCsSEO, which showed an increase in the average size and

Table 1. Potential Z, average size and polydispersity index of the nanoparticles obtained.

Treatment	Potential Z (Mv)	Diameter (nm)	PDI
NpCs	+ 43.8 ± 11.25	361.9 ± 31.4	0.35
NpCsSEO	+ 40.2 ± 7.5	516.9 ± 45.5	0.74
NpCsCEO	+ 22.9 ± 0.3	29.3 ± 0.9	0.3
NpCsTEO	+ 22.7 ± 0.8	19.7 ± 0.9	0.2

NpCS: chitosan nanoparticles, NpCsSEO, NpCsCEO and NpCsTEO are nanoparticles of chitosan with TEO, SEO and CEO, respectively. Values represent the mean ± standard deviation of three replicates.

PDI. Our results differ from those of Keawchaoon & Yoksan (2011); Woranuch & Yoksan (2013) and Sotelo-Boyás et al. (2017), who found an increase in the average size after loading EOs. The zeta potential of NpCs and NpCsSEO were higher than those from NpCsTEO and NpCsCEO. These high values implies a particle surface positively charged, good stability, in addition to a potential functionality. They can be considered high, which implies a particle surface positively charged, good stability, in addition to a potential functionality. The interactions between the amino groups of the chitosan and the OH groups of the EO could be responsible for the increase of this value.

3.2 Evaluation of the in vitro antimicrobial activity

The resarzurin reductive assay was carried out to evaluate the proliferation. This is a cell permeable redox indicator, which is used to monitor viable cell number. The viable cells with active metabolism reduce resazurin into resorufin, which is pink and fluorescent (Riss et al., 2004). The antibacterial response to EOs alone and incorporated in chitosan.

3.3. E. coli ATCC 25922

In the proliferation test for *E. coli* after 24 h of incubation, statistical differences were found (p < 0.05, Table 2). *E. coli* was not inhibited in TEO, CEO and chitosan whereas in the other treatments its proliferation was low. The higher concentration of EOs in the nanoparticles caused moderate inhibition 10.0, 11.0, and 26.0% (NpCsCEO, NpCsSEO, and NpCsTEO, respectively), and they were also lower than the obtained with gentamicin (39.3%). The lower CI_{50} calculated was for the NpCsTEO and NpCsCEO (580 and 683, respectively), which were determined at 95% of confidence intervals using a Probit analysis

Table 2. Proliferation percentage of human pathogenic bacteria in presence of the individual essential oils (μg/mL) and nanoencapsulated in chitosan.

Treatment	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*Enterococcus* sp.	*S. aureus*
MHB	100[a]	100[a]	100[a]	100[a]	100[a]
Gentamicin	70.3±2.1d	100[a]	87.6±0.6[ef]	31.9±0.5[l]	80.8±12.5[fg]
Chitosan					
125	100±0.0[a]	100±0.9[a]	93.7±0.8[bc]	91.9±3.4[bc]	99.4±0.7[ab]
250	100±5.1[a]	93.7±1.9[bc]	100±2.9[a]	98.1±2.5[ab]	92.8±1.7[bcde]
500	100±1.5[a]	90.9±6.3[cde]	100±1.9[a]	80.6±4.2[ef]	91.4±0.6[cde]
SEO					
125	100±0.0[a]	97.7±0.8[abc]	90.8±2.9[abc]	89.0±1.7[ab]	100±0.3[a]
250	100±3.0[a]	58.7±5.5[bcd]	100±0.7[f]	75.0±4.8[g]	100±1.4[abcd]
500	100±15.5[a]	84.8±2.8[def]	100±2.2[h]	46.7±3.0[ef]	94.2±1.7[abcd]
CEO					
125	100±0.0[a]	98.2±5.3[abc]	99.4±5.9[ab]	94.5±3.4[abc]	100±0.9[a]
250	100±5.5[a]	81.0±6.3[g]	100±0.7[a]	75.8±1.2[fg]	100±1.6[a]
500	100±5.0[a]	75.2±4.2[h]	100±2.2[a]	39.7±7.7[k]	100±3.5[a]
TEO					
125	100±0.0[a]	94.7±4.8[ab]	95.8±3.2[cde]	97.2±0.5[cd]	100±0.7[a]
250	100±5.1[a]	91.7±3.1[ij]	83.8±1.0[ef]	71.6±4.7[fg]	94.9±3.6[a]
500	82.4±6.3[bc]	86.7±4.3[fg]	62.8±1.0[h]	81.3±3.1[jk]	96.6±5.3[abcd]
NpCs					
125	95.7±1.3[a]	47.6±5.3[l]	88.5±0.6[def]	78.7±4.8[efg]	75.4±3.0[g]
250	95.5±2.2[a]	30.0±2.8[m]	67.6±4.2[h]	57.0±1.5[hi]	76.5±5.4[g]
500	76.8±5.3[b]	15.9±4.3[n]	65.4±0.2[h]	6.1±3.1[n]	66.6±1.7[h]
NpCsSEO					
125	99.2±1.4[a]	86.0±7.0[efg]	95.4±3.5[abc]	83.4±3.4[de]	87.0±5.5[ef]
250	87.4±0.b[2]	56.9±4.6[kl]	93.3±3.3[bcd]	62.8±9.7[h]	63.3±4.9[h]
500	75.9±6.3[cd]	49.2±5.5[kl]	75.1±3.4[g]	29.6±8.5[l]	67.5±3.3[h]
NpCsCEO					
125	100±0.0[a]	53.8±4.8[jk]	100±3.2[a]	60.4±0.5[h]	97.6±0.7[abc]
250	100±7.1[a]	44.1±3.1[l]	100±1.0[a]	52.3±4.7ij	78.1±3.6[g]
500	77.5±6.3[c]	20.1±4.1[n]	100±1.0[a]	13.7±3.1[m]	86.9±5.3[ef]
NpCsTEO					
125	100±0.0[a]	95.4±2.2[abc]	100±2.0[a]	63.0±14.1[h]	91.9±1.5[cde]
250	82.2±15.7[bc]	74.6±1.3[h]	100±1.8[a]	41.7±2.2[k]	98.3±1.0[abc]
500	60.7±3.9[e]	60.2±2.4[i]	100±2.9[a]	24.7±0.2[l]	90.4±6.3[de]

MHB = Mueller Hinton Broth, SEO = *S. molle*, TEO = thyme and CEO = Cinnamon essential oils. NpCs: chitosan nanoparticles, NpCsSEO, NpCsCEO and NpCsTEO are nanoparticles of chitosan with TEO, SEO and CEO, respectively. Values represent the mean ± standard deviation of three replicates. Same lowercase letter in the superscript indicate no statistical difference (P <0.05) among treatments for each bacteria.

with NCSS 97 statistical program (NCSS, LLC., Kaysville, UT, USA). This agrees to Chen et al. (2009) findings, because they reported that NpCs grafted with two essential oils components, eugenol or carvacrol, had more antibacterial activity than NpCs against *E. coli*. In addition, they mentioned that modification of chitosan nanoparticles with the essential oil components via the Schiff base reaction involved the amine group of chitosan, and it might be expected that the antibacterial properties of the chitosan will be compromised such as occurred in our study.

A study of Saei-Dehkordi et al. (2010) and Tiwari et al. (2009) reported that among the organisms they evaluated, the Gram negative bacteria were the most resistant and the Gram positive were the most susceptible ones. This does not match totally with our finding, just for *E. coli* and *Enterococcus sp*. It has been reported previously (Sandri et al., 2007) that the resistance of the Gram negative bacteria could be due to the presence of their impermeable outer membrane that provide hydrophilic properties to lipophilic compounds such as the essential oils. In the other hand, the no existence of this barrier in the Gram-positive bacteria favors the contact of the essential oil with the phospholipid bilayer of the cell membrane. Thus might cause an increase in the ion permeability and leakage of intracellular constituents or an impairment of the bacteria enzyme systems (Yi et al., 2014; Nakayama et al., 2013). In addition, the inhibitory effect of phenols could be explained by interactions with the cell membrane of microorganisms and is often correlated with the hydrophobicity of the compounds (Sikkema et al., 1995; Weber & de Bont, 1996). For instance, Lambert et al. (2001) found that oregano essential oil induces permeability alteration in *P. aeruginosa* and *S. aureus* membranes with a consequent leakage of protons, phosphates and potassium.

3.4 S. aureus ATCC 25923

From the response of *S. aureus* to the treatments it was found that, there is significant difference among them (p < 0.05). The NpCs (500 µg/mL) and NpCsSEO (250 and 500 µg/mL) reduced the proliferation by 35.0% while gentamicin only caused 19.2%, which indicate that nanoparticles were more effective (Table 2). In addition, our data showed that CEO did not produced any effect in this bacteria, along with TEO (125 and 250 µg/mL) and SEO (125 µg/mL). All the CI_{50} were higher than the obtained with the NpCsSEO (1017 µg/mL). However, according to a study by Keawchaoon & Yoksan (2011), chitosan nanoparticles with carvacrol exhibited antimicrobial activity against *S. aureus, B. cereus, and E. coli* compared to chitosan nanoparticles. They also demonstrated that this activity was more effective against Gram positive bacteria which does not match with our results. Thymol, carvacrol, linalool and γ-terpinene are the main components of thyme essential oil (Akrout et al., 2010) and they have shown antimicrobial activity. Those compounds are terpenoids and their antimicrobial activity is related to their functional groups, and the hydroxyl groups on the phenolic terpenoids along with the presence of delocalized electrons (Ben Arfa et al., 2006; Lambert et al., 2001). In addition, Ben Arfa et al. (2006) mentioned that thymol and carvracol are capable to disintegrating the cell outer membrane.

3.5 Enterococcus sp. (vancomicin resistant)

The antimicrobial activity of the treatments against *Enterococcus* sp. is shown in Table 2. It can be seen that they showed inhibitory effect on the proliferation of this microorganism. We found that it was the most sensitive bacteria from all the above tested. The highest antiproliferative activity was obtained with the nanoparticles at 500 µg/mL (NpCs >NpCsCEO>NpCsTEO and NpCsSEO, 6.1, 13.0, 29, 30%, respectively) compared to gentamicin (31.9%) and the EOs alone except for CEO at 500 µg/mL which activity was closer to the gentamicin. The CI_{50} for SEO, CEO, TEO, NpCs, NpCsTEO and NpCsCEO were 414, 472, 233, 317, 195 and 193 µg/mL, respectively. The synergy among chitosan and the components of each EO can be easily observed. Unlu et al. (2010) and Ribeiro-Santos et al. (2017) stated that cinnamaldehyde is the major compound in cinnamon EO which has proven to be effective against some species of both Gram positive and negative bacteria (Friedman et al., 2002; Ooi et al., 2006). Ribeiro-Santos et al. (2017) found that *Cinnamomum cassia* EO exhibited great antimicrobial activity against *E. coli*, *S. aureus* and other microorganisms. Furthermore, cinnamaldehyde and eugenol caused damage to the cell wall of the bacteria (Di Pasqua et al., 2007) and cinnamaldehyde reduces the activity of enzymes causing protein denaturation and increases the cell wall permeability, thereby permitting leakage of cell components resulting in cell death (Tiwari et al., 2009).

3.6 K. pneumoniae BLEE

As shown in Table 2, gentamicin and chitosan (125 µg/mL) did not show antiproliferative activity. This could be due to its resistance to extended spectrum β-lactamases antibiotics that *K pneumoniae* possesses. Kader & Kumar (2005) found isolates of *Klebsiella* highly resistant to gentamicin that agrees to our results. Also, Díaz et al. (2004) reported that 65 of 100 strains isolated from several hospitals of different Chilean cities resulted gentamicin resistant. A dose-response was observed in most of the treatments. The highest antiproliferative activity was observed with the NpCs followed by the NpCsCEO and NpCsSEO which inhibition ranged from 50 to 84.8%. The lower CI_{50} were for the NpCs, NpCsCEO, NpCsSEO and NpCsTEO (115, 161, 422 and 610 µg/mL, respectively).

3.7 P. aeruginosa carbapenemase producer

As can be seen in Table 2 for *P. aeruginosa* the treatments of SEO, TEO, NpCs, and NpCsSEO at 250 and 500 µg/mL were statistically significant (p < 0.05). Proliferation of this bacteria was high in most of the treatments and to gentamicin. Thyme and *S. molle* EOs produced similar inhibition in the proliferation followed by NpCs. Martins et al. (2014) and López-Meneses et al. (2018) reported that the main components in the leaves of *S. molle* EO are D-limonene, o-cimene, β-phellandrene, δ-cadinene and caryophillene. Martins et al. (2014) found that leaf EO was effective against *Staphylococcus epidermidis*, *S. aureus*, *Enterococcus faecalis* while fruit EO was more effective against *S. aureus* and *E. faecalis*. Also, both EOs were effective against antibiotic-resistant Gram bacteria, *P. aeruginosa* and *Salmonella entiritidis* serovar Typhimurium. Also, Mendonça-Rocha et al. (2012), demonstrated that EO of *S. molle* has high inhibitory effect

against *S. aureus* ATCC 25923 strain, for *E. coli* ATCC 25922 the inhibitory effect was smaller, however, it was strong when the undiluted crude extract (950 mg/mL) was applied. Also, they found that *P. aeruginosa* ATCC 27853 strain was less sensitive for this oil. These results agrees to our findings. The lowest CI_{50} was for Cs, CEO and NpCsCEO (24, 33 and 690 µg/mL, respectively).

4 Conclusions

In general terms, the chitosan nanoparticles with EOs of cinnamon, thyme and *S. molle* showed the highest antimicrobial effect than the chitosan and EOs alone. Even that chitosan have been reported to exhibit high bactericidal effects against Gram positive, there were no marked trends in inhibitory effects of NpCsEOs against both types of bacteria. *Enterococcus* sp. was the most sensitive bacteria and *S. aureus* the most resistant. NpCS, NpCsCE and NpCsTEO were capable to inhibit growth of *Enterococcus* sp. and *K. pneumoniae*. This study demonstrated that chitosan nanoparticles with essential oils were effective against some foodborne pathogens and seems to have a beneficial impact on microbial reduction. These finding implied that NpCsEOs could be used as an antimicrobial agent in the food industry.

Acknowledgements

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