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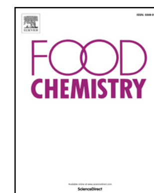

Improving the antifungal activity of clove essential oil encapsulated by chitosan nanoparticles

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ABSTRACT

Encapsulation of clove essential oil (CEO) by chitosan nanoparticles (ChNPs) was performed, using an emulsion-ionic gelation technique to improve the antifungal efficacy of CEO. The mass ratios of chitosan (Ch) to tripolyphosphate (TPP), 1:1, for unloaded ChNPs and 1:1:1 for Ch to TPP to CEO, for CEO-loaded ChNPs (CEO-ChNPs), were selected as optimum formulations based on dynamic light scattering and ultraviolet-visible spectroscopy. The presence of CEO in optimum CEO-ChNPs, was evidenced by Fourier transform infrared spectroscopy. Particle size distribution, of around 40 and 100 nm for the most optimum unloaded and oil-loaded ChNPs, was obtained by field emission-scanning electron microscopy. *In vitro* release studies of CEO-ChNPs revealed a controlled release during 56 days. The nano-encapsulated CEO demonstrated a superior performance against *Aspergillus niger*, isolated from spoiled pomegranate, compared with ChNPs and free oil. Therefore, this study revealed that CEO-ChNPs can be used as a promising natural fungicide in agriculture and food industry.

1. Introduction

The susceptibility of fruits and vegetables to postharvest diseases, caused by fungal pathogens, has resulted in using synthetic fungicides in order to control these diseases (Usall, Ippolito, Sisquella, & Neri, 2016); however, the need for natural safe fungicides as alternatives to commercial ones has recently increased due to pathogenic resistance towards commercial fungicides and public concern about food contamination from commercial fungicidal residues (Amiri, Dugas, Pichot, & Bompeix, 2008). In this regard, plant essential oils (EOs), which are generally recognized as safe, have been revealed to possess a broad spectrum of fungicidal activities against postharvest pathogens and have hence been considered as biodegradable safe natural alternatives over the past decade (Linde, Combrinck, Regnier, & Virijevic, 2010; Tajkarimi, Ibrahim, & Cliver, 2010).

Among the EOs, clove essential oil (CEO) has the ability to control postharvest contamination of various agricultural commodities, including cereals, oil seeds, fruits and nuts, resulting from the accumulation of ochratoxin A, an important mycotoxin produced by plant pathogens such as *Aspergillus niger* and associated with carcinoma, nephropathy and immunosuppressive diseases (Passone, Girardi, & Etcheverry, 2012). This oil and its main component, eugenol, with activity against fungal pathogens such as *Botrytis cinerea* and *Penicillium expansum* and also *Aspergillus* spp., such as *Aspergillus niger* (*A. niger*) and *Aspergillus flavus*, have been considered as natural fungicides (Amiri et al., 2008; Passone, Girardi, Ferrand, & Etcheverry, 2012; Passone et al., 2012). CEO has been extracted from the buds of *Syzygium aromaticum* and is widely used in the flavouring industry, fragrance and cosmetics. The major ingredients of CEO are eugenol (4-allyl-2-methoxy phenol), the phenylpropanoid, eugenyl acetate, the monoterpene ester and β-caryophyllene, a sesquiterpene. This oil can be applied as a food preservative due to its antibacterial, antifungal, antioxidant, insecticidal and antiviral properties (Chaieb et al., 2007; Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015). However, the antimicrobial property of CEO is considerably limited due to its highly volatile and slightly water-soluble constituents, such as eugenol (Woranuch & Yoksan, 2013; Sebaaly et al., 2015).

To solve these problems, nano-encapsulation has been recently developed as an efficient technique for protecting EOs from evaporation and oxidation (Beyki et al., 2014), offering prolonged activity for encapsulated compounds through controlled release (Yoksan, Jirawutthiwongchai, & Arpo, 2010), improving the stability and hence antimicrobial bioactivity of unstable compounds during food processing and storage (Fang & Bhandari, 2010) and improving the water-solubility and bioavailability of lipophilic compounds (Arulmozhi, Pandian, & Mirunalini, 2013). In this regard, chitosan (Ch) has recently achieved much attention in the encapsulation of bioactive compounds and EOs due to its nontoxicity, biocompatibility, biodegradability and

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antimicrobial properties, and also its ability to form gels, films and particles (Keawchaoon & Yoksan, 2011; dos Santos et al., 2012; Wang, Wu, Qin, & Meng, 2014; Beyki et al., 2014).

Among several techniques, ionic gelation is a mild, simple and organic solvent-free approach for the formation of stable nanosize particles. This approach is based on interaction between positively charged polymers such as Ch and polyanions such as pentasodium tripolyphosphate (TPP) which lead to the formation of inter- and intra-molecular cross-linkages without using high temperatures and toxic crosslinking agents (Keawchaoon & Yoksan, 2011; Woranuch & Yoksan, 2013; Saharan et al., 2013). Chitosan nanoparticles (ChNPs), formed by ionic gelation, have been reported as useful for loading of sensitive bioactive compounds, such as carvacrol (Keawchaoon & Yoksan, 2011), ellagic acid (Arulmozhi et al., 2013), eugenol (Woranuch & Yoksan, 2013) and chlorogenic acid (Nallamuthu, Devi, & Khanum, 2015), and also eucalyptus (Ribeiro et al., 2013), oregano (Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013), summer savory (Feyzioglu & Tornuk, 2016) and lime (Sotelo-Boyás, Correa-Pacheco, Bautista-Baños, & Corona-Rangel, 2017) EOs.

Although the loading of CEO into soybean phospholipid-based liposomes has been recently studied (Sebaaly et al., 2015), the instability of liposomes, rapid release of the entrapped drug, high costs (materials and process) and poor loading efficacy of the drug are major problems of the liposomes which have to be considered (Keawchaoon & Yoksan, 2011; Rodríguez, Martín, Ruiz, & Clares, 2016). To our knowledge, there is no study on the encapsulation of CEO by ChNPs to overcome the evaporation problems of this volatile oil under *in vitro* conditions. Therefore, in the current study, we focussed on the loading of CEO into ChNPs, using the two-step approach of emulsion-ionic gelation, to improve the antifungal activity of oil against *A. niger* through the controlled release of oil.

2. Materials and methods

2.1. Materials

Medium molecular weight ch (75–85% degree of deacetylation, CAS # 9012-76-4) and TPP (CAS # 7758-29-4; technical grade) were purchased from Sigma-Aldrich (Germany). Tween 80, citric acid monohydrate, tri-sodium citrate dehydrate, sodium hydroxide and potato dextrose agar (PDA) were supplied by Merck-Chemicals Co. (Germany). Glacial acetic acid, hydrochloric acid and ethanol (absolute) were acquired from Scharlab, S.L. (Spain). Clove (*Eugenia caryophyllata*) essential oil mainly composed of 77.2% eugenol, 8.31% eugenyl acetate and 7.19% β-caryophyllene, was obtained from Barij Essence Pharmaceutical Co. (Iran). All chemicals were applied as received without any purification.

2.2. Methods

2.2.1. Preparation of oil-loaded and unloaded particles

The oil-loaded particles were prepared, based on the two-step approach of droplet formation and solidification, according to methods of Keawchaoon and Yoksan (2011) and Hosseini et al. (2013), with some modifications. Briefly, the droplet formation was first achieved by the oil-in-water emulsion technique in Ch solution. Then, droplet solidification was performed by TPP solution through the ionic gelation approach to form spontaneous nanoparticles (NPs). For this purpose, two concentrations of Ch (0.3 and 0.5% (w/v)) in aqueous acetic acid solution (1% (v/v)) were produced by stirring at a temperature of 25°C overnight to form aqueous phases. After pH adjustment to 4.6 using 9 N NaOH, the prepared solutions were filtered by Büchner funnel and Whatman 42 paper. Then, Tween 80 (HLB 15.9, 1% (w/v)) was added to the aqueous solutions as a surfactant and stirred at 25 °C for 30 min to obtain homogeneous mixtures. The different concentrations of oil were then gradually dropped in aqueous solutions prepared by two concentrations of Ch to produce eight different mass ratios of Ch to CEO as follows: 1:0.25, 1:0.5, 1:0.75, 1:1, 1.6:0.25, 1.6:0.5, 1.6:0.75 and 1.6:1. At the same time, the agitation was done at 700 rpm for 10 min at 25 °C to prepare oil-in-water emulsions. TPP solution (0.3% (w/v)) was then produced in distilled water and flush-mixed with prepared emulsions to obtain two mass ratios of Ch to TPP of 1:1 and 1.6:1. The mixtures were subsequently subjected to agitation for 30 min to effect crosslinking. The final pH of the mixtures should be 4.6. The same procedure without oil addition was applied for unloaded particles. The spontaneous formed particles were collected by centrifuge (SIGMA 8 K, Germany) at $10{,}000 \times g$ for 35 min at 4 °C and washed several times with aqueous Tween 80 solution 1% (v/v), then dispersed in distilled water and treated by ultrasonic homogenizer (TOPSONICS, UP400, Iran) at 60 W for 6 min with a sequence of 3 s sonication and 7 s rest. The homogenized dispersions were then kept at 4°C until further analysis. A part of the prepared dispersions was freeze-dried at −40 °C for 24 h, using a freeze dryer (Dena Vacuum Industry Co., LTD, 5005, Iran), and stored at −30 °C.

2.2.2. Particle size and surface charge measurements

The mean particle size and surface charge of freshly prepared NPs were measured by photon correlation spectroscopy (PCS) assembly and laser doppler anemometry (LDA) in a dynamic light scattering (DLS) instrument (Zetasizer 3000 HS, Malvern Instruments, UK), respectively. Results were represented as the means of three measurements ± SD (standard deviation).

2.2.3. Encapsulation efficiency (EE), loading capacity (LC) and yield determination

The percentage of encapsulated CEO for CEO-loaded ChNPs (CEO-ChNPs), prepared using both initial mass ratios of Ch to TPP of 1:1 and 1.6:1, was determined by ultraviolet-visible (UV–vis) spectrophotometry (SP-UV 500DB spectrophotometer, Spectrum Instruments, Canada) according to the methods of Rahaiee, Shojaosadati, Hashemi, Moini, and Razavi (2015), as well as Feyzioglu and Tornuk (2016), with some modifications. 10 mg/ml of CEO-ChNPs dispersions were lysed through boiling NPs inside aqueous hydrochloric acid solution (2 M, 5 ml) at 95 °C for 30 min; then 1 ml of ethanol was added into the cooled mixture and the whole centrifuged. ChNPs were also prepared as blank samples in the same manner. The absorbance of CEO within the supernatant was measured at 282 nm (maximum absorption wavelength). Subsequently, total amount of loaded CEO was calculated by a standard curve which was prepared using the absorbance of different concentrations of CEO in absolute ethanol at 282 nm. Measurements were done in triplicate. The encapsulation efficiency (EE) and loading capacity (LC) of CEO were calculated through the following formulas (Rahaiee et al., 2015; Feyzioglu & Tornuk, 2016):

$$EE\,(\%) = \frac{Total\ weight\ of\ loaded\ CEO}{Initial\ weight\ of\ CEO} \times 100 \quad (1)$$

$$LC\,(\%) = \frac{Total\ weight\ of\ loaded\ CEO}{Weight\ of\ freeze{-}dried\ NPs} \times 100 \quad (2)$$

NPs yield was calculated from the weight of freeze-dried NPs (W1) and the sum of dry weight of initial materials (W2) through the following formula (Rahaiee et al., 2015):

$$Nanoparticle\ Yield\,(\%) = \frac{W_1}{W_2} \times 100 \quad (3)$$

Measurements were done in triplicate.

2.2.4. Fourier transform infrared (FTIR) characterization

FTIR spectra of Ch powder, CEO and CEO-ChNPs, prepared by initial mass ratio of Ch to TPP to CEO of 1:1:1, were acquired using 16 scans, at a resolution of 4 cm^{-1} over wave numbers that ranged from 400 to 4000 cm^{-1} with a FTIR spectrometer (Equinox 55, Bruker,

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