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Microencapsulation of *Gaultheria procumbens* essential oil using chitosan-cinnamic acid microgel: Improvement of antimicrobial activity, stability and mode of action

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ABSTRACT

The present study was undertaken to investigate the efficacy of chitosan-cinnamic acid based microencapsulated *Gaultheria procumbens* L. essential oil (GPEO) against *Aspergillus flavus* (EC-03), aflatoxin B_1 secretion and its mode of action. Scanning electron microscopy (SEM) and particle size analyzer analysis revealed that the microencapsulated GPEO exhibited an even spherical shape and particle size ranged between 7.00 and 90.0 μm. During chemical characterization (GC-MS), methyl salicylate (96.25%) was identified as the major component of GPEO. Microencapsulated GPEO exhibited strong antifungal and aflatoxin B_1 suppressor activity than the uncapsulated GPEO and completely inhibited growth and toxin production at 1.00 μL/mL. The mode of action of microencapsulated GPEO was elucidated targeting ergosterol content in the cell membrane, the release of cellular ion contents and morphological alteration in *A. flavus*. The results demonstrate the potential of chitosan-based encapsulating material for the improvement of the antimicrobial efficacy as well as stability of GPEO.

1. Introduction

Nowadays, the application of microencapsulation technology has received increased attention of the food industries as a tool to protect the unpleasant tastes and odours of essential oils based food preservatives with controlled vapour release and enhanced efficacy. A large number of natural as well as synthetic molecules viz., polysaccharides (starch, cellulose and derivatives, chitin, chitosan, dextran and alginate), proteins (casein, albumin and gelatine), and synthetic polymers (polyvinyl alcohol, polylactate, polyglycolate, polyesters and polyamines) have already been explored for their efficiency as encapsulating materials in drug delivery (Kaur & Kaur, 2014; Nitta & Numata, 2013). Among all, chitosan obtained by alkaline deacetylation of chitin has recently gained momentum of interest by the food industries as a coating material because of its biodegradable, biocompatible, low mammalian toxicity, mucoadhesive and better film forming properties (Cota-Arriola, Onofre Cortez-Rocha, Burgos-Hernandez, Marina Ezquerra-Brauer, & Plascencia-Jatomea, 2013). Chitosan-based nanoparticles are currently being used as nanocarriers of anti-cancerous drugs and therapeutic enzyme carriers (Khalili et al., 2015; Sotelo-Boyas, Correa-Pacheco, Bautista-Banos, & Corona-Rangel, 2017; Zamora-Mora et al., 2017). Further, chitin is the second most abundant polymer after cellulose. Therefore, the production of chitosan is economically viable for large scale application (Zamora-Mora et al., 2017).

Aspergillus flavus is one of the cosmopolitan moulds, causing significant deterioration of food grains and their shelved products. Its toxic metabolites aflatoxins have attracted the attention of food industries *in view* of their genotoxic, teratogenic and carcinogenic potential causing high human and animal health risk (International Agency for Research on Cancer (IARC), 1993; Prakash, Singh, Kedia, Singh, & Dubey, 2012). Currently, a large number of synthetic preservatives have been widely used to control the mold and mycotoxin contamination in food items. In view of the recent consumer awareness towards green consumerism, the use are synthetic preservatives are discouraged. Therefore, currently, food industries have paid more interest in natural food preservatives to reduce or eliminate moulds growth and aflatoxin contamination.

In the past few decades, essential oils of traditionally used aromatic plants and their bioactive compounds have been extensively studied as promising alternatives of synthetic preservatives.

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Owing to their inherent biological activity, favourable safety profile, ephemeral and biodegradable nature most of the essential oils are kept in generally recognised as safe (GRAS) categories by U.S., FDA (Prakash & Kiran, 2016; U.S. Code of Federal Regulations, 2016). Although, plant essential oils exhibit promising preservative potential they have some technological drawbacks such as high volatility, reactivity, poor water solubility, uneven dispersal, unstability and undesirable effects on organoleptic taste of food system (Prakash & Kiran, 2016). In addition, EOs are also sensitive to ambient oxygen, temperature and light which may change their antimicrobial activity. Hence, these limitations need to be overcomed for their wider application in the food system. In this context, the use of microencapsulation technology has proved its significance to transform the academic research output to the industrial application in the real food system with enhanced bioefficacy and stability (Cota-Arriola et al., 2013; Prakash & Kiran, 2016; Sao Pedro, Cabral-Albuquerque, Ferreira, & Sarmento, 2009).

Gaultheria procumbens L. commonly known as wintergreen oil is an aromatic plant of family Ericaceae. Its application as an insecticidal, antimicrobial, antileishmanial, antioxidant and antidiabetic agent has been explored previously (Kiran & Prakash, 2015). Therefore, in the present investigation, it has been thought to desirable to develop chitosan-cinnamic acid based microencapsulation of *Gaultheria procumbens* L. essential oil (GPEO) and its assessment against *Aspergillus flavus* (EC-03) and aflatoxin B_1 secretion. In addition, the study also elucidates its detailed mode of action targeting the ultrastructure changes, ergosterol content and imbalance of cellular ion contents in *A. flavus* exposed to microencapsulated GPEO.

2. Material and methods

2.1. Chemicals and equipment

The chemicals and reagents used in the study were procured from Sigma Chemical Co. (St. Louis, MO, USA), Hi-media, and Sisco Research Lab (Mumbai, India). The low molecular weight of chitosan, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and cinnamic acid were procured from Sigma (Germany). Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were purchased from Hi-media (India). The gas chromatograph-mass spectrometer was from PerkinElmer (Turbomass Gold, USA) and the spectrophotometer from Shimadzu (UV-1800).

2.2. Extraction of essential oil and its characterization

The leaves of *G. procumbens* EO were subjected to hydro-distillation to obtain the EO. The leaves were thoroughly washed in double distilled water (three times) to avoid the contaminants and then subjected to hydro-distillation (3 h) in a Clevenger's apparatus (Prakash et al., 2012). The inherent chemical profile of EO was elucidated by GC-MS analysis (PerkinElmer equipped with a Turbo mass Gold mass spectrometer). The separation was carried out under the following conditions using PerkinElmer Elite-5 column (column length = 30 m, inner diameter = 0.25 mm, film thickness = 0.25 mm), Oven: Initial temp 70 °C for 2 min, ramp 3 °C/min to 250 °C, hold 10 min, Inj = 250 °C, Split = 20:1, Carrier Gas = He, Solvent Delay = 4.00 min, Transfer Temp = 180 °C, Source Temp = 160 °C, Scan: 40–400Da. The injection volume of 1 μl (1:100 dilute in acetone). The identification of eluted compounds was based on their retention indices comparison of relative to n-alkanes (C_8-C_{22}) analyzed under the same condition. Further, their identity was confirmed by matching of their mass spectral pattern available with Wiley, NIST and NBS mass spectral libraries or with published data in the literature (Adams, 2007).

2.3. Preparation of the chitosan-cinnamic acid micro-gel and encapsulation of GPEO

The microencapsulation of *Gaultheria procumbens* essential oil using chitosan-cinnamic acid microgel was done following the method of Beyki et al., 2014 with slight modification. 0.5 g of low molecular weight of chitosan was dissolved in 1% aqueous acetic acid solution (100 mL). Thereafter, 85 mL of methanol was gently added to the solution under continuous stirring at 250 rpm for 20 min at room temperature (27±2 °C). The prepared stock solution was made acidic (pH 3.5–4.0) and sonicated for 5 min. Another stock was prepared by adding EDC (669 μL) to cinnamic powder (317.5 mg). Cinnamic-EDC solution was then added to 75 mL of the chitosan solution under continuous stirring (250 rpm) at room temperature and kept for 24 h. After incubation, the solution was adjusted to pH 8–9 by using sodium hydroxide to precipitate the microgel. The prepared microgel was centrifuged at 4618.45 g for 15 min to recover the precipitated microgel. The precipitate was washed with double distilled water and ethanol (three times) to remove the impurities. The obtained precipitate was dried under vacuum for 24 h and re-suspended by using acetic acid solution under magnetic stirring till pH (3.5–4.0). The resulting opalescent chitosan-cinnamic acid micro-gel was then filtered through a membrane filter (Whatman) and kept for further analysis. Thereafter, GPEO (5.0 μL/mL) was then added to the prepared emulsion (0.5% w/v) and sonicated at (70 Hz for 5 min) using a probe type sonicator.

2.4. Characterization of microencapsulated GPEO

2.4.1. Particle size and morphology of microencapsulated GPEO

Particle size and morphology of microencapsulated GPEO were observed following the method described by Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013 with slight modification. The microencapsulated GPEO was suspended in deionised water and kept in an ultrasonic water bath for 5 min. Average particle size of microencapsulated GPEO was characterised by laser particle size analyzer using deionised distilled water as the dispersion medium. Morphology of the microencapsulated GPEO was examined by scanning electron microscopy. The frozen microencapsulated GPEO was dissolved in deionised water and sonicated for 5 min. One drop of the solution was placed on a glass plate and dried at room temperature. The samples were subjected to gold coating and observed under a scanning electron microscope to study the morphology (LEO 435VP, UK) connected to video copy processor (Mitsubishi, Japan).

2.4.2. Determination of encapsulation efficiency and loading capacity

The content of GPEO-loaded in chitosan-cinnamic acid based encapsulation was determined by UV–vis spectrophotometer. Microencapsulated GPEO (50 mg) were dissolved in aqueous hydrochloric acid solution (2 M, 4 mL) boiled at 95 °C for 30 min. Thereafter, 2 mL hexane was added to the solution mixture and kept for centrifugation at 4618.45g for 5 min at 25 °C (Hosseini et al., 2013). The content of GPEO was observed by measuring the optical density of supernatant at 306 using UVvis spectrophotometer (Model 1650-PC, Shimadzu, Kyoto, Japan). Parallel to treatment a blank (solution mixture containing chitosan microgel without GPEO) was kept. The amount of GPEO was calculated by using calibration curve of free GPEO in hexane ($R^2 = 0.996$). Experiment was performed in triplicate

Encapsulation efficiency (EE) = Total amount of loaded GPEO/ Initial amount of GPEO × 100