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Abstract

In an attempt to improve the solubility and permeability of Standardized Bacopa Extract (SBE), a complexation approach based on phospholipid was employed. A solvent evaporation method was used to prepare the SBE-phospholipid complex (Bacopa Naturosomes, BN). The formulation and process variables were optimized using a central-composite design. The formation of BN was confirmed by photomicroscopy, Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), and Powder X-ray Diffraction (PXRD). The saturation solubility, the in-vitro dissolution, and the ex-vivo permeability studies were used for the functional evaluation of the prepared complex. BN exhibited a significantly higher aqueous solubility compared to the pure SBE (20-fold), or the physical mixture of SBE and the phospholipid (13-fold). Similarly, the in-vitro dissolution revealed a significantly higher efficiency of the prepared complex (BN) in releasing the SBE (> 97%) in comparison to the pure SBE (~ 42%), or the physical mixture (~ 47%). The ex-vivo permeation studies showed that the prepared BN significantly improved the permeation of SBE (> 90%), compared to the pure SBE (~ 21%), or the physical mixture (~ 24%). Drug-phospholipid complexation may thus be a promising strategy for solubility enhancement of bioactive phytoconstituents.

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The role of phospholipid as a solubility- and permeability-enhancing excipient for the improved delivery of the bioactive phytoconstituents of *Bacopa monnieri*

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Abstract

In an attempt to improve the solubility and permeability of Standardized Bacopa Extract (SBE), a complexation approach based on phospholipid was employed. A solvent evaporation method was used to prepare the SBE-phospholipid complex (Bacopa Naturosome, BN). The formulation and process variables were optimized using a central-composite design. The formation of BN was confirmed by photomicroscopy, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), and Powder X-ray Diffraction (PXRD). The saturation solubility, the *in-vitro* dissolution, and the *ex-vivo* permeability studies were used for the functional evaluation of the prepared complex. BN exhibited a significantly higher aqueous solubility compared to the pure SBE (20-fold), or the physical mixture of SBE and the phospholipid (13-fold). Similarly, the *in-vitro* dissolution revealed a significantly higher efficiency of the prepared complex (BN) in releasing the SBE (>97 %) in comparison to the pure SCE (~42 %), or the physical mixture (~47 %). The *ex-vivo* permeation studies showed that the prepared BN significantly improved the permeation of SBE (>90 %), compared to the pure SBE (~21 %), or the physical mixture (~24 %). Drug-phospholipid complexation may thus be a promising strategy for solubility enhancement of bioactive phytoconstituents.

Keywords

Excipients, Phytoconstituents, Complexation, Solubility, Permeability

Introduction

Recent years have seen a dramatic increase in the popularity of natural products in the management of several ailments (Sikarwar et al., 2008). Among other challenges, the poor bioavailability of the bioactive phytoconstituents is accepted as a major limitation to the use of natural products in mainstream pharmacotherapy. Attributes such as high molecular size, poor aqueous solubility, and lower plasma membrane permeability of pharmacologically active phytoconstituents are known to result in overall poor bioavailability of these components, thus limiting their pharmaceutical applications (Husch et al., 2013; Manach et al., 2004).

A major rate-limiting factor in the development of pharmaceutical drug products from the bioactive phytoconstituents is improving their solubility and permeability, thereby optimizing their bioavailability. Preparing drug-phospholipid complexes is among the several recently explored, and promising approaches aimed at improving the bioavailability of drugs. Several studies have reported an improvement in the solubility, permeability, and subsequently the systemic absorption and bioavailability of active phytoconstituents by forming their aggregates with phospholipid molecules (forming *Naturosomes*, *Phytosomes*, or *Herbosomes*) (Husch et al., 2013; Khan et al., 2013). The pharmacological profiles have also been shown to successfully improve by using drug-phospholipid complexation approaches (Freag et al., 2013; Kennedy et al., 2007; Mukherjee et al., 2011; Pathan and Bhandari, 2011; Zaidi et al., 2011). Additionally, fundamental advantages and significant breakthroughs for clinical use have been observed for several phytoconstituents by improving their oral availability, owing to the amphiphilic characteristics of *Naturosomes* (Fricker et al., 2010; Kidd, 2009). *Naturosomes* technology have been successfully used to deliver herbal extracts as well as phytochemicals with significant improvements in pharmacokinetic as well as in pharmacodynamic functionalities (Kennedy et al., 2007; Kidd and Head, 2005; Maiti et al., 2006; Maiti et al., 2009).

Bacopa monnieri Linn is a plant native to the Indian subcontinent, and has been described in ancient text as being used to sharpen intellect and attenuate several mental deficits (Aguiar and Borowski, 2013; Sairam et al., 2002). Current literature also reports its usefulness in the treatment of anxiety, improving cognitive functions, memory enhancement, neuroprotection, and hepato-protection (Calabrese et al., 2008; Janani et al., 2009; Rastogi et al., 2012). The major pharmacologically active constituents reported in *Bacopa monnieri* are bacopasides (triterpenoid

saponins) such as bacoside A3, bacopaside I, bacopaside II, bacoside A (bacopasaponine C), and a jujubogenin isomer of bacopasaponine C (Sivaramakrishna et al., 2005; Zhou et al., 2007).

Safety, efficacy, and quality are the cornerstones of any pharmaceutical product application. In recent years, the goal of developing a quality product, and minimizing its functional variability have been successfully achieved via adopting a Quality by Design (QbD) principles. QbD has been described earlier as a scientific, risk-based, holistic, and proactive approach to pharmaceutical development (Dave et al., 2015). This approach includes a sound science-based understanding of the predefined objectives, formulation and process variables, and a thorough assessment and management of risks with respect to the product quality.

Several pharmaceutical and nutraceutical products containing extracts of *Bacopa monnieri* are commercially available (Pravina et al., 2007). However, the current literature lacks studies on the issue of the low aqueous solubility of its extract, and any approaches to improve the solubility. In a recent study, the authors have demonstrated success in improving the solubility and permeability of a standardized extract of *Centella asiatica* via preparation of its complex with a hydrogenated soy phosphatidylcholine (Phospholipon[®] 90H)(Saoji et al., 2015a). In the present study, this approach was used to assess the feasibility of improving the aqueous solubility of Standardized Bacopa Extract (SBE).

The primary objective of this study was to prepare and optimize the SBE-Phospholipon[®] 90H complex by a modified solvent evaporation method. The formulation and process variables were optimized by the means of a central composite design. The prepared complex was characterized for its physicochemical properties using photo microscopy, Scanning Electron Microscopy (SEM), particle size distribution and zeta potential, Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA), and Powder X-Ray Diffractometry (PXRD). The functional properties of the prepared complex were evaluated by saturation solubility studies, in-vitro dissolution studies, and ex-vivo permeation studies. These properties of the prepared complex were compared to that of the pure SBE and a physical mixture (1:1) of SBE and Phospholipon[®] 90H.

Materials and methods

Materials

The Standardized Bacopa Extract (SBE), containing approximately 40% w/w bacopasides was obtained from Arjuna Natural Extracts Ltd, Kerala, India. The identity and purity of the SBE

composition were confirmed by HPLC analysis. The hydrogenated soy phosphatidylcholine i.e. Phospholipon[®] 90H obtained from Lipoid, Ludwigshafen, Germany. All other chemicals and reagents used were of analytical grade.

Analytical method for the triterpenes present in SBE

The concentrations of the triterpene saponins present in SBE i.e. bacoside A (a mixture of bacoside A3, bacopaside II, jujubogenin isomer of bacopasaponine C, and bacopasaponine C) were determined using a modified, reverse-phase high performance liquid chromatography (RP-HPLC) method previously described by Phrompittayarat et al. (Phrompittayarat et al., 2007). Briefly, the HPLC system (Model: Prominence, Shimadzu Corporation, Kyoto, Japan) with LC solution software, equipped with a LC-20AD HPLC pump, a manual rheodyne sample injector, and a SPD-M20A detector were utilized for the separation. The mobile phase composed of phosphoric acid (0.2%): acetonitrile (65:35 v/v), pH adjusted to 2.8 with 3M Sodium hydroxide, and a flow rate of 1.5 mL/min. A Micra- NPS RP18 column (33×8.0×4.6 mm, 1.5µm) was used as a stationary phase, and the detector wavelength was 205 nm at room temperature. The trailing/asymmetry factor, theoretical plates, and the relative standard deviation (RSD) were calculated to monitor the system suitability of the chromatographic setup. The calibration curves were obtained by plotting the peak areas versus concentration of the standard solution. For each component of the extract (bacopaside), seven concentrations of standard solution were analyzed. The implemented chromatographic method was validated by analyzing several relevant validation characteristics such as linearity, accuracy, and precision.

Preparation of Bacopa Naturosome (BN)

A slightly modified solvent evaporation method, previously described by Bhattacharyya et al. was used to prepare the BN (Bhattacharyya et al., 2014a). Briefly, different ratios of Phospholipon[®] 90H and SBE, i.e. 0.5:1, 1:1, 1.75:1, 2.5:1, or 3:1 were added to a 100 mL round bottom flask containing 40 mL ethanol. The reactions were carried out at various controlled temperatures, i.e. 40°C, 44°C, 50°C, 56°C, or 60°C, and for different durations, i.e. 1h, 1.4h, 2h, 2.6h or 3 h. The resultant clear solution was evaporated to about 2-3 mL. Excess amount of n-hexane was added to this mixture with continuous stirring. The formed BN was then precipitated, filtered, and dried under a vacuum to remove any traces of the solvents. The prepared BN was stored at room temperature in amber colored bottles, flushed with nitrogen.

Quality by Design (QbD) based design of experiments

The Critical Quality Attributes (CQA) of the product, i.e. the drug entrapment efficiency of the BN was studied systematically for the joint influence of the formulation and the process variables such as, phospholipid-to-drug ratio (X_1 , w:w), reaction temperature (X_2 , °C), and the reaction time (X_3 , h) by applying a QbD-based approach using central composite design. Using this design, 20 possible combinations of experimental trials were carried out to analyze the influence of the above three factors (Yue et al., 2010; Yue et al., 2008). The responses were evaluated employing a statistical model incorporating the interactive and polynomial terms using the equation I below:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (I)$$

Where, Y is the dependent variable, b_0 is the arithmetic mean response of the 20 runs, and b_i is the estimated coefficient for the factor X_i . The average response due to a change in the level of one factor at a time is represented by the main effects (X_1 , X_2 , and X_3). The changes in the response when all factors were simultaneously changed are represented by the interaction terms (X_1X_2 , X_2X_3 , and X_1X_3). The on-linearity was evaluated by including the polynomial terms (X_1^2 , X_2^2 , and X_3^2). The tables, 1 and 2 shows the levels of the evaluated factors, and the composition of the trial batches, respectively.

Entrapment efficiency of BN

The entrapment efficiency, i.e. the total bacopaside content entrapped in the prepared BN was analyzed using a combination of methods previously described in the literature (Bhattacharyya et al., 2013; Tan et al., 2012). Briefly, ~100 mg of BN was dispersed in 10 mL chloroform. The prepared BN and any unused Phospholipon® 90H both were easily dissolved in the chloroform. The non-entrapped SBE (free bacopasides) in the mixture was insoluble, and collected as a sediment. This non-entrapped SBE was then separated via filtration and assayed using the HPLC procedure described above. The entrapment efficiency of BN was calculated using the equation (II) below:

$$\text{Entrapment efficiency (\%)} = \frac{C_t - C_f}{C_t} \times 100 \quad (II)$$

Where, C_t = Total concentration of SBE, and C_f = SBE contained in the filtrate.

Determination of SBE content in BN

The SBE content in the prepared BN was determined by HPLC method described above. The drug content was calculated using equation (III) below, previously described by Bhattacharya et al. (Bhattacharyya et al., 2014b).

$$\text{Drug content (\%)} = \frac{\text{Amount of drug in CN}}{\text{Amount of CN}} \times 100 \quad (\text{III})$$

Physico-chemical characterization of CN

Photomicroscopy

The prepared BN was morphologically characterized using photomicroscopy. Briefly, a suspension containing approximately 100 mg of the prepared BN powder was transferred to a glass tube and diluted with 10 mL Phosphate Buffer Saline (PBS, pH 7.4). The suspended dispersion was then mounted on a clear glass slide, and photomicrographs were recorded using an optical microscope equipped with a camera (Model: DM 2500, Leica Microsystems, Germany) at 20x magnification.

Scanning Electron Microscopy (SEM)

The prepared BN was also evaluated by SEM to characterize the surface morphology. Briefly, the BN powder obtained from the optimized formulation batches was sprinkled on a double-sided carbon tape, and the tape was placed on a brass stub. The auto fine coater (Model: JFC1600, Jeol Ltd., Tokyo, Japan) was used to coat the powder surface with a thin layer of palladium. The SEM photomicrographs of the palladium coated samples were obtained on a Scanning Electron Microscope (Model: JSM-6390LV, Jeol Ltd, Tokyo, Japan) operating at 10 KV accelerating voltage, and equipped with a digital camera.

Particle size and zeta potential analysis

The prepared BN was analyzed for particle size distribution and zeta potential with dynamic light scattering on a nanoparticle analyzer (Model: nano Partica SZ-100, Horiba Instruments, Irvine, CA) equipped with a Helium-Neon laser (5mW), with a wavelength output of 633 nm. The samples were measured at an angle of 90°, at 25 °C. Water was used as a dispersant, and the sample run-time was at least 40-80 seconds. The zeta potential was calculated using the Smoluchowski's equation from the electrophoretic mobility of naturosomes (Sze et al., 2003). All measurements were carried out in triplicate.

Fourier Transform Infrared (FTIR) Spectroscopy

An FTIR spectrophotometer (Model: IR Prestige-21, Shimadzu, Japan) equipped with an attenuated total reflectance (ATR) accessory was used to record the infrared spectra of pure SBE,

Phospholipon[®] 90H, the physical mixture of SBE with Phospholipon[®] 90H(PM), and the prepared BN. The samples were analyzed in a diffuse reflectance mode using KBr pellets. In order to remove any influence of the residual moisture present in the sample on the quality of the spectra, all samples were subjected to vacuum drying prior to sample analysis. The spectra for all samples were obtained in the wavelength region of 4000 to 400 cm⁻¹ (Dhore et al., 2016).

Thermal analysis

Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) techniques were used to analyze the thermal behavior of SBE, Phospholipon[®] 90H, the physical mixture (50:50) of SBE and Phospholipon[®] 90H (PM), and the prepared BN. A differential scanning calorimeter (Model: Q20, TA Instruments, Inc., New Castle, DE, USA) was used to determine and compare the peak-transition/peak-onset temperatures of the tested samples. The analysis was carried out under a purge of dry nitrogen gas (50 mL/min). The instrument was calibrated for the heat flow and the heat capacity using a high-purity indium standard. Accurately weighed, 5 mg samples were hermetically sealed in aluminum cells using a crimper. The samples were subjected to a single heating cycle from 0°C to 400°C at a heating rate of 10°C/min (Saoji et al., 2015b).

The thermogravimetric analysis of samples, i.e. SBE, Phospholipon[®] 90H, PM and BN, were carried out using a thermogravimetric analyzer (Model: TGA Q50, TA Instruments, Inc., New Castle, DE, USA). TGA analysis was carried with a nitrogen flow rate of 60 mL/min through the furnace. Accurately weighed samples (~5 mg) were subjected to a single heating cycle from 0°C to 400°C at a heating rate of 10°C/min. The data obtained from the thermal analysis of the samples were analyzed using the software (Universal Analysis, V.4.5A, build 4.5.0.5, TA Instruments, Inc., New Castle, DE, USA).

Powder x-ray diffraction (PXRD)

A powder x-ray diffractometer (Model: D2 Phaser, Bruker AXS, Inc., Madison, WI, USA) equipped with a Bragg-Brentano geometry ($\theta/2\theta$) optical setup was used to determine the polymorphic state of the tested samples, i.e. SBE, Phospholipon[®] 90H, PM and BN. A Bruker AXS' compound silicon strip technology based LYNXEYE[™] detector was used to monitor the diffraction pattern of the samples. An operating voltage and amperage of 30 mV and 10 mA respectively, was maintained for proper functioning of monochromatic CuK β radiation ($\lambda=1.5406 \text{ \AA}$). The diffraction patterns of samples were recorded at a step-angle of 0.2° 2 θ with the diffraction angle increasing from 2° to 90° and a count time of 0.5 seconds.

Functional evaluation of CN

Apparent solubility analysis

The apparent solubility of pure SBE and the SBE incorporated in the prepared BN were analyzed and compared using the method previously described by Singh et al. (Singh et al., 2013). Briefly, excess of pure SBE or BN were placed in sealed glass containers containing 10 mL of water or n-Octanol. The dispersion was continuously agitated 25°C for 24 hours. The dispersion was then centrifuged for 30 min at 4000 RPM. The supernatant was collected and filtered through a membrane filter (0.45µ) to remove any insoluble particulate matter. Appropriate dilutions were prepared by mixing this filtrate with the mobile phase. These samples were then measured for SBE concentration at an absorbance wavelength of 205 nm, using the RP-HPLC method described above.

In-vitro drug release (dissolution)

The release profiles of pure SBE and the prepared BN were determined and compared with the in-vitro dissolution study, using the method previously described by Zhang et al. (Zhang et al., 2014). Briefly, the study was carried out using a six-station dissolution test apparatus, type II (Model: TDT-06T, Electrolab India Pvt. Ltd., India), and the dissolution medium consisted of 900 mL phosphate buffer (pH-6.8), maintained at 37°C ± 0.5°C. Accurately weighed samples of pure SBE (50 mg) or the prepared BN (≈50 mg SBE) were placed on the surface of the medium. The dissolution was carried out at 100 RPM, for a period of 11 hours. Samples (10 mL) were withdrawn at hourly intervals, and the dissolution vessels were replenished with an equal volume of fresh medium to maintain the sink conditions. The withdrawn samples were filtered through a membrane filter (0.45 µm), diluted appropriately with the mobile phase, and analyzed using the RP-HPLC method described above.

Dissolution efficiency (DE)

The dissolution behavior of pure SBE and the prepared BN was further analyzed by calculating and comparing their dissolution efficiencies (DE) at the end of 11 hours. Anderson et al. previously described a method for comparing the dissolution profiles of molecules based on the differences in the areas under the dissolution curve between any given time points. The dissolution efficiencies for the tested samples were calculated using the equation (IV) below.

$$DE = \frac{\int_{t_1}^{t_2} y. dt}{y_{100} \times (t_2 - t_1)} \times 100 \quad (IV)$$

Where, y is the percentage of dissolved drug. DE is the area under the dissolution curve between time points t_1 and t_2 expressed as a percentage of the curve at maximum dissolution, y_{100} , over the same time period. The area under the dissolution curve was calculated using a model independent trapezoidal method by employing the equation (V) below.

$$AUC = \sum_{i=1}^{i=n} \frac{(t_i - t_{i-1})(y_{i-1} + y_i)}{2} \quad (V)$$

Where t_i is the i^{th} time point, y_i is the percentage of dissolved drug at time t_i .

Ex-vivo permeability

An apparatus based on *everted* rat intestine, previously described by Dixit et al. was employed for the evaluation of ex-vivo permeability of the SBE (Dixit et al., 2012). The apparatus consisted of two glass tubes held together by a joint with the open, tapering ends facing each other. An inbuilt bulge at the end of each tube facilitated the mounting of the tissue. The apparatus was designed to be conveniently set up in a 250 mL glass beaker. The entire assembly was placed in a glass beaker after mounting the everted rat intestinal segment. The interior of the glass tubes served as the receiver compartment, whereas the space between the apparatus assembly and the beaker served as the donor compartment.

Prior approvals were obtained from the Institutional Animal Ethics Committee (IAEC) for the handling and use of the experimental animals in the study. Overnight fasted Sprague-Dawley[®] rats (200-250 g) were euthanized by cervical dislocation. The abdomen was exposed by giving a midline incision, and the ileo-cecal junction was identified after carefully maneuvering the intestine. A section of the intestine (jejunum, ~7 cm) was isolated from the mesenteric attachments carefully without damaging the intestine. The isolated segment was thoroughly washed with Krebs solution, and everted using a glass rod. The everted segment was transferred to a petri dish containing fresh Krebs's solution. For the permeability experiments, a 6 cm segment was used.

The everted piece of intestine was mounted between the two tapered ends of the perfusion apparatus. The perfusion apparatus was filled with Krebs solution and placed in the glass beaker (250 mL) containing SBE, PM, or BN (~100 µg/mL SBE) in 250 mL Krebs solution. The whole assembly was placed on a constant temperature (37 °C ± 2°C) magnetic stirrer, and the contents stirred at 25 RPM. The Krebs solution was continuously aerated with carbogen (Oxygen: Carbon dioxide (95:5) mixture). The samples from within the apparatus were

collected at 15 min intervals up to three hours and analyzed by the RP-HPLC to estimate the permeability.

Preliminary pharmacological evaluation

Animals: Male Swiss albino mice (4 months old) were housed in groups of 6 mice/cage under controlled room temperature (25 ± 2 °C), relative humidity (50- 70% RH), and maintained under natural light/dark cycle. Food (Trimurti feeds, Nagpur, India) and water was given *ad libitum*, except during experimentation. All experiments were conducted during between 9:00 AM and 3.00 PM. All experimental procedures and protocols were carried out under strict ethical guidelines, and were duly approved by Institutional Animal Ethics Committee, Department of Pharmaceutical Sciences, R. T. M. Nagpur University, Nagpur, Maharashtra, India.

Tail Suspension Test: The potential antidepressant effects of pure SBE and the prepared SBE-Phospholipid complex (BN) were evaluated using the '*Tail Suspension Test*' described earlier by Steru et al., with some modifications (Steru et al., 1985). The doses used in this study were similar to those reported to be effective in the animals (Sairam et al., 2002). The animals were administered saline (control), imipramine (10 mg/kg, intraperitoneal), pure SBE (40 mg/kg), or the prepared SBE-Phospholipid complex (equivalent to 40 mg/kg) orally, for a period of five days. The animals were then suspended from their tails, 50 cm above the floor, by using an adhesive tape placed at 1 cm from the tip of the tail. The animals were left suspended for 6 minutes and the immobility was observed for the final 4 minutes. The animals were considered immobile if they remained suspended passively without any body movements. This procedure was repeated for five days. On the fifth day, the animals were observed for immobility 30 minutes after the administration of the test material (blank, standard, or the drug) (Sairam et al., 2002).

Forced Swim Test: The antidepressant effects of pure SBE and the prepared SBE-Phospholipid complex (BN) were additionally evaluated using the '*Forced Swim Test*' in mice, another well-documented method commonly utilized for evaluating the antidepressant effect of drugs (Porsolt et al., 1977). The doses used in this study were similar to those reported to be effective in the animals (Sairam et al., 2002). The animals were administered saline (control), imipramine (10 mg/kg, intraperitoneal), pure SBE (40 mg/kg), or the prepared SBE-Phospholipid complex (equivalent to 40 mg/kg) orally, for a period of five days. The animals were individually forced to swim in a cylindrical glass tank (20 cm in diameter $\times$ 25 cm tall) containing water at a height

of 10 cm, maintained at 25 ± 2 °C, for the period of 6 min. While excluding the initial 2 minutes of observed vigorous activity, the immobility time was evaluated for the final 4 minutes. The animals were considered immobile if they floated passively in water, making only necessary movements to keep their heads above the water level. This procedure was repeated for five days. On the fifth day, the animals were observed for immobility 30 minutes after the administration of the test material (blank, standard, or the drug) (Sairam et al., 2002). A decrease in the duration of immobility was considered as an indication of the pharmacological effectiveness of the treatment.

Statistical analysis

The statistical analysis of the behavioral data from the *Tail Suspension Test* and *Forced Swim Test* was carried out by one-way analysis of variance (ANOVA), followed by *post hoc* Bonferroni's multiple comparison test. The data are presented as mean $\pm$ SEM. The results were considered to be significant at $p < 0.05$.

Results and discussion

Preparation of Bacopa Naturosome (BN)

A preliminary investigation of the studied process parameters revealed that the factors phospholipid-to-drug ratio, reaction temperature, and the reaction time highly influenced entrapment efficiency of the prepared naturosomes. The results of the entrapment efficiency (% w/w) are shown in Table 2. The measured values from the experimental trials revealed a wide range (~53 - 88, % w/w) of entrapment efficiencies in the prepared BN. The fitted polynomial equations relating the response (entrapment efficiency, % w/w) to the transformed factors are shown in Figure 1. The polynomial equations could be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries, i.e., positive or negative. The results from the Figure 1 indicated that the coefficients b_1 , b_2 , b_3 , b_{11} , b_{12} , b_{13} , b_{22} , b_{23} , and b_{33} were statistically significant ($p < 0.05$). The value of correlation coefficient (R^2) was observed to be 0.9457, indicating a good fit to the quadratic model. The multiple regression analysis also revealed that the coefficients b_1 , b_2 , and b_3 were positive. These results indicated that the entrapment efficiency of the prepared BN increased with increasing levels of X_1 , X_2 , and X_3 . The analysis shown in the Table 3 further indicated that the quadratic model was statistically significant ($F_{critical}$ value = 19.38; $p < 0.001$).

The data from all the 20 batches of central composite design were used for generating interpolated values using Design-Expert[®] (V9, Stat-Ease, Inc., Minneapolis, MN). Based on the employed central composite design, the changes in the entrapment efficiencies (% w/w) as a function of varying levels of X_1 , X_2 , and X_3 are exhibited in the form of response surface, and contour plots in Figure 2. The response surface and contour plots also indicated a strong influence of the studied factors X_1 , X_2 , and X_3 on the entrapment efficiencies of the prepared BN. In general, the higher levels of X_1 , X_2 , and X_3 were observed to be favorable conditions for optimizing the entrapment efficiency. Thus, based on the multiple regression model and the above observations, the optimal set of conditions for the preparation of BN were found to be 3:1, 60°C, and 3 hours for the phospholipid: drug ratio, the reaction temperature, and the reaction time, respectively.

Validation of the model

The robustness of the developed model were evaluated by preparing an additional batch of BN, using the model-optimized formulation and processing conditions. This new batch was prepared using a phospholipid: drug ratio of 3:1, the reaction temperature of 60°C, and a reaction time of 3 hours. The table 4 shows the values of the entrapment efficiency of the prepared BN as predicted by the model, as well as that achieved from the prepared validation batch. The entrapment efficiency for the batches of BN prepared under the optimized conditions was found to be 87.09 %. These results were found to be comparable to the model-predicted value, i.e. 85.67 %, indicating the validity and the robustness of the developed model. The calculated bias (%) based on the equation VI below, was found to be -1.65%, further supporting the robustness of the model (Qin et al., 2010).

$$Bias (\%) = \frac{\text{predicted value} - \text{observed value}}{\text{predicted value}} \times 100 \quad (VI)$$

Physico-chemical characterization of the prepared CN

Photomicroscopy and Scanning electron microscopy (SEM)

Figures 3 and 4 shows the surface morphological characteristics of the SBE or the prepared BN, as observed by photomicroscopy (Figure 3) and scanning electron microscopy (Figure 4). The surface morphologies of the SBE and the prepared BN are compared at 20x magnification in the figure 3. The SBE particles were exhibited as thin, mostly transparent, needle-shaped crystals (Fig. 3A). The particles of the prepared BN appeared to be larger, opaque, irregularly-shaped,

and non-crystalline with a porous surface (Fig. 3B). The SBE particles appeared to be either entrapped, or partially amorphized in the prepared BN.

Further analysis of the surface morphology of the prepared BN by SEM is shown in the Figure 4 (A, B, and C). The electron micrographs at different magnifications i.e. 1000x (4A), 3000x (4B), and 6000x (4C) revealed that the BN particles were composed of irregular sized and shaped vesicles of Phospholipon[®] 90H, with the SBE particles possibly intercalated within the lipid layers.

Particle size and zeta potential analysis

The figure 5 shows the particle size distribution and the zeta potential values of the prepared BN. From the analysis, the mean particle size of the BN prepared using the optimized conditions was found to be $395 \text{ nM} \pm 11 \text{ nM}$. There exists an inverse relationship between the surface area/volume (SA/V) and the particle size of most pharmaceutical particles. Thus, the release of the entrapped drug is greatly facilitated due to the smaller particle size of the BN. Additionally, the smaller size of the BN also facilitates their penetration into, and permeation through most physiological barriers. It has been previously suggested that particles larger than 5 μm are typically taken up via the lymphatic system, while particles smaller than 500 nm typically traverse biological membranes via endocytosis (LeFevre et al., 1978; Savic et al., 2003).

Another parameter commonly used to assess the physical stability of the naturosomes is the zeta potential. For the prepared BN, the observed zeta potential value was found to be $-37.6 \pm 1.1 \text{ mV}$. These results are in accordance with previously published results stating that the zeta potential values higher than -30 mV are acceptable, and indicative of good physical stability (Freitas and Müller, 1998).

Fourier transform infrared Spectroscopy (FTIR)

The figure 6 (A-D) shows the FTIR spectra of pure SBE (Fig. 6A), Phospholipon[®] 90H (Fig. 6B), the physical mixture of SBE with Phospholipon[®] 90H (Fig. 6C), and the prepared BN (Fig. 6D). The spectrum of pure SBE showed alkyl stretching peaks at $\sim 2944 \text{ cm}^{-1}$ and $\sim 2882 \text{ cm}^{-1}$. A bending peak was also observed at $\sim 1446 \text{ cm}^{-1}$. The ketone functional groups present in the SBE were represented by a moderate peak at $\sim 1717 \text{ cm}^{-1}$. The aromatic nature of the basic ring nucleus of the triterpenes present in the SBE was represented as the aromatic stretching peak in the region of $\sim 1514 \text{ cm}^{-1}$. The aromatic nucleus also contained a side chain with an alkene carbon, and represented by a broad stretching peak around 3309 cm^{-1} . The C-O bonds

corresponding to the furanosyl and pyranosyl group in the ring are exhibited by the peaks around 1077 cm^{-1} and 1028 cm^{-1} , respectively.

The FTIR spectrum of Phospholipon[®] 90H revealed the characteristic C-H stretching band of the long-chain fatty acid at $\sim 2918\text{ cm}^{-1}$ and $\sim 2850\text{ cm}^{-1}$. The spectrum also exhibited the carbonyl stretching band in the fatty acid ester at $\sim 1738\text{ cm}^{-1}$, a P=O stretching band at $\sim 1236\text{ cm}^{-1}$, a P-O-C Stretching band at 1091 cm^{-1} , and a $\text{-N}^+(\text{CH}_3)_3$ stretching band at 970 cm^{-1} .

In the spectrum of the prepared BN, however, the alkene side-chain stretching band appeared to have shifted to $\sim 3443\text{ cm}^{-1}$ from 3309 cm^{-1} observed in the spectrum of pure SBE. This indicated a possible interaction of the phytoconstituents of the SBE with Phospholipon[®] 90H. Further, a new absorbance peak was observed at $\sim 1654\text{ cm}^{-1}$, likely due to the formation of weak bonds between the amide groups present in the phospholipid and the SBE. The band representing the ketone functional group in the SBE spectrum also appeared to be shifted in the BN spectrum. Additionally, the band representing the aromatic ring stretching at 1514 cm^{-1} disappeared in the BN spectrum, possibly due to weakening, or shielding by the phospholipid molecule. These observations further supported the interactions between the phospholipid and the SBE, and possible formation of the complex. The SBE components are likely packed in the hydrophobic cavity of the phospholipid, and held together by van der Waals forces, and other hydrophobic interactions (Jena et al., 2014).

Thermal analysis

Thermal analytical techniques such as DSC and TGA are commonly employed to characterize material properties and analyze the interactions, if any, between the components of a formulation. Such interactions are usually demonstrated in the form of endothermic/exothermic peaks that appear, disappear, or change. Measuring changes in enthalpies also provide insights with respect to these interactions. The DSC thermograms of pure SBE, PM, BN, and pure Phospholipon[®] 90H are shown in the figure 7A, 7B, 7C, and 7D, respectively.

The DSC thermogram of pure SBE revealed a broad endothermic peak between $50\text{-}100^\circ\text{C}$, possibly due to the dehydration (water evaporation). This thermogram also showed an endothermic peak around 128°C corresponding to the melting of the phytoconstituents. A broad endothermic peak was observed around 185°C and may be attributed to the phase transition of SBE from a gel to a liquid crystalline state. The thermogram of Phospholipon[®] 90H showed two major endothermic peaks at 125°C and 182°C , respectively. The first peak is likely due to the

movements of a polar head group of phospholipid, whereas, the second peak could be due to the phase transition from a gel to a liquid crystalline state, and the carbon-chain in the phospholipid may have perhaps undergone melting, isomeric or other crystal changes (Semalty et al., 2012). In the thermogram of the physical mixture (PM) of the SBE and Phospholipon[®] 90H, two endothermic peaks were observed at ~86 °C and ~122 °C. These two peaks likely corresponded to the melting of SBE and Phospholipon[®] 90H, and an interaction between these components might have resulted in a shift in their respective melting points. The thermogram of the prepared BN exhibited a broad endothermic peak between 50 °C and 85 °C. This peak appeared to be consisting of three partially fused, endothermic peaks at 54 °C, 63 °C and 81 °C, respectively. This peak also differed significantly compared to the corresponding peaks observed in the thermograms of pure SBE and pure Phospholipon[®] 90H. A reduction in the enthalpy and melting points is shown to reduce the crystallinity and enhance the solubility of the drugs (Singh et al., 2012). As some of the key peaks observed with SBE and Phospholipon[®] 90H were observed to disappear from the thermogram of the prepared BN, and the phase transition temperature was observed to be lower than that of pure Phospholipon[®] 90H, these observations indicate the possible formation of SBE-Phospholipid complex (BN). These findings are in accordance with previously reported results. The interactions between the SBE and Phospholipon[®] 90H can be attributed to a combination of forces such as hydrogen bonding and/or van der Waals interactions, and can be considered as an indication of drug amorphization and/or complex formation, as supported by IR spectroscopy (Khan et al., 2014; Li et al., 2013; Zhang et al., 2015). The interactions of the SBE with the polar region of Phospholipon[®] 90H are likely followed by the intercalation of the SBE within the hydrophobic domains of the phospholipid molecules. This could result in a sequential decrease in the free hydrophobic domains of the phospholipid, and the subsequent disappearance of the second endothermic peak of Phospholipon[®] 90H, along with a reduction in the phase transition temperature (Singh et al., 2014). The results obtained from the TGA studies (data not shown), were consistent and complementary to the DSC results. The weight loss (%) as a function of increasing temperature was observed to be the slowest for the prepared SBE-Phospholipon[®] 90H complex in comparison to that observed with the pure SBE, or the physical mixture of SBE and Phospholipon[®] 90H, indicating a relatively higher thermal stability of the prepared BN.

Powder x-ray diffraction (PXRD)

The x-ray diffraction patterns of pure SBE, Phospholipon[®] 90H, PM, and BN are shown in the Figure 8A, 8B, 8C, and 8D, respectively. The diffractogram of the pure SBE exhibited several sharp peaks at $45^{\circ}2\theta$, $40^{\circ}2\theta$, $31^{\circ}2\theta$, $28^{\circ}2\theta$, and $27^{\circ}2\theta$. These types of peaks are typical of highly crystalline materials. Phospholipon[®] 90H revealed a single, relatively sharp peak at $21^{\circ}2\theta$. The diffractograms of the physical mixture (PM) showed peaks that appeared to be a combination of pure SBE and Phospholipon[®] 90H, while also retaining most of the individual peaks of SBE. In the diffractogram of the prepared BN, however, only one major peak around $20^{\circ}2\theta$ was observed. This peak appeared as a fused peak, and the crystalline peaks observed with pure SBE were absent. The results were in agreement with previously reported studies on the phospholipid complexes of mitomycin-C and curcubitacin B (Cheng et al., 2015; Hou et al., 2013). The disappearance of the crystalline peaks of pure SBE thus further corroborated the hypothesis of the formation of SBE-phospholipid complex. It may thus be inferred that SBE is associated in a molecularly dispersed, or in a partially amorphized state within the carrier (Phospholipon[®] 90H) (Semalty et al., 2010).

Functional evaluation of CN

Apparent solubility

Table 5 shows the estimated apparent solubilities of the pure SBE, the physical mixture of SBE and Phospholipon[®] 90H (PM), and the prepared Bacopa naturosome (BN). The lipophilic nature of the SBE was evident from its poor aqueous solubility ($\sim 12 \mu\text{g/mL}$), and a comparatively higher solubility in n-octanol ($\sim 160 \mu\text{g/mL}$). The aqueous solubility of SBE in the physical mixture (PM) was not found to be significantly different compared to that of pure SBE ($\sim 18 \mu\text{g/mL}$). The aqueous solubility of the prepared SBE-Phospholipon[®] 90H complex (BN) however, was found to be significantly higher compared to either that of the pure SBE, or the SBE in the physical mixture (PM). BN showed over 20-fold enhancement in the aqueous solubility compared to that of pure SBE, and over 13-fold enhancement compared to that of the SBE in the physical mixture. Partial amorphization (reduced molecular crystallinity) of the drug, and the overall amphiphilic nature of the BN may likely explain this increase in the solubility of the prepared complex (Maiti et al., 2007; Xia et al., 2013). The Phospholipon[®] 90H molecule and the hydrophobic moiety of SBE may form a quasi-stable bonding; and the phospholipid molecule may possibly enwrap the non-polar groups in the SBE, thus enhancing its aqueous solubility (Kidd, 2009; Tan et al., 2012).

***In-vitro* drug release (dissolution)**

The Figure 9 shows the dissolution profiles of the pure SBE, the physical mixture of SBE and Phospholipon[®] 90H (PM), and the prepared Bacopa naturosomes (BN) in the phosphate buffer (pH 6.8). The pure SBE exhibited the slowest rate of dissolution, i.e. only about 42% w/w SBE was released at the end of 11-hour dissolution period. Similarly, the dissolution rate of SBE in the PM also appeared to be slower, and not significantly different from that of pure SBE, i.e. ~47% w/w SBE was released at the end of 11 hours. The dissolution profile of the BN, however, showed a significantly higher rate of SBE release from the prepared SBE-Phospholipon[®] 90H complex (BN). At the end of 11 hours, over 97% w/w SBE was released from the BN, following a near zero-order release. Crystal morphology and the wettability of solids are known to influence the dissolution rate; and the partially amorphized state of the SBE in the prepared BN, and subsequent improvement in the aqueous solubility may explain the improved dissolution of the SBE in BN (Freag et al., 2013; Semalty et al., 2010). The relatively higher amorphous nature of the prepared complex may have had a positive impact on the cumulative release of the SBE.

Dissolution efficiency (DE)

The dissolution efficiencies calculated from the in-vitro dissolution studies in phosphate buffer (pH-6.8), for the pure SBE, the physical mixture of SBE and Phospholipon[®] 90H (PM), and the prepared SBE- Phospholipon[®] 90H complex (BN) are shown in Table 6. At the end of 11 hours, pure SBE exhibited a dissolution efficiency of ~22%. The dissolution efficiency of SBE in the physical mixture showed a marginal, but a statistically significant ($p < 0.01$) increase, compared to the pure SBE. This increase could be attributed to the amphiphilic nature of the phospholipids, and their solubilizing characteristics. The prepared SBE- Phospholipon[®] 90H complex (BN) however, revealed a significant ($p < 0.001$) improvement in the dissolution efficiency compared to that of either pure SBE, or the SBE in the physical mixture. At the end of 11 hours, over 2.5-fold increase in the DE was seen for the prepared BN. This observed increase in the release of SBE from BN can be attributed to the partial amorphization of SBE in the prepared complex, and subsequent improvement in the aqueous solubility of SBE.

***Ex-vivo* permeability**

Figure 10 shows the results of the three-hour, *ex-vivo* permeability analysis of the pure SBE, the physical mixture of SBE and Phospholipon[®] 90H (PM), and the prepared SBE- Phospholipon[®] 90H complex (BN), as performed with the *everted* intestine method. The relative permeabilities of the tested samples across the everted intestinal membrane appeared to follow a similar pattern

to that observed with the *in-vitro* dissolution studies. Among the samples evaluated, the pure SBE exhibited the slowest rate and extent of permeation, i.e. only about 21% w/w SBE permeation was observed at the end of three hours. The permeation of SBE from the physical mixture was not found to be significantly different from that observed with pure SBE. The rate and extent of SBE permeation from the prepared SBE- Phospholipon[®] 90H complex (BN) was found to be significantly higher compared to that observed with either pure SBE, or the physical mixture. At the end of three hours, over 90% w/w of SBE was observed to be permeated through the intestinal membrane. The amphiphilic nature of the phospholipids may lend itself for them to function as surfactants, assisting the drug to traverse across the membrane. Considering the observed increase in the apparent aqueous solubility of SBE, an increase in the rate and extent of SBE release, and the improved permeability of SBE from the prepared BN, complex formation of drugs, with phospholipids as carriers can be employed as a potential alternative strategy to enhance the delivery of SBE or other similar phytoconstituents.

Preliminary pharmacological evaluation

The preliminary antidepressant-like activity of the prepared SBE-Phospholipon[®] 90H complex (BN) was analyzed using established behavioral models (Porsolt et al., 1977; Steru et al., 1985). The results of the *Tail Suspension Test* and *Forced Swim Test* are shown in the Figure 11(A) and Figure 11(B), respectively. As shown in the Figure 11 (A), a five-day treatment of mice with imipramine (10 mg/kg), pure SBE (40 mg/kg), or the prepared SBE-Phospholipon[®] 90H complex resulted in a significant decrease in the duration of immobility in the *Tail Suspension Test*. The observed decrease in the immobility was found to be 44.8%, 23.4%, and 46.9% for imipramine, pure SBE, and the SBE-Phospholipon[®] 90H complex, respectively [one-way ANOVA, $F(3, 23) = 17.08$, $p < 0.0001$]. The *post hoc* Bonferroni's multiple comparison test revealed that the prepared SBE-Phospholipon[®] 90H complex (BN) elicited antidepressant-like effects similar to those observed with the known antidepressant, imipramine ($p < 0.05$). Interestingly, these effects of the prepared BN were also observed to be significantly better compared to pure SBE ($p < 0.05$).

The antidepressant-like effects of pure SBE and the prepared BN were also confirmed using *Forced Swim Test*. As shown in the Figure 11 (A), a five-day treatment of mice with imipramine (10 mg/kg, i.p.), pure SBE (40 mg/kg), or the prepared SBE-Phospholipon[®] 90H complex resulted in a significant decrease in the duration of immobility in this test. The

immobility was found to be decreased by 43.9%, 22.8% and 45.6%, for imipramine, pure SBE, and the SBE-Phospholipon[®] 90H complex, respectively [$F(3, 23) = 16.53, p < 0.0001$]. The *post hoc* Bonferroni's multiple comparison test revealed that the prepared SBE-Phospholipon[®] 90H complex (BN) elicited antidepressant-like effects similar to those observed with the known antidepressant, imipramine ($p < 0.05$). Interestingly, these effects of the prepared BN were also observed to be significantly better compared to pure SBE ($p < 0.05$). The improved pharmacological activity of the prepared complex observed with both tests can be attributed to the increased solubility, permeability, and possibly overall bioavailability of SBE from the complex.

The duration of immobility measured in the above behavioral tests in mice have been known to reflect a state of 'behavioral despair and variants' or 'failure to adapt to stress' (Willner and Muscat, 1991). A functional deficiency of the cerebral monoaminergic transmitters such as norepinephrine, 5-hydroxy tryptamine, and/or dopamine, located at the synapses is thought to be the predominant contributor of depression (Schildkraut, 1965). The antidepressant-like activity of Bacopa extract is reported to be due to its adaptogenic effects via normalization of various stress parameters and monoaminergic levels, resulting in the restoration of normal monoaminergic neurotransmitters (Rai et al., 2003). Bacopasides, which are the major constituents of Bacopa, are also reported to exhibit antidepressant effects by improving the vital neurotransmitter activities (Chatterjee et al., 2010). Additionally, the neurochemical studies following the behavioral despair tests have previously revealed an improved brain antioxidant activity (Liu et al., 2013; Mannan et al., 2015). Thus, the observed antidepressant effects of Bacopa extract in the present study might be related to both, the antioxidant activation and the noradrenergic neurotransmitter activation.

Conclusions

The feasibility of enhancing the aqueous solubility of the SBE was attempted via preparing its complex with phospholipids (Bacopa Naturosome, BN) in the current study. The formulation and process variables were optimized using a central composite design. The physicochemical and functional characteristics of the prepared BN were evaluated, and compared to that of pure SBE. The successful formation of a vesicular SBE-Phospholipon[®] 90H complex was ascertained via FTIR, DSC, PXRD, photomicroscopy, and the SEM studies. The solubility analysis, the *in-vitro* drug release studies (dissolution), and the permeability analysis across the everted rat intestine

(*ex-vivo*) indicated that, the aqueous solubility, the drug release, and the membrane permeation of the SBE from the prepared BN showed a significant improvement as a result of complex formation.

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Figure 1: Pareto diagrams for the estimation of effects. The effects presenting probability values higher than 0.05 are not considered as statistically significant.

Figure 2: The response surface plots and the contour plots of entrapment efficiency (Y , %) as a function of the ratio of Phospholipon[®] 90H and SBE (X_1 , w:w), the reaction temperature (X_2 , °C), and the reaction time (X_3 , h).

Figure 3: The photo-microscopic images of (A) pure SBE, and (B) the SBE- Phospholipon[®] 90H complex at a magnification of 20X.

Figure 4: The SEM photomicrographs of SBE- Phospholipon[®] 90H complex.

Figure 5: (A) Particle size distribution, and (B) Zeta potential profiles of the SBE- Phospholipon[®] 90H complex

Figure 6: The FTIR spectra of (A) pure SBE, (B) Phospholipon[®] 90H, (C) the physical mixture (1:1) of SBE and Phospholipon[®] 90H, and (D) SBE- Phospholipon[®] 90H complex.

Figure 7: DSC thermograms of (A) pure SBE, (B) Phospholipon[®] 90H, (C) the physical mixture (1:1) of SBE and Phospholipon[®] 90H, and (D) SBE- Phospholipon[®] 90H complex.

Figure 8: The X-ray diffractograms of (A) pure SBE, (B) Phospholipon[®] 90H, (C) the physical mixture (1:1) of SBE and Phospholipon[®] 90H, and (D) SBE- Phospholipon[®] 90H complex.

Figure 9: The *in-vitro* dissolution profiles of pure SBE, the physical mixture (1:1) of SBE and Phospholipon[®] 90H, and the SBE- Phospholipon[®] 90H complex.

Figure 10: The *ex-vivo* permeability profiles of pure SBE, the physical mixture (1:1) of SBE and Phospholipon[®] 90H, and the SBE- Phospholipon[®] 90H complex.

Figure 11: *Tail Suspension Test* (A) and *Forced Swim Test* (B) in mice. Animals were treated with saline (control), imipramine (10 mg/kg, i.p.), pure SBE (40 mg/kg), or the SBE- Phospholipon[®] 90H complex (equivalent to SBE 40 mg/kg). Data expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.001$ vs control; *** $p < 0.05$ vs pure SBE.

Fig. 1

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Fig. 10

Fig. 11

Table 1: Coded levels and “Real” values for each factor under study.

Variables	Levels				
	-1.7	-1	0	+1	+1.7
<i>Independent</i>	Real values				
Phospholipid : drug ratio (X_1 , w:w)	0.5	1.0	1.8	2.5	3.0
Reaction temperature (X_2 , °C)	40.0	44.0	50.0	56.0	60.0
Reaction time (X_3 , h)	1.0	1.4	2.0	2.6	3.0
<i>Dependent</i>					
Entrapment efficiency (Y , % w/w)					

Table 2: Central composite design formulation batches with respective entrapment efficiencies.

Batches	X_1	X_2	X_3	Entrapment efficiency* (% w/w)
F1	-1	+1	+1	79.2 ± 0.9
F2	-1	-1	+1	80.3 ± 1.3
F3	+1.7	0	0	87.7 ± 1.0
F4	+1	-1	+1	85.3 ± 1.0
F5	0	0	-1.7	63.2 ± 1.1
F6	-1.7	0	0	58.3 ± 1.2
F7	0	0	+1.7	86.9 ± 1.3
F8	+1	+1	+1	87.2 ± 1.2
F9	0	-1.7	0	72.7 ± 0.9
F10	-1	-1	-1	53.1 ± 1.3
F11	-1	+1	-1	73.1 ± 1.1
F12	0	+1.7	0	86.5 ± 1.1
F13	+1	+1	-1	88.2 ± 1.0
F14	+1	-1	-1	83.3 ± 1.3
F15- F20	0	0	0	85.8 ± 1.0

* Values represent mean ± standard deviation (n=3)

Table 3: ANOVA of the Quadratic Model.

Source	Sum of squares	Degrees of freedom	Mean square	$F_{critical}$	p value
Regression	1976.8	9	219.7	19.4	0.001
Residual	113.4	10	11.3		
Total	2090.2	19			

Table 4: Comparison of the observed and predicted entrapment efficiency (%) values of SBE-Phospholipon[®] 90H complex (BN), prepared under predicted optimum conditions

Response Variable	Predicted Value	Observed value*	Bias (%)
Entrapment efficiency (%)	85.7	87.1 ± 1.2	-1.7

*: Values represent mean ± standard deviation (n=3)

Table 5: Solubility profiles of pure SBE, the physical mixture (1:1) of SBE and Phospholipon[®] 90H (PM), and the SBE- Phospholipon[®] 90H complex (BN).

Sample	Aqueous solubility (µg/ml)*	n-Octanol solubility (µg/ml)*
Pure SBE	11.9 ± 0.7	160.4 ± 1.8
PM	18.3 ± 0.4	173.2 ± 1.1
BN	249.4 ± 2.1	198.2 ± 2.1

*: Data expressed as mean values and standard deviations (±SD); n = 3

Table 6: Calculated dissolution efficiencies of pure SBE, the physical mixture (1:1) of SBE and Phospholipon[®] 90H (PM), and the SBE- Phospholipon[®] 90H complex (BN) in phosphate buffer (pH-6.8).

Time (h)	Dissolution efficiency (DE) (%)		
	Pure SBE	PM	BN
11	22.0 ± 1.1	24.2 ± 1.0*	56.4 ± 1.7**

All values are mean ± SD (n = 3). The level of significance is $p < 0.05$, * implies $p \leq 0.01$, ** implies $p \leq 0.001$ as compared to pure SBE.

Graphical abstract