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Nanoliposomal encapsulates of piperine-rich black pepper extract obtained by enzyme-assisted supercritical carbon dioxide extraction

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ABSTRACT

Piperine-rich black pepper extract obtained from α-amylase-assisted supercritical carbon dioxide extraction (at 300 bar, 60 °C, 135 min total extraction time and a flow rate of gaseous CO_2 of 2 L/min) was encapsulated as nanoliposomes for enhanced storage stability and to allow sustained release of piperine. Soya phosphatidylcholine: Tween 80:: 1:1.2 and 2% (w/w) concentration of black pepper extract contributed to an encapsulation efficiency of 78.6%. This nanoliposome (29.75 ± 0.84 nm) when compared to its counterpart formulated with pure piperine, exhibited similar size and morphology. *In vitro* release profiles of piperine followed Higuchi model of first order kinetics. However, the former possessed higher antioxidant potency (1.10 times) and better storage stability (2.4 times higher at 4 ± 1 °C and 7.8 times higher at 70 ± 2 °C) compared to the nanoliposome, formulated with pure piperine. Nanoliposomes of piperine-rich extract of black pepper holds promise as nutraceuticals in designer food applications.

1. Introduction

Black pepper has wide applications in food processing, pharmaceutical and cosmetic products (Ferreira and Meireles, 2002). The extract of black pepper and its pungent bioactive principle piperine, reportedly possess several physiological effects. We had successfully enhanced the yield and phytochemical potencies (antioxidant activity, total phenolic content, reducing power and anti-inflammatory activity) of piperine-rich extract employing α-amylase-assisted supercritical carbon dioxide (SC-CO_2) extraction of black pepper (Dutta and Bhattacharjee, 2015). These extracts have promising applications in food industries as natural antioxidants and also as food preservatives. Since piperine is highly photosensitive in solution (Ravindran and Kallupurackal, 2001), it could possibly undergo degradative changes owing to its high content in the extracts. Therefore, there is a necessity to protect these piperine-rich extracts from light, heat, oxygen and other environmental hazards for long term storage-stability.

Microencapsulation by spray drying is a popular method of rendering enhanced stability to native extracts as has been reported by several authors. We have reported on encapsulation of 1,8-cineole-rich SC-CO_2 extract of small cardamom using maltodextrin and gum arabic as wall materials (Dutta and Bhattacharjee, 2016). However, high pungency [80,000 Scoville heat unit, estimated in accordance with the method reported by Ranganna (1986)] of black pepper extract could possibly impede usage of its spray dried powder form in food and therapeutic applications. This prompted us to explore formulation of nanoliposomes using the piperine-rich SC-CO_2 extract to ensure sustained release of the bioactive and concomitantly minimizing its degradation during storage. Liposomes are closed, continuous, vesicular structures composed mainly of phospholipid bilayers that incorporate hydrophilic molecules inside the aqueous core and lipophilic molecules in their bilayer (Mozafari and Mortazavi, 2005; Riaz, 1996). They have promising applications in food industries for encapsulation and controlled release of food constituents besides enhancing the bioavailability, stability and shelf-life of its sensitive ingredients (Mozafari et al., 2008). Use of nanoliposomal vehicles for spiceuticals in food engineering is scarce.

The specific objectives of this work were: optimization of encapsulation parameters for formulation of stable nanoliposomes using enzyme-assisted SC-CO_2 extract of black pepper and standard piperine as core material; physicochemical characterization of both nanoliposomes; *in vitro* release study of piperine and storage studies for either nanoliposome.

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2. Materials and methods

2.1. Materials

Malabar Garbled black pepper was procured from Spices Board, Cochin, India. Standard piperine (97% pure), α-amylase from *Bacillus licheniformis* (lyophilized powder, 500–1500 units/mg protein, 93–100% SDS PAGE) and soya phosphatidylcholine were procured from Sigma, India; Silica gel 60 (F_{254}) coated Al plates, Tween 80 and Triton X-100 were procured from E-Merck, Mumbai, India; Dialysis membrane (cellulose membrane, molecular weight cut off 12,000 Da) was procured from Himedia, India. Food grade CO_2 was procured from BOC India Ltd., India. All chemicals used in this work were of AR grade.

2.2. Enzyme-assisted SC-CO_2 extraction of black pepper extract

A laboratory scale 'SCF Green Technology SPE-ED SFE 2' model (Applied Separations, Allentown, USA) was used for enzyme-assisted SC-CO_2 extraction of piperine-rich extract from black pepper. 20 g (batch size optimized by preliminary trials) of ground black pepper ($d_p = 0.42 \pm 0.02$ mm) was mixed with lyophilized α-amylase in optimized ratio (enzyme: black pepper powder:: 1: 5000) and subjected to SC-CO_2 extraction in accordance with the method reported by Dutta and Bhattacharjee (2015), at the optimized condition of 300 bar, 60 °C, 135 min (static time 120 min + dynamic time 15 min) and a flow rate of gaseous CO_2 of 2 L/min.

2.3. Estimation of piperine content of black pepper extract

Estimation of total piperine content of black pepper extract was conducted by high performance thin layer chromatography (HPTLC) (Camag Linomat 5, Camag, Switzerland) using standard piperine as reference, according to the method reported by Dutta and Bhattacharjee (2015). Piperine content of the extract was determined from the standard curve of piperine ($R_f = 0.27$). α-amylase-assisted SC-CO_2 extract of black pepper having yield of piperine as 1.36 ± 0.04 mg/g dry black pepper (Dutta and Bhattacharjee, 2015) was used for formulation of nanoliposomes.

2.4. Gas chromatography-mass spectrometry (GC-MS) analysis of black pepper extract

Black pepper extract was analyzed by GC-MS for identification of its chemical constituents. A Polaris Q Mass Spectrometer coupled with Trace GC Ultra Gas Chromatography and DB-5 MS fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) (Thermo Scientific, USA) was employed. The GC injector and MS transfer line temperatures were set at 230 °C and 270 °C, respectively and split less mode was selected. The oven temperature was programmed as follows: isothermal hold at 85 °C for 3 min, 85–200 °C at 2 °C/min and held at 200 °C for 1 min. It was then increased to 250 °C at 10 °C/min and held finally for 10 min. The carrier gas was He at a flow rate of 1 mL/min. The injected volume of extract was 1 μL. The ionization of the sample was performed in the EI mode (70 eV) and the acquisition mass range was set at 40–600 amu. Identification of components of the extract was based on its computer matching with the NIST (2007) library and Adams (2007).

2.5. Formulation of nanoliposomes

Nanoliposomes encapsulating black pepper extract were formulated by probe sonication method. Soya phosphatidylcholine (S) and Tween 80 (T) in different ratios (1:0.6, 1:0.9 and 1:1.2, w/w basis) were dissolved in 2 mL ethanol in screw-capped amber-colored glass vials to develop the lipid phase. Thereafter, black pepper extract was dissolved in the lipid phase. Optimization of lipid phase (S: T ratio) was conducted keeping the concentration of black pepper extract constant at 1% (w/w). Ethanol was evaporated from the solution by purging a gentle stream of nitrogen, to obtain a thin film of the lipid phase at the bottom surface of the vial. The dried lipid film was then rehydrated in 4 mL phosphate buffer saline (PBS, 0.01 M, pH 7.2) and vortexed for 10 min to obtain a multilamellar liposomal suspension. This suspension of liposomes was then subjected to probe sonication (diameter of probe: 0.5 mm; LabSonic M, Sartorius Stedium India Pvt. Ltd., Bangalore, India) at 30 kHz for 5 min (with intervals of 2 min) for 6 to 7 times, at 15–20 ± 1 °C, in accordance with the method reported by Memoli et al. (1995) to reduce the size and lamellarity and to finely disperse the liposomes. Concentration of black pepper extract for stable nanoliposomes was investigated at optimized S: T ratio with different concentrations of black pepper extract at 1%, 2%, 4% and 8%, w/w basis.

After ultrasonication, the nanoliposome suspension was subjected to dialysis using phosphate buffer (pH 7.0, 0.05 M) at 4 ± 1 °C, for 24 h, to separate the non-encapsulated black pepper extract from the nanoliposome. The dialysis bag (cellulose tube) containing 1 mL of liposome suspension was placed in 100 mL buffer solution at 4 ± 1 °C with continuous gentle stirring. The solution buffer was replaced by fresh buffer at predetermined time intervals (2, 4 and 6 h). After 24 h, the nanoliposome was recovered from the dialysis bag and stored in amber colored screw capped vials in an inert atmosphere of nitrogen at 4 °C in the dark, until further analyses.

The S: T ratio and concentration of black pepper extract at which clear and stable nanoliposome was obtained, was considered as the optimized formulation. Nanoliposomes were subsequently formulated using the same formulation, with the extract and standard piperine for comparative evaluation of the two formulations.

2.6. Optimization of encapsulation parameters

Optimization of encapsulation parameters, i.e. composition of lipid bilayer (S: T) and concentration of black pepper extract in the liposomal suspension was conducted primarily based on the stability of the nanoliposome (judged by visual observation). The stable liposomes were further analyzed for %EE and the liposome with highest %EE was considered as the 'best' nanoliposome (BN) in our study. Nanoliposome formulated with piperine (PN) and BN were further analyzed.

2.7. Estimation of encapsulation efficiency of nanoliposomes encapsulating black pepper extract

Estimation of %EE of nanoliposomes was conducted by quantification of piperine encapsulated in the nanoliposomes. Nanoliposomes recovered from dialysis bags were treated with Triton X-100 (10% v/v, 1:1:: Triton X-100:nanoliposome) to rupture the lipid bilayer of the nanoliposomes. Liposome solution containing Triton X-100 was analyzed by HPTLC to estimate the piperine content of the nanoliposomes. %EE of nanoliposomes was calculated in accordance with Pezeshky et al. (2016).

$$\%EE = \frac{\text{piperine encapsulated in nanoliposomes}}{\text{initial piperine supplied for encapsulation}} \times 100 \qquad (1)$$