Innovative Food Science and Emerging Technologies 28 (2015) 73–80

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Nanoencapsulation of *Zataria multiflora* essential oil preparation and characterization with enhanced antifungal activity for controlling *Botrytis cinerea*, the causal agent of gray mould disease

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ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 21 December 2014
Accepted 27 December 2014
Available online 8 January 2015

Keywords:
Zataria multiflora
Essential oil
Chitosan nanoparticles
Encapsulation
Antifungal activity

ABSTRACT

This study was undertaken to investigate the nanoencapsulation of *Zataria multiflora* essential oil (ZEO) in chitosan nanoparticles (CSNPs) in order to enhance antifungal activity and stability of the oils against one isolate of *Botrytis cinerea* Pers., the causal agent of gray mould disease. ZEO was encapsulated by an ionic gelation technique into CSNPs with an average size of 125–175 nm as observed by transmission electron microscopy (TEM). From UV-vis spectrophotometry results, the drug encapsulation and loading efficiency of ZEO decreased from 45.24% to 3.26% and from 9.05% to 5.22%, respectively, upon increasing initial ZEO content from 0.25 to 1 g/g chitosan. *In vitro* release studies also demonstrated a controlled and sustained release of ZEO for 40 days. The superior performance of ZEO when encapsulated by CSNPs under both *in vitro* and *in vivo* conditions in comparison with unmodified ZEO against *B. cinerea* was revealed. The *in vivo* experiment also showed that the encapsulated oils at 1500 ppm concentration significantly decreased both disease severity and incidence of *Botrytis*-inoculated strawberries during 7 days of storage at 4 °C followed by 2–3 more days at 20 °C. These findings revealed the promising role of CSNPs as a controlled release system for EOs in order to enhance antifungal activities.

Industrial relevance: Application of plant essential oil (EOs) treatment at pre- or postharvest stage has been considered as an alternative treatment to the use of synthetic fungicides to prevent fruit postharvest decay and to extend the storage life while retaining the overall quality of different fresh commodities. Although EOs have proved to be good antimicrobial agents, their use for maintaining fruit quality and reducing fungal decay is often limited due to their volatile compounds which can easily suffer degradation under the action of heat, pressure, light and oxygen. Furthermore, they are insoluble in water, and for certain applications a controlled release is required. In this regard, nano-size carriers provide more surface area and can possibly upgrade solubility, enhance bioavailability and improve controlled release and targeting of the encapsulated food ingredients, in comparison to micro-size carriers. These findings revealed the promising role of CSNPs as a controlled release system for EOs in order to enhance their antimicrobial activities.

1. Introduction

The pathogen *Botrytis cinerea* Pers. Fr. (known as "gray mould fungus") causes serious losses in more than 200 plant species worldwide (Jarvis, 1977), including important crops and harvested commodities, such as grapevine, tomato, strawberry, raspberry, blackberry, cucumber, cut flowers and ornamental plants (Droby & Lichter, 2007). With increasing international trade in cold-stored produce, this fungus has attained great importance because it can grow effectively over long periods at just above freezing temperatures in products such as kiwifruit, apples, pears and strawberries (Williamson, Tudzynski, Tudzynski, & van Kan, 2007). The synthetic fungicides provide the primary means for controlling postharvest decay of fruit (Soylu, Kurt, & Soylu, 2010). Although the synthetic fungicides are effective, their continued or repeated application has significant drawbacks, including cost, handling hazards, contamination of fruits and vegetables with fungicide residues, and threats to human health and the environment (Paster & Bullerman, 1988). These adverse effects have led to intensified worldwide research efforts to develop safe and biodegradable alternatives as natural fungicides to replace synthetic chemicals (Costa et al., 2000).

The application of plant essential oil (EOs) treatment at pre- or postharvest stage has been considered as an alternative treatment to the use of synthetic fungicides to prevent fruit postharvest decay and to extend the storage life while retaining the overall quality of different fresh

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http://dx.doi.org/10.1016/j.ifset.2014.12.011
1466-8564/

commodities (Aloui et al., 2014). Among a wide variety of EOs, *Zataria multiflora* Boiss EOs (ZEO) appear as promising natural compounds for controlling postharvest decay in fruits. Quantitatively, the most abundant components in hydro-distilled ZEO are oxygenated monoterpenes (approximately 70%) followed by monoterpene hydrocarbons, sesquiterpenes hydrocarbons and oxygenated sesquiterpenes (Sajed, Sahebkar, & Iranshahi, 2013). They have been proven to have antifungal properties against several postharvest phytopathogens, including *B. cinerea* and species of *Aspergillus*, *Rhizopus* and *Penicillium* (Abdollahi, Hamzehzarghani, & Saharkhiz, 2011; Mohammadifar et al., 2012).

Although EOs have proved to be good antimicrobial agents, their use for maintaining fruit quality and reducing fungal decay is often limited due to their volatile compounds which can easily suffer degradation under the action of heat, pressure, light and oxygen. Furthermore, they are insoluble in water, and for certain applications a controlled release is required (Martín, Varona, Navarrete, & Cocero, 2010). Hence, controlled or sustained delivery is crucial to obtain maximum benefits of using EOs as antimicrobial agents. Nano-/micro-encapsulation technology of these compounds can be a practical and efficient approach to solve some of these problems such as the physical instability and enhance their bioactivity, while at the same time, controlling fruit postharvest decay, by lowering the diffusion processes and maintaining high concentrations of active molecules on the surface of the fruit (Aloui et al., 2014). In this regard, nano-size carriers provide more surface area and can possibly upgrade solubility, enhance bioavailability and improve controlled release and targeting of the encapsulated food ingredients, in comparison to micro-size carriers (Mozafari et al., 2006).

Recently, chitosan (CS) has attracted a great attention in the encapsulation of bioactive compounds because of its general recognition as safe (GRAS) and its advantageous biological properties, such as biodegradability, biocompatibility and nontoxicity, as well as ability to form films, membranes, gels, beads, fibers and particles (Keawchaoon & Yoksan, 2011). Several studies on antimicrobial activities of CS alone or in combination with other natural agents have been recently carried out (Ajun, Yan, Li, & Huili, 2009; Aloui et al., 2014). In particular, in our previous study, CS in combination with ZEO showed strong *in vitro* and *in vivo* antimicrobial activity against some pathogenic bacteria and fungi (under publication). The CS nanoparticles (CSNPs) has shown its capacity for the loading and delivery of sensitive bioactive compounds such as lipophilic drugs (Ajun et al., 2009), polyphenolic compounds (Keawchaoon & Yoksan, 2011), proteins (Avadi et al., 2010), genes (Csaba, Köping-Höggård, & Alonso, 2009) and vitamins (Luo, Zhang, Whent, Yu, & Wang, 2011). However, to our knowledge, preparations of ZEO combined in CSNPs with *in vitro* release have not been studied. The present study was set to encapsulate hydrophobic ZEO in CSNPs using ionic gelation technique in order to enhance antifungal activity and stability of the oils against *B. cinerea*.

2. Materials and methods

2.1. Materials

Chitosan derived from crab shell with a molecular weight of 684 kDa and deacetylation degree of ~85% was purchased from Sigma (Germany). ZEO (98%) was purchased from Magnolia Co (IRAN). Pentasodium triphosphate (TPP), acetic acid glacial and sodium hydroxide were purchased from Merck (Germany). All chemicals used were of analytical grade and used as received. *B. cinerea* (IRAN 1304C) was obtained from the Iranian Research Institute of Plant Protection (IRIPP).

2.2. Preparation of ZEO-loaded CSNPs

ZEO-loaded CSNPs (ZEO@CSNPs) were prepared by an ionic gelation technique as described by Keawchaoon and Yoksan (2011) with slight variation. Briefly, CS (0.3% w/v, 50 mL) was dispersed in an aqueous solution of glacial acetic acid (1%, v/v) at ambient temperature overnight, followed by ultrasonication (MISONIX Inc. S-4000, USA) for 4 min at 60 W. ZEO was gradually dropped into the stirring mixture of CS solution for 30 min to obtain an oil-in-water emulsion. Various contents of ZEO, i.e., 0, 375, 750, 1500 and 3000 ppm, were used to obtain different weight ratios of CS to ZEO of 1:0, 1:0.25, 1:0.50, 1:0.75 and 1:1.00, respectively. A TPP solution (0.3% w/v, 20 mL) was prepared in water and its pH was adjusted to 5.6 using 1 N NaOH. ZEO@CSNPs were spontaneously obtained by the addition drop-wise of TPP solution into an o/w emulsion under constant stirring at room temperature for 60 min. The final pH of mixture solution was ~5.0. The resultant suspensions were subjected to particle-size analysis. Further, the formed particles were collected by centrifugation (Tomy Kongyo Co. LTD. Suprema 25, JAPAN) at 27000×*g* for 14 min at 25 °C and washed with distilled water to remove free ZEO. The obtained particles were dispersed in distilled water and kept at 4 °C.

2.3. Characterization of ZEO-loaded CSNPs

Morphological characteristics of CSNPs and ZEO@CSNPs were examined by high-resolution transmission electron microscope (TEM) (Hitachi, H-600) at an accelerating voltage of 100 kV. Samples were immobilized on copper grids. They were dried under reduced pressure at an ambient temperature overnight prior to TEM observation and then were examined using a TEM without being stained. Particle size was determined by dynamic light scattering (DLS) using a photon correlation spectroscopy (PCS) assembly (Zetasizer 3000 HS, Malvern Instruments, UK). Zeta potential was measured by laser Doppler anemometry on the same instrument. Results are reported as the mean of three measurements ± SD (standard deviation). UV-vis absorption spectra were recorded over a wavelength ranging from 220 to 400 nm by a TECAN spectrometer (infinite M200 Pro, Switzerland).

2.4. Determination of encapsulation efficiency and loading capacity

The percentage of ZEO encapsulated was determined after lysis of the prepared NPs with hydrochloric acid solution and alcohol, as described previously (Keawchaoon & Yoksan, 2011). The concentration of ZEO in absolute alcohol was determined spectrophotometrically at 230 nm (maximum absorption wavelength of ZEO) using a UV-visible spectrophotometer in triplicate. Encapsulation efficiency (EE) was calculated through the following relationship:

$$\%\mathrm{EE} = \frac{\text{Mass of loaded ZEO}}{\text{Mass of initial ZEO}} \times 100 \quad (1)$$

Loading capacity (LC) was articulated as

$$\%\mathrm{LC} = \frac{\text{Mass of loaded ZEO}}{\text{Mass of sample}} \times 100 \quad (2)$$

2.5. Nanoparticle yield determination

Each nanoparticle sample was centrifuged as described in Section 2.2, and the residue was lyophilized. Nanoparticle yield was calculated from the weight of the lyophilized nanoparticles (W_1) and the sum of the initial dry weight of starting materials (W_2) as using the following formula:

$$\text{Percentage yield}(\%) = \frac{W_1}{W_2} \times 100 \quad (3)$$

Results are reported as the mean of three measurements ± SD.