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...
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-links 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, & Kuman, 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 dextrine 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 Barjir 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 0.05% (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 × 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 Rahaeie, Shojaoosadi, 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 (Rahaeie et al., 2015; Feyzioglu & Tornuk, 2016):

\[
EE (\%) = \frac{\text{Total weight of loaded CEO}}{\text{Initial weight of CEO}} \times 100
\]  
\[
LC (\%) = \frac{\text{Total weight of loaded CEO}}{\text{Weight of freeze-dried NPs}} \times 100
\]

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 (Rahaeie et al., 2015):

\[
\text{Nanoparticle Yield (}\%) = \frac{W_1}{W_2} \times 100
\]

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,
Germany). Samples were prepared by crushing dried NPs with KBr and squeezing them to form disks.

2.2.5. Field emission-scanning electron microscopy (FE-SEM) observation

The morphology of ChNPs and CEO-ChNPs, prepared using mass ratios of Ch to TPP of 1:1 and 1:1:1, respectively, was studied by FE-SEM (MIRA 3, TESCAN, Czech Republic). Freshly prepared NPs were diluted with distilled water and one drop of diluted dispersions was dried at room temperature. The dried NPs were coated with gold and then examined.

2.2.6. In vitro release studies

The release kinetics of CEO from CEO-ChNPs, prepared using two mass ratios of Ch to TPP to CEO (1:1:0.25 and 1:1:1), were measured spectrophotometrically in citrate buffer solutions (pH 3 and 5) for a period of eight weeks according to the methods of Keawchoaon and Yoksan (2011) and Hosseini et al. (2013), with some modifications. After centrifugation and water decanting of 10 mg/ml of NPs dispersions, 10 ml of citrate buffer solutions (pH 3 and 5) were added to the centrifuge tubes containing wet NPs and the obtained mixtures were agitated by a vortex (GENIUS 3, IKA VORTEX, Germany), followed by incubation at 4 °C for 2 months. On different days of incubation (1-2-3-4-7-10-16-24-32-40-48 and 56th day), the samples were centrifuged and their supernatants were analyzed at 282 nm. Measurements were done in triplicate. The cumulative percentage of released-CEO was measured with the following formula (Hosseini et al., 2013):

\[
\text{Cumulative released−CEO} (\%) = \frac{\text{cumulative amount of released−CEO at each sampling day}}{\text{initial amount of loaded−CEO in the sample}} \times 100
\]  

(4)

2.2.7. Molecular identification of A. niger isolated from spoiled pomegranate

Black infected pomegranate fruits were collected from an orchard in Ismaiel Abad village, Saveh, Iran in winter. Dried fruits were transported to the laboratory and cut with a sterilized blade. Three small samples of black spores were transferred directly through a sterile needle onto the centre of sterilized chloramphenicol potato dextrose agar (PDA) Petri dishes containing 15 ml of PDA and then incubated at 25 °C for 5 days.

The new cultures of A. niger were acquired by sub-culturing of 5 mm mycelium disks from the edge of obtained fungal colonies onto the centre of water agar (WA) plates after incubation at 25 °C for 24–48 h. In a sterile hood, a single hypha from the edge of a fresh isolate of A. niger was found under a sterilized dissecting microscope, then a hyphal tip just before the last branching point was cut with the aid of a sterilized minuten pin. The hyphal tip was transferred onto the centre of a new PDA plate, then incubated in darkness (Jensen et al., 2013).

DNA extraction was subsequently performed according to Liu, Coloe, Baird, and Pedersen (2000), with some modifications, while extraction accuracy was checked by agarose gel electrophoresis. The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR), using the primers of ITS-1 F (CTT GGT CAT TTA GAG GAA GTA A) (Gardes & Bruns, 1993) and ITS-4 (TCC TCC GAT TAT GGA TAT GC) (White, Bruns, Lee, & Taylor, 1990). Polymerase chain reaction (PCR) product of A. niger isolate was sequenced in the Macrogen company of South Korea by DNA analyzer (ABI PRISM 3730XL, USA) and the sequencing result was edited by ChromasPro version 1.7.6 (Technelysium Pty Ltd., Australia) and EditSeq version 5.01 (DNASTAR Inc., USA). For sequencing, 10 ml of citrate buffer solutions (pH 3 and 5) was added to the centrifuge tubes containing wet NPs and the obtained mixtures were agitated by a vortex (GENIUS 3, IKA VORTEX, Germany), followed by incubation at 4 °C for 2 months. On different days of incubation (1-2-3-4-7-10-16-24-32-40-48 and 56th day), the samples were centrifuged and their supernatants were analyzed at 282 nm. Measurements were done in triplicate. The cumulative percentage of released-CEO was measured with the following formula (Hosseini et al., 2013):

\[
\text{Cumulative released−CEO} (\%) = \frac{\text{cumulative amount of released−CEO at each sampling day}}{\text{initial amount of loaded−CEO in the sample}} \times 100
\]  

(4)

2.2.8. Effect of Ch, CEO and prepared particles on inhibition of A. niger growth

The pour-plate technique for antifungal assays was used according to Askarne et al. (2012), with some modifications. Ch, free CEO, and also prepared ChNPs and CEO-ChNPs, using both initial mass ratios of Ch to TPP, 1:1 and 1:6:1, were examined against A. niger. Various concentrations (0.187–3 mg/ml) of tested materials were mixed with sterilized PDA plates. After solidification, 5 mm mycelium disks were cut from the edge of A. niger plates with the aid of a sterile scalpel and transferred onto the centre of PDA plates sealed with parafilm, then incubated at 25 °C for 5–7 days until the growth of the control reached the edge of the plates. The experiments were done in triplicate and percentage of antifungal index for each treatment was calculated using the following formula (Askarne et al., 2012):

\[
\text{Antifungal index (\%)} = \left(\frac{C − T}{C}\right) \times 100
\]

where C and T are radial growth (mm) of control and treated plates of A. niger, respectively.

2.3. Statistical analysis

The one-way analysis of variance (ANOVA) was done through IBM SPSS Statistics version 23. To determine the statistical differences among the mean values of treatments with 95% significance level, Duncan’s multiple range tests were used.

3. Results and discussion

3.1. Preparation of oil-loaded and unloaded particles

CEO-ChNPs was prepared, based on the two-step approach of droplet formation and solidification. Droplet formation was first achieved by the oil-in-water emulsion technique in Ch solution. Then, each droplet was solidified by the ionic gelation method in which the interaction between protonated amino groups of the Ch molecule surrounding the oil droplet, with polyphosphate groups of the TPP molecule, caused spontaneous formation of NPs with positive charge (Keawchoaon & Yoksan, 2011; Woranuch & Yoksan, 2013). The particle size of prepared particles was studied by the DLS technique (Table 1). The morphology and chemical structure of optimum CEO-ChNPs were compared by the FE-SEM technique (Fig. 1).

Table 1

<table>
<thead>
<tr>
<th>Ch/TPP:CEO mass ratio (w/w)</th>
<th>Z-average diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:6:1:1.00</td>
<td>148.43 ± 0.39</td>
</tr>
<tr>
<td>1:6:1:0.25</td>
<td>1287.70 ± 0.60</td>
</tr>
<tr>
<td>1:6:1:0.50</td>
<td>1145.33 ± 9.14</td>
</tr>
<tr>
<td>1:6:1:0.75</td>
<td>1121.53 ± 12.64</td>
</tr>
<tr>
<td>1:6:1:1</td>
<td>1059.47 ± 9.78</td>
</tr>
<tr>
<td>1:1:0.0</td>
<td>129.83 ± 0.57</td>
</tr>
<tr>
<td>1:1:0.25</td>
<td>571.17 ± 1.67</td>
</tr>
<tr>
<td>1:1:0.50</td>
<td>493.37 ± 6.68</td>
</tr>
<tr>
<td>1:1:0.75</td>
<td>401.30 ± 4.69</td>
</tr>
<tr>
<td>1:1:1</td>
<td>264.47 ± 0.71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ch/TPP:CEO mass ratio (w/w)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:6:1:1.00</td>
<td>39.75 ± 1.83</td>
</tr>
<tr>
<td>1:6:1:0.25</td>
<td>31.38 ± 0.18</td>
</tr>
<tr>
<td>1:6:1:0.75</td>
<td>27.82 ± 1.48</td>
</tr>
<tr>
<td>1:6:1:1</td>
<td>26.66 ± 0.09</td>
</tr>
<tr>
<td>1:1:0.25</td>
<td>24.95 ± 0.36</td>
</tr>
<tr>
<td>1:1:0.75</td>
<td>22.45 ± 0.90</td>
</tr>
</tbody>
</table>

Results of both Z-average diameter and Zeta potential followed by the column with various letters are significantly different at P < 0.05 according to the Duncan test. Results are represented as means ± SD.
investigated by FE-SEM (Fig. 2) and FTIR spectroscopy (Fig. 1). The UV–vis spectrum of CEO in absolute ethanol was recorded over wavelengths ranging from 200 to 400 nm and two absorption peaks at 226 and 282 nm were related to eugenyl acetate and eugenol, respectively (Patil, Agrawal, Mahire, & More, 2016). To prepare a standard curve of CEO, the peak of eugenol, the main component of CEO, was considered as the maximum absorption wavelength to determine encapsulation efficiency, loading capacity (Table 2) and in vitro release studies (Fig. 3).

### 3.2. Particle size and surface charge measurements

The effect of two various mass ratios of Ch to TPP (increasing Ch concentration) on average size and surface charge of unloaded particles was investigated by the DLS technique and is presented in Table 1. Although the size, ranging from 1 to 100 nm, is the main feature of NPs, the “nano” prefix is usually used for particles with sizes up to several hundred nanometers (Rodríguez et al., 2016). From the Table 1, the average size and positive surface charge of ChNPs increased significantly (P < 0.05) from 129 to 148 nm and from +31 to +39 mV, respectively, with increase in Ch to TPP mass ratio from 1:1 to 1.6:1. Higher average size and more positive surface charge could be explained by more complete ionic crosslinking as a result of higher protonation of amino groups (Woranuch & Yoksan, 2013). The results were in accordance with Gan, Wang, Cochrane, and McCarron (2005) and Nallamuthu et al. (2015) who observed an increase in both average size and zeta potential of ChNPs with increasing Ch to TPP mass ratio. Moreover, the effect of increasing CEO concentration on average size and surface charge of CEO-loaded particles was investigated by the DLS technique. The average size was measured at two mass ratios of Ch to TPP (1.6:1 and 1:1) and the surface charge was measured at a constant mass ratio of Ch to TPP of 1:1 (Table 1). The results showed that, by loading of CEO into ChNPs, the average size increased significantly (P < 0.05); however, the zeta potential decreased significantly (P < 0.05), as seen in the Table 1. The decrease in zeta potential value might be a result of less availability of free amine groups on the surface of NPs due to the possible interaction between them and EO (Sotelo-Boyás et al., 2017). The observed increase in average size accompanied

### Table 2

<table>
<thead>
<tr>
<th>Ch:CEO mass ratio (w/w)</th>
<th>UV-vis spectrophotometry</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EE (%)</td>
<td>LC (%)</td>
</tr>
<tr>
<td>1:0.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:0.25</td>
<td>45.77 ± 1.25d</td>
<td>3.36 ± 0.13c</td>
</tr>
<tr>
<td>1:0.50</td>
<td>36.67 ± 0.54e</td>
<td>4.85 ± 0.19b</td>
</tr>
<tr>
<td>1:0.75</td>
<td>33.33 ± 1.46eh</td>
<td>6.07 ± 0.60a</td>
</tr>
<tr>
<td>1:1</td>
<td>31.00 ± 1.78e</td>
<td>6.18 ± 0.20a</td>
</tr>
<tr>
<td>1.6:0.0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>1.6:0.25</td>
<td>43.99 ± 1.09d</td>
<td>–</td>
</tr>
<tr>
<td>1.6:0.50</td>
<td>35.99 ± 0.66e</td>
<td>–</td>
</tr>
<tr>
<td>1.6:0.75</td>
<td>32.90 ± 1.81e</td>
<td>–</td>
</tr>
<tr>
<td>1.6:1</td>
<td>31.00 ± 2.18e</td>
<td>–</td>
</tr>
</tbody>
</table>

Results followed by the columns with various letters are significantly different at P < 0.05 according to the Duncan test. Results are represented as means ± SD.
by decrease in zeta potential was also reported after loading carvacrol (Keawchaoon & Yoksan, 2011), eugenol (Woranuch & Yoksan, 2013), ellagic acid (Arulmozhi et al., 2013) and lime EO (Sotelo-Boyás et al., 2017) into ChNPs. As seen in the Table 1, with increasing concentration of CEO, both average size and zeta potential of CEO-loaded particles decreased significantly (P < 0.05). The reduction in average size could be a result of greater packing of polymer chains, due to the high number of amino groups in Ch, responsible for the interaction with the drug (Russo et al., 2014). The reduction in both average size and zeta potential of particles with increase in the concentration of CEO, was in accordance with previous studies conducted on the loading of NPs with various concentrations of ascorbyl palmitate (Yoksan et al., 2010), foscarnet (Russo et al., 2014) and summer savory EO (Feyzioglu & Tornuk, 2016).

Fig. 2. DLS graphs and FE-SEM images; DLS graphs for size distribution of optimum (A) unloaded and (B) oil-loaded ChNPs. DLS graphs for zeta potential of optimum (C) unloaded and (D) oil-loaded ChNPs. FE-SEM images for optimum (E) unloaded and (F) oil-loaded ChNPs.
3.3. Encapsulation efficiency (EE), loading capacity (LC) and yield determination

The percentage of encapsulation efficiency (EE) for various concentrations of CEO in prepared CEO-loaded particles, with initial mass ratios of Ch to TPP both of 1:1 and 1.6:1, were calculated by UV-vis spectrophotometry at 282 nm (Table 2). The data revealed that in spite of different average size, no significant difference (P < 0.05) existed between EE values of CEO. CEO-loaded particles which was prepared using both mass ratios, showed a similar trend of decrease in EE as a result of increase in initial CEO concentration (Table 2). Considering no significant difference (P < 0.05) in EE values, the percentages of particle yield and loading capacity (LC), only for lower size CEO-loaded particles prepared using initial mass ratio of Ch to TPP of 1:1, measured by weight determination of freeze-dried particles, are presented in Table 2. As seen in the Table, EE of CEO decreased significantly (P < 0.05) from 45.8 ± 1.25 to 31.0 ± 1.78 upon increase in initial CEO concentration so that the maximum percentage of EE was obtained for the sample prepared using a minimum concentration of CEO. The limitation in encapsulation for a higher initial concentration of CEO might be related to the saturation of CEO loaded into ChNPs (Yoksan et al., 2010). As seen in Table 2, yield percentage also tended to decrease with loading of EO into ChNPs, as well as an increase in EO concentration. A similar trend was also found from the study on loading of carvacrol (Keawchaoon & Yoksan, 2011) and foscarnet (Russo et al., 2014) by ChNPs. Contrary to EE, percentage of LC increased significantly (P < 0.05) from 3.36 ± 0.13 to 6.18 ± 0.20 with an increase in initial concentration of CEO (Table 2). Decrease in EE and increase in LC are in agreement with previous studies on loading of various compounds by ChNPs such as bovine serum albumin (Xu & Du, 2003), aspirin and probucol, in combination (Ajun, Yan, Li, & Huili, 2009), ascorbyl palmitate (Yoksan et al., 2010), oregano EO (Hosseini et al., 2013) and strawberry polyphenols (Pulicharla, Marques, Das, Rouissi, & Brar, 2016).

Fig. 3. In vitro release profiles of CEO from prepared CEO-ChNPs with initial mass ratios of (A) Ch to TPP to CEO, 1:1:1 and (B) Ch to TPP to CEO, 1:1:0.25 in two citrate buffer solutions with pH 3 and 5. In each treatment, vertical bars refer to standard deviation of three replicates.
3.4. Selection of optimum NPs

The lowest size CEO-ChNPs with appropriate zeta potential and high amount of entrapped oil, prepared using initial mass ratio of Ch to TPP to CEO of 1:1:1, were selected as optimum oil-loaded ChNPs. The optimum unloaded ChNPs were also prepared using an initial mass ratio of Ch to TPP of 1:1. The size distribution and zeta potential of optimum unloaded and oil-loaded ChNPs, measured by DLS technique, is shown in Fig. 2(A)–(D). In the lowest size CEO-ChNPs, the high amount of entrapped oil could increase the possible interaction between EO and amine groups of Ch and result in greater packing of the polymer chains and less availability of free amine groups on the surface of NPs and subsequently less crosslinking density (Woranuch & Yoksan, 2013; Russo et al., 2014; Sotelo-Boyás et al., 2017). Moreover, the lowest size CEO-ChNPs, with the high amount of entrapped oil and subsequently less crosslinking density, could result in higher burst and diffusion release of oil due to greater surface-to-volume ratio, resulting in greater release of the oil adsorbed on the surface of NPs, as well as greater gradient of concentration, resulting in the diffusion in high amount of oil loaded near the surface of NPs due to better particle swelling and high dissolution rate of polymer matrix near the surface of NPs (Anitha et al., 2011; Keawchoaon & Yoksan, 2011; Hosseini et al., 2013). Therefore, the lowest size CEO-ChNPs with greater surface-to-volume ratio, high amount of entrapped oil, less crosslinking density, higher burst and diffusion release of oil and consequently higher antifungal activity were selected for further characterization.

3.5. Fourier transform infrared (FTIR) characterization

Chemical structure of Ch powder, pure CEO and optimum CEO-ChNPs was characterized by FTIR technique as shown in Fig. 1. The spectrum of Ch showed several peaks at 3450–3245 cm\(^{-1}\) due to O–H and N–H stretching, 2866 cm\(^{-1}\) due to C–H stretching, 1731 cm\(^{-1}\) due to C=O stretching of amide I, 1585 cm\(^{-1}\) due to N–H bending of amide II, 1374 cm\(^{-1}\) due to C–N stretching, 1153 cm\(^{-1}\) due to \(\delta(–(1–4))\) glycosidic linkage, 1065 cm\(^{-1}\) due to C–O–C stretching of glucose ring, 1026 cm\(^{-1}\) due to C–O stretching and 896 cm\(^{-1}\) due to the vibration of the pyranose ring (Fig. 1(A)) (Yoksan et al., 2010; Woranuch & Yoksan, 2013). The spectrum of pure CEO showed high numbers of peaks indicating the existence of different volatile compounds and characterized by several peaks at 3524 cm\(^{-1}\) due to O–H stretching, 2923–2841 cm\(^{-1}\) due to C–H stretching, 1607, 1511 and 1430 cm\(^{-1}\) due to C=C–C stretching of aromatic ring, 1265 and 1033 cm\(^{-1}\) due to C–O vibration, 915 cm\(^{-1}\) due to O–H bending, also 850 and 793 cm\(^{-1}\) due to C=H bending of aromatic ring (Fig. 1(B)) (Woranuch & Yoksan, 2013; Feyzioglu & Tornuk, 2016; Sotelo-Boyás et al., 2017). In the case of NPs, compared with the spectrum of Ch (Fig. 1(A)), the peak at 3450–3245 cm\(^{-1}\) became wider indicating the enhancement of hydrogen bond. Moreover, the N–H bending peak of amide II shifted from 1585 to 1571 cm\(^{-1}\) and new peaks were monitored at 1088 and 1251 cm\(^{-1}\) due to the stretching vibration of PO\(_2\) groups and P=O, respectively. These changes might imply the complex formation through electrostatic interaction between ammonium groups of Ch and phosphoric groups of TPP (Fig. 1(C)) (Yoksan et al., 2010). In the spectrum of NPs, one peak at 1731 cm\(^{-1}\) related to the spectrum of Ch, and also several peaks, at 1511, 1265, 1033, 915 and 850 cm\(^{-1}\), related to the spectrum of CEO, were also monitored. The presence of these peaks might indicate the presence of CEO in the Ch matrix. In addition, compared with the spectrum of Ch (Fig. 1(A)), the incorporation of CEO resulted in a significant increase in the intensity of the peaks at 2923–2866 cm\(^{-1}\) due to C–H stretching and 1418 cm\(^{-1}\) due to C=C–C stretching vibration of aromatic ring. The increased intensity of these peaks in the spectrum of NPs might be a result of possible interaction between CEO and Ch matrix (Fig. 1(C)) (Keawchoaon & Yoksan, 2011; Hosseini et al., 2013; Feyzioglu & Tornuk, 2016; Sotelo-Boyás et al., 2017).

3.6. Field emission-scanning electron microscopy (FE-SEM) observation

The morphology and size distribution of optimum unloaded and oil-loaded ChNPs were observed by FE-SEM (Fig. 2(E) and (F)). The obtained images showed spherical shapes with smooth surfaces, as well as the stability of NPs during the steps of the preparation process. The size distribution obtained by FE-SEM indicated that ChNPs were smaller than CEO-ChNPs, possibly due to the loading of CEO into ChNPs (Fig. 2(E) and (F)). As mentioned in Table 1, the size distributions measured by DLS technique for the ChNPs and CEO-ChNPs, prepared using initial mass ratio of Ch to TPP of 1:1 and Ch to TPP to CEO of 1:1:1, were 129.83 and 268.47 nm, respectively, while the size distributions measured by FE-SEM (true radius of the particles) were around 40 and 100 nm for the most optimum unloaded and oil-loaded ChNPs, respectively; therefore the nano-size range was also examined by FE-SEM images (Fig. 2(E) and (F)). The lower size distribution measured by FE-SEM might be related to the dehydration of NPs during the preparation process while using DLS technique, the hydrodynamic diameter of NPs in aqueous dispersion was measured; thus the higher size distribution measured by the DLS technique might be due to self-aggregation among individual NPs and/or swelling of the Ch layer, surrounding single NPs (Keawchoaon & Yoksan, 2011; Rahaiee et al., 2015). These findings were in accordance with the results previously reported by Woranuch and Yoksan (2013) and Hosseini et al. (2013).

3.7. In vitro release studies

In vitro release profiles of CEO from CEO-ChNPs, prepared using different initial mass ratios of Ch to TPP to CEO of 1:1:0.25 and 1:1:1, were investigated spectrophotometrically at 282 nm for 56 days in two citrate buffer solutions with pH of 3 and 5 (Fig. 3). In vitro release studies were determined to confirm the success of CEO encapsulation, investigate the release mechanism of CEO from NPs and to determine the optimum pH for releasing of CEO, which was necessary for further application of NPs as a fungicide. Initial rapid release was observed during the first 10 days in both NPs with different CEO concentrations (Fig. 3). The amount of oil released was near 80% and more than 50% in citrate buffer solutions with pH 3 and 5, respectively for CEO-ChNPs with initial mass ratio of Ch to TPP to CEO of 1:1:1 (Fig. 3(A)). This amount for CEO-ChNPs with initial mass ratio of Ch to TPP to CEO of 1:1:0.25 was near 30% and more than 20% in citrate buffer solutions with pH 3 and 5, respectively (Fig. 3(B)). The mechanism of rapid oil release at this stage might be related to the diffusion of CEO molecules adsorbed on the surface of NPs, and also the diffusion of high amounts of oil loaded near the surface of NPs due to the high dissolution rate of polymer matrix near the surface (Anitha et al., 2011). In the next stage, from day 10 to 30, in comparison with initial rapid release, the release of CEO dramatically decreased and eventually from day 30 to 56 it reached a plateau in both NPs, with different CEO concentrations (Fig. 3). The decrease in oil release at this stage might be a result of reduction in diffusion or concentration gradient of CEO between NPs and media (Keawchoaon & Yoksan, 2011) and the subsequent release near zero might be due to the inability of buffer solutions to break the compact structures of NPs (Agnihotri, Mallikarjuna, & Aminabhavi, 2004). The final amount of CEO released from low size or optimum NPs containing high oil concentration (initial mass ratio of Ch to TPP to CEO of 1:1:1) after 56 days was more than 80% and about 58% in citrate buffer solutions with pH 3 and 5, respectively (Fig. 3(A)). This amount for high size NPs containing low oil concentration (initial mass ratio of Ch to TPP to CEO of 1:1:0.25) was more than 30% and about 25% in citrate buffer solutions with pH 3 and 5, respectively (Fig. 3(B)). Therefore, small or optimum NPs with high oil concentration in high acidic medium exhibited the highest release amount of CEO which was up to 80% (Fig. 3(A)). The results revealed that the amount of CEO released might be affected by oil concentration, particle size and pH of medium. As earlier mentioned, higher amount of oil could result in a greater gradient of concentration and hence higher release (Keawchoaon & Yoksan, 2011).
Furthermore, lower size of NPs could result in greater surface-to-volume ratio and subsequently greater release of oil adsorbed on the surface of NPs (Hosseini et al., 2013). In addition, higher acidic medium could result in better swelling and partial dissolution of hydrophilic NPs, caused by ionic repulsion of protonated free amino groups on one Ch chain with neighboring chains and subsequently higher diffusion of entrapped oil, compared to lower acidic medium (Zhang, Mardyani, Chan, & Kumacheva, 2006). The data show that CEO-ChNPs could be suitable for controlled release of CEO to create a gradual antifungal effect for a long time. Initial rapid release (and later sustained release) was also reported for ascorbyl palmitate (Yoksan et al., 2010), carvacrol (Reawchaoon & Yoksan, 2011), oregano EO (Hosseini et al., 2013) and strawberry polyphenols (Pulicharla et al., 2016).

3.8. Molecular identification of A. niger isolated from spoiled pomegranate

The edited nucleotide sequence of ITS-rDNA region of the isolated fungus, associated with black infected pomegranate fruit, was 99% similar to the reference sequences of A. niger present in NCBI (https://www.ncbi.nlm.nih.gov/). The fungal isolate was used for in vitro antifungal assays of NPs and deposited in the GenBank under accession No. MF540907.

3.9. Effect of Ch, CEO and prepared particles on inhibition of A. niger growth

The percentage of antifungal index for Ch, free CEO, ChNPs, obtained using initial mass ratio of Ch to TPP of 1:1 and CEO-loaded
of growth significantly (P < 0.05) decreased (Fig. 4(A)). For the other tested compounds, between 0.187 and 1.5 mg/ml, the mycelial growth of A. niger decreased significantly (P < 0.05); however, between 1.5 and 3 mg/ml, no significant decrease (P < 0.05) was observed in fungal mycelial growth under in vitro conditions (Fig. 4(A)). The results showed the antifungal activity to be as follows: CEO-loaded particles > ChNPs > CEO > Ch (Fig. 4(A)). As seen in the Figure, in vitro antifungal activity against A. niger showed that, despite the different average sizes of CEO-loaded particles, prepared using two mass ratios of Ch to TPP, there was no significant difference (P < 0.05) between the percentages of their antifungal indices, probably due to their similar values of EE (%). Moreover, the results showed that the mycelial growth of A. niger could not be completely inhibited by free CEO, even at concentrations as high as 3 mg/ml, while, after loading of oil into ChNPs, the encapsulated CEO could completely inhibit the fungal growth at 1.5 mg/ml (Fig. 4(A)). The use of high concentrations of EO for inhibiting microbial decay in food products can severely affect their taste; however, the encapsulation can minimize the alteration of organoleptic properties by lowering the used concentration (Hsieh, Mau, & Huang, 2001; Doni, Amunziani, Sessa, & Ferrari, 2011). Overall, CEO-ChNPs showed greater inhibitory activity than did free CEO against A. niger (Fig. 4(A)). The superior performance of CEO-ChNPs over free CEO, might be due to the controlled release of the preserved volatile oil from ChNPs during the experiment, leading to better inhibitory effect, as well as the inhibitory effect of ChNPs itself (Beyki et al., 2014). Previous studies on nano-encapsulation of Mentha piperita EO and foscarnet also resulted in the superior performance of these encapsulated antifungal and antiviral compounds against A. flavus and human immunodeficiency virus type 1 (HIV-1) at lower concentrations (Beyki et al., 2014; Russo et al., 2014).

4. Conclusions

CEO-ChNPs were synthesized by a two-step process, including formation of an oil-in-water emulsion and ionic gelation of emulsion droplets. Interaction of CEO with ChNPs was confirmed by FTIR spectroscopy. The average size, surface charge, encapsulation efficiency, loading capacity and yield percentage of CEO-ChNPs could be controlled by mass ratios of Ch to CEO. ChNPs were also synthesized by the ionic gelation technique to investigate the effect of Ch to TPP mass ratios on the average size and surface charge of NPs. FE-SEM images, obtained from most of the optimum ChNPs and CEO-ChNPs, showed spherical shapes and size ranges of around 40 and 100 nm, respectively. Although the amount of CEO released from lower size or optimum CEO-ChNPs with higher concentration of CEO was relatively high in both buffer solutions (pH 3 and 5), more acidic media revealed faster release. A. niger associated with spoiled pomegranate, was isolated and identified by molecular characterization, and then deposited in the GenBank. The encapsulated EO revealed superior performance, compared with free EO, against the identified A. niger isolate under in vitro conditions, probably due to the controlled release of the preserved volatile oil from ChNPs during the experiment, leading to better inhibitory effect, as well as the inhibitory effect of ChNPs itself. Considering the impressive antifungal activity of CEO-ChNPs, the application of these NPs as a natural fungicide to extend the shelf life of fresh-cut fruits and vegetables is proposed.
Biodeterioration & Biodegradation, 70, 82–88.