

Preparation & characterization of embelin–phospholipid complex as effective drug delivery tool

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Abstract The aim of the present study was to prepare an embelin–phospholipid complex (EPC) formulation in an attempt to enhance the water solubility and to characterize the new developed formulation. Embelin, due to water insolubility causes poor bioavailability by oral route. To improve the bioavailability and prolong its duration in body system, its phospholipid complexes were prepared by a simple and reproducible method. EPC was formulated by mechanical dispersion method using ethanol as a reaction medium, embelin and phospholipids were dissolved into the medium, after that organic solvent was removed under vacuum condition and EPC was formed. The complex formation was confirmed by carrying out FTIR, $^1\text{H-NMR}$, XRD, DSC and microscopical studies. Solubility and in vitro studies were carried out to ascertain the solubility and dissolution pattern of free and complexed embelin. Content of embelin in EPC was found to be 92.44% (w/w). FTIR, $^1\text{H NMR}$, DSC and XRD data confirmed the formation of embelin phospholipid complex. Water solubility of embelin was improved from 3 to 42 $\mu\text{g/mL}$ in the prepared complex. *n*-Octanol solubility were also altered for free embelin and EPC from 2.3 to 39 $\mu\text{g/mL}$. Unlike the free embelin, which showed a total of only 19% drug release at the end of 120 min, EPC showed 99.80% release at the end of 120 min of dissolution study in distilled water. Microscopical characterization of the developed formulation also showed the entrapment of embelin in the lipid core showing complex structure, which was further, supported by change in surface morphology of embelin on microscopical examination.

Hence, the present findings demonstrate that complexing embelin with phospholipid can be further explored for improved therapeutic implications.

Keywords Embelin · Phospholipid complex · Solubility · XRD · DSC · FTIR

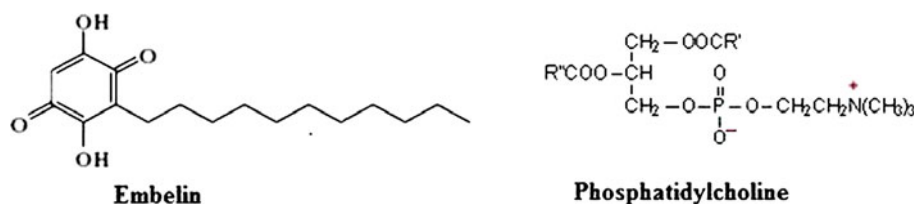
Introduction

Herbal medicine has been used for the prevention and treatment of various health ailments from time immemorial [1]. The belief that natural medicines are much safer than synthetic drugs has gained popularity in recent years and led to tremendous growth of phytopharmaceutical usage and development of phytomedicine formulation. Herbal drugs have been explored to formulate a wide variety of dosage forms [2]. Even after advancement, certain limitations still exist for formulating herbal dosage forms such as poor solubility of the bioactive constituents of phytomedicines (e.g., anthocyanidins from bilberry, catechins from green tea, silymarin from milk thistle), lack of potential of reaching the drug to the target site, dose optimization, pre systemic metabolism etc. [3]. Most of the biologically active constituents of plants are non polar or water insoluble molecules. It has been observed that complexation with certain other clinically useful nutrients substantially improves the bioavailability of such extracts and their individual constituents [4]. The nutrients so helpful for enhancing the absorption are the phospholipids. Phospholipids are complex molecules that are used in all known life forms to make cell membranes.

Phospholipids are compound lipids derived from glycerol (Phosphoglycerides) or Sphingosine (sphingomyelin) (Fig. 1). Being amphiphatic molecules, phospholipids in

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Fig. 1 Chemical structure of pure embelin & phosphatidylcholine



aqueous medium form minute, closed bilayered vesicles, which serve as permeability barriers. Phospholipids occur naturally in cell membrane and serve to maintain integrity and acts as precursor for synthesis of platelet activating factor and arachidonic acid and second messengers in signal transduction. Phospholipids occur naturally in egg yolk and soybeans [5–7]. Phospholipid complex are known to improve the solubility, stability, site specificity and hence, therapeutic efficacy of hydrophobic drugs. These phospholipid form micelles when come into contact of aqueous system and change into self-assembled nanosized (20–60 nm), spherical structures with a hydrophobic core capable of solubilising a considerable amount of highly water insoluble drugs. Several studies have indicated the beneficial role of phospholipids in enhancing the therapeutic efficacy of some bioactive molecules having poor oral absorption. Naringenin, [8], ginkgo biloba [9], oxy-metrine [10], quercetin [11], curcumin [12], marsupsin [13] have poor aqueous solubility and oral absorption but when formulated as a phospholipid complex, enhanced aqueous solubility and improved pharmacokinetic profile were observed.

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), the active constituent of the fruits of *Embelia ribes* (Family: Myrsinaceae) commonly known as ‘Vidanga’ is a component of herbal drug and possess wide range of medicinal properties. Aqueous extract of the fruits possess antibacterial [14] and antifertility activities [15]. In a preliminary study, Tripathi [16] reported the antihyperglycemic activity of decoction of the *Embelia ribes* fruits in glucose-fed albino rabbits. Bhandari et al. [17] reported the potential of ethanolic extract of *Embelia ribes* in diabetic dyslipidemia and protection from lipid peroxidation in tissues in streptozotocin-induced diabetes in rats. Embelin revealed antifertility [18], analgesic [19], anthelmintic [20], antimicrobial [21], anti-inflammatory, antioxidant and antitumour properties [22] and also been reported as a potential effective suppressor of tumor cell survival, proliferation, angiogenesis, invasion, and inflammation in experimental animals. [23–25]. Further, cardioprotective effect of an ethanol extract of *Embelia ribes* fruits was reported on isoproterenol-induced myocardial infarction in albino rats [26]. In addition, aqueous extract of *Embelia ribes* Burm fruits revealed protective effect in middle cerebral artery occlusion-induced focal cerebral ischemia in rats [27].

Despite the promising pharmacological effects of embelin, poor oral bioavailability due to low aqueous solubility is responsible for its unfavourable pharmacokinetics-pharmacodynamic profile which has restricted its use in the management of human ailments [28, 29]. Poor oral absorption [30] due to its extremely low aqueous solubility or extensive pre-systemic metabolism may be responsible for the unfavorable pharmacokinetics of this molecule.

Keeping in view, the promise of embelin as a therapeutically active agent and its poor oral absorption, it is pertinent to develop a novel formulation of embelin, which can increase its oral absorption and enhance its therapeutic activity.

Hence, the aim of the present study was to prepare phospholipid complex of embelin (EPC) to overcome the problems like poor solubility associated with the embelin and to characterize the phospholipid complex with respect to various parameters like solubility studies, structural changes by FTIR, NMR, DSC, microscopy and in vitro dissolution profile.

Material & methods

Materials

Embelin (98% pure) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Soya phosphatidylcholine (Phospholipon 90H) was obtained as a gift sample from LIPOID, GmbH Germany. All other chemicals used were of analytical grade.

Methods

Preparation of embelin–phospholipid complex

The complex was prepared with embelin and phospholipon 90H at a molar ratio of 1:0.5 to 1:3. Weighed amount of embelin and phospholipon 90H were taken in a 500 mL round bottom flask with 250 mL of ethanol. The mixture was kept at a temperature not exceeding 60 °C for 2 h on magnetic stirrer. The resultant solution was evaporated by spray drying method to remove organic solvent. The resultant embelin–phospholipid complex (EPC) (yield 82%, w/w) was kept in an amber colored glass bottle

flushed with nitrogen and stored in refrigerator (8–15 °C) and was verified through microscopic analysis.

Ultra high performance liquid chromatography method for estimation of embelin

Stock solution of standard embelin was prepared by dissolving 10 mg of standard embelin in 100 mL of methanol and was sonicated for 10 min to get the clear solution. The solution was filtered through 0.22 μ syringe filter and stored at –20 °C and brought to room temperature before use. Ultra high performance liquid chromatography (UHPLC) analysis was performed on a Waters Acquity UHPLC system (Milford, MA, USA) equipped with a binary solvent manager, an auto sampler, column manager composed of a column oven, a pre-column heater and a photodiode array detector. Five microliter of final analytical solution was injected into a Waters Acquity BEH C18 (50 mm \times 2.1 mm, 1.7 μ L) UHPLC column kept at 50 °C and the chromatographic separation was performed by gradient elution. The mobile phase consisting of a mixture of A: 0.1% Tri fluoro acetic acid (TFA) in water (pH 3.6) and B: methanol, with the flow rate of 0.5 mL/min was employed. The analysis was performed at 288 nm wavelength with total run time of 5 min. The LOD and LOQ data for the Embelin in the solution was found 10 ng/ μ L and 33 ng/ μ L respectively. Data acquisition, data handling and instrument control were performed by Empower Software v1.0. The details of the gradient elution programme for the analysis of embelin is mentioned in Table 1.

Calibration curve for embelin

A stock solution of embelin (100 ppm) was prepared by dissolving an appropriate amount of embelin in methanol. Working standard solution (10 ppm) of embelin was prepared daily by dilution of stock solution with methanol. To prepare the calibration standards, increased concentrations of working standard solutions to give embelin concentrations of 1–10 μ g/mL Calibration standards were processed

Table 1 Gradient elution programme for the analysis of embelin

S. no.	Time (min)	Flow rate ml/min	A (%)	B (%)	Curve
1	0.00	0.50	60	40	6
2	1.00	0.50	60	40	6
3	2.00	0.50	50	50	6
4	3.00	0.50	50	50	6
5	4.00	0.50	65	35	6

A = 0.1% TFA in water (pH 3.6); B = Methanol

according to sample preparation procedure and were analyzed by UHPLC method.

Determination of embelin entrapment efficiency in EPC

The proportion of encapsulated embelin was determined by centrifuging a 10 mL of formulation at 15000 \times g for 1 h at room temperature. The EPC was separated from supernatant and sonicated with distilled water to measure encapsulated embelin content at 288 nm. The percentage entrapment efficiency was calculated by the equation given below.

$$\%E = (ED/AD) \times 100\%$$

where %E, AD and ED are percentage entrapment efficiency, amount of added drug and amount of encapsulated drug respectively.

Apparent solubility study

Apparent solubility was determined by adding excess of embelin and EPC to 10 mL of purified water and in *n*-octanol in sealed glass containers at room temperature. The liquid was agitated for 24 h on rotator shaker then centrifuged for 15 min at 5,000 \times g to remove excessive embelin. The supernatant was filtered through 0.22 μ m membrane filter (Millipore, USA) then 1 mL filtrate was mixed with 9 mL of methanol to prepare dilutions and these samples were measured at wavelength of 288 nm by using UV-spectrophotometer and the concentration was calculated by using calibration curve.

Microscopy

To detect the physical appearance and surface morphology of the prepared complex, microscopy of complex was performed at 100 \times microscope by using Motic microscope (Motic SD digital microscope) operated with version 2.0 of Motic® *Images Plus* software.

Differential scanning calorimetry

Differential scanning calorimetric (DSC) analysis was used to characterize the thermal behavior of the pure embelin, phopholipon 90H, physical mixture of embelin & phospholipon 90H and prepared EPC. DSC thermograms were obtained using an automatic thermal analyzer system (Pyris 6 DSC, Perkin Elmer, USA) based upon Pyris thermal analysis software (v 4.01). Temperature calibration was performed using indium calibration reference standard (transition point: 156.60 °C) as a standard. Samples were crimped in a standard aluminium pans (BO14-3020 25 μ L, 0.15 mm pans) and heated at a heating rate of 10 °C min⁻¹

in the range of 30–200 °C under constant purging of dry nitrogen at 30 mL min⁻¹. An empty pan, sealed in the same way (as the sample) was used as a reference. The peak transition onset temperature of embelin, phospholipon 90H, EPC and physical mixture of embelin and phospholipon 90H were determined and compared.

Fourier-transform infra-red spectroscopy studies

Fourier-transform Infra-red (FT-IR) spectra of pure embelin, phospholipon 90H, physical mixture of embelin & phospholipon 90H and prepared EPC were obtained using a FTIR-8300 spectrophotometer (Shimadzu, Japan). A total of 2% (w/w) of sample, with respect to the potassium bromide (KBr) disc, was mixed with dry KBr. The mixture was ground or triturated into fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi for 30 s. Each KBr disc was scanned at 4 mm/s at a resolution of 2 cm over a wave number region of 4000–400 cm⁻¹ using IR solution software (ver. 1.10). The characteristic peaks were recorded for different samples and compared.

X-ray diffraction studies

Crystalline state in pure embelin, phospholipon 90H, physical mixture of embelin: phospholipon 90H and prepared EPC was evaluated in by powder X-ray diffractometry (Bruker AXS D8 Advance Diffractometer). A voltage of 40 kV and a current of 30 mA for the generator were used, with Cu as the tube anode material. The solids were exposed to a Cu-K α radiation ($\alpha_1 = 1.54060 \text{ \AA}$ and $\alpha_2 = 1.54439 \text{ \AA}$, with an α_1/α_2 ratio of 0.5), over a range of 2θ angles from 3° to 60°, at an angular speed of 0.04° (2θ) min⁻¹, using divergence and receiving slits of 1 θ and 0.1 θ ; respectively. The obtained XRD spectra were compared with respect to degree of crystallinity.

¹H-NMR spectroscopy

¹H-NMR (300 MHz C6D6, δ in ppm) spectroscopy of uncomplexed embelin and embelin–phospholipon 90H complex was carried out by using Bruker DRX-300 (300 MHz FT-NMR with low and high temperature facility, –90 °C to +80 °C) at Indian Institute of Technology (I.I.T.) Delhi, India.

In vitro release study of embelin and EPC

In vitro release of embelin and EPC was measured using dialysis method using cellophane membrane (6 kD). Activation of cellophane membrane was done in order to

remove sulphur and other impurities. Activated membrane was stored in PBS pH 7.4 until used. Embelin, 2 mg, and 2 mg of EPC was transferred to sample holder of two different diffusion cells (made up of laboratory standard glass material having total dissolution media volume of 25 mL) which receiving compartment is containing 18 mL of distilled water. The whole set is placed on a magnetic stirrer adjusted to constant speed of 150 rpm at 25 °C. At predetermined time interval (5, 15, 30, 45, 60 and 120 min) for 120 min, 1 mL of release medium was withdrawn for analysis and was compensated by same volume of fresh dissolution medium. Samples were measured at λ_{max} 288 nm.

Statistical method

ANOVA followed by Dunnett's-test were used to determine the statistical significance level at $p < 0.05$.

Results & discussion

In the present study embelin–phospholipid complex was prepared by a simple and reproducible method. UHPLC analysis was carried out for the quantification of the embelin as an analytical tool. Chromatogram showed retention time (R.T.) at 2.3 min (Fig. 2). Equation for linearity as per calibration curve was found to be in the linearity range of 1–10 $\mu\text{g/ml}$ ($y = 30762x + 101.4$; $R^2 = 0.999$) for the embelin pure solution. The very small retention time and value of R^2 (0.999) showed that the developed quantification method is less time consuming, cost effective and accurate. The entrapment efficiency for the different prepared EPC, 1: 0.5, 1:1, 1:2, 1:3 was determined as 31.8, 46.4, 60.2 and 83.4% respectively. The formulation optimization was done with respect to

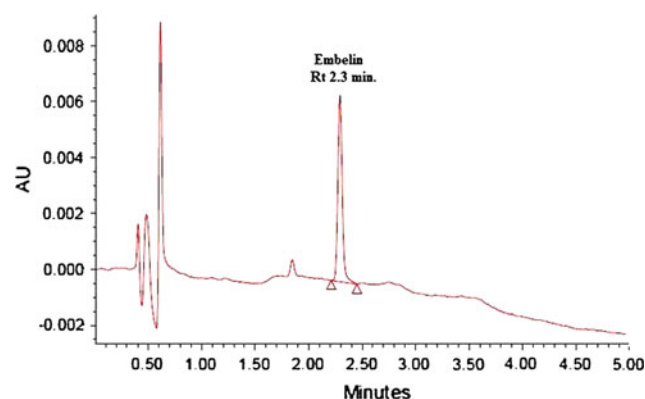


Fig. 2 UHPLC chromatogram of embelin

entrapment efficiency and aqueous solubility of the drug. In our study it was found that as the ratio of lipid increases in the formulation, the entrapment efficiency increases with the enhanced aqueous solubility. The enhanced entrapment of drug is may be due to the increase lyphophilic content that favour the encapsulation of higher concentration of lipophilic drug embelin.

Content of embelin in complex and apparent solubility study

Content of embelin in the complex (1:3), as estimated by UHPLC, was found to be 92.44% (w/w). The formed complex has higher solubility of drug in water and *n*-octanol than embelin and physical mixture of embelin and phospholipid. Apparent solubility study results showed average apparent solubility of embelin and EPC were 3, 42 $\mu\text{g/mL}$ in water and 2, 39 $\mu\text{g/mL}$ in *n*-octanol. The data suggested that solubility of EPC in *n*-octanol and water was increased multiple times as compared to pure embelin (Figs. 3 and 4).

The low solubility of drug alone and in physical mixture is due to its crystalline and high lipophilic nature. Complexing the embelin with phospholipid (EPC 1:3), imparts the amorphous character to the system and also moderates the lipophilic character along with the amphiphilic surfactant nature of the phospholipid. It is well established concept that the moderate lipophilic molecules with amorphous character have better solubility and the dissolution profile. The result of solubility and drug content were also supporting the same theory.

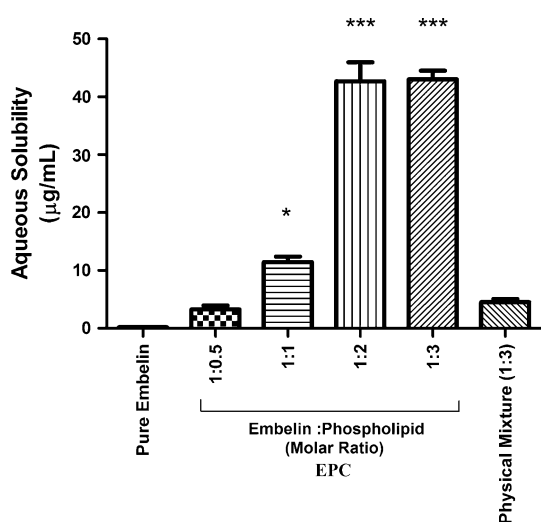


Fig. 3 Aqueous solubility study of embelin, EPC, physical mixture data represented as $n = 3$ and analysed by Dunnett's test as compared to pure embelin (control) at $* p < 0.05$

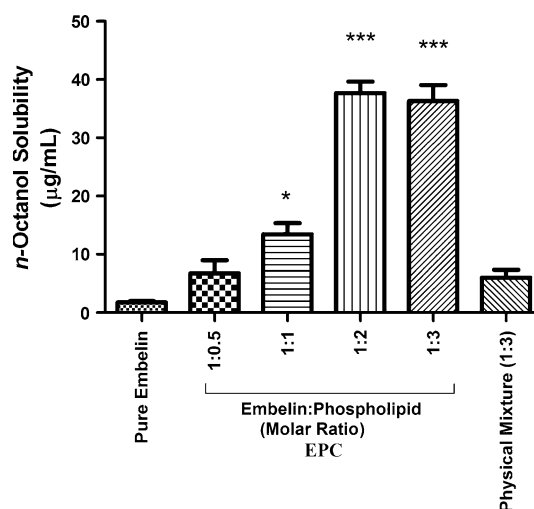


Fig. 4 *n*-octanol solubility study of embelin, EPC, physical mixture data represented as $n = 3$ and analysed by Dunnett's test when compared with pure embelin (control) at $* p < 0.05$

Embelin–phospholipid complex characterization

Morphology & microscopy studies

The microscopy the pure embelin and EPC was performed at stage microscope at 100 \times resolution. The pure embelin existing in the form of irregular crystal structures showing sharp edges. However EPC were well-identified perfect spheres and seen in disperse and aggregate collection. EPC were found in the diameter range of 0.05–0.5 μm . The microscopy of the EPC shows the encapsulation of embelin in the lipid layer (Fig. 5).

FT-IR studies

Spectrum of embelin showed bands at 3398 (OH), 3001 (CH structure) and 1776(C=O) cm^{-1} which disappeared after formulating it in phospholipid complex form. Physical mixture of embelin phosphatidylcholine spectrum showed intact bands of embelin and phosphatidylcholine thus there no chemical interaction between embelin and phosphatidylcholine as found. Whereas in EPC spectrum bands appearing at 3434.60, 2956, 1,804.62 showed modifications which confirmed the chemical interaction (Fig. 6).

¹H-NMR studies

¹HNMR spectrum of embelin was analysed in deuterated chloroform. A broad singlet peak was observed at δ 7.74 ppm assigned for hydroxyl protons protons (D_2O exchangeable). At δ 5.99 ppm. a singlet peak was found confirming a single ring proton. A triplet at δ value 2.45–2.41 ppm was assigned for two aliphatic protons nearest to the ring. One more triplet was observed at δ value

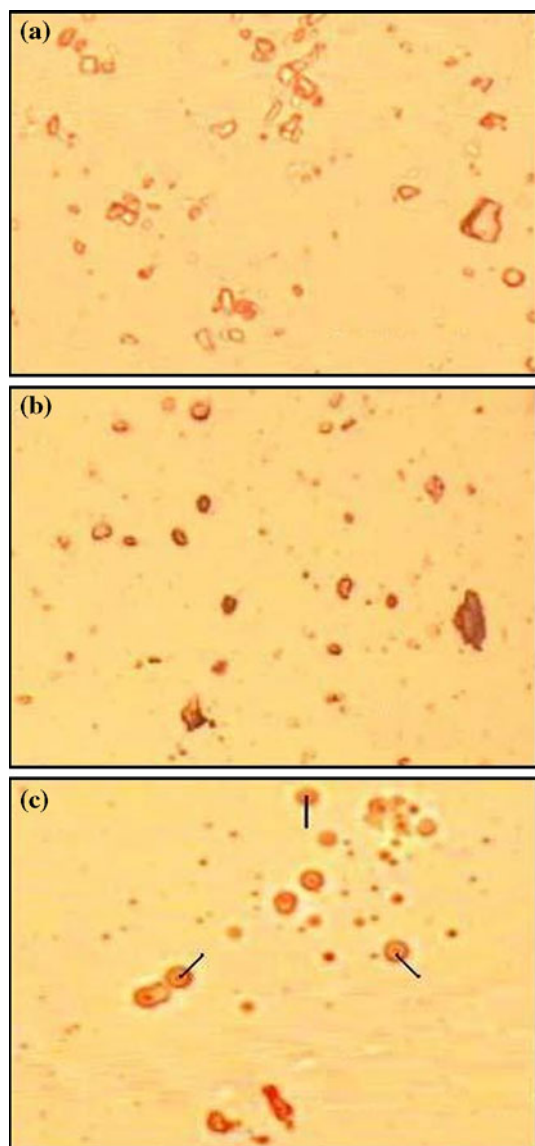


Fig. 5 Microscopic studies of **a** embelin **b** physical mixture **c** EPC

0.88–0.85 ppm and assigned for three terminal aliphatic protons. Remaining all eighteen protons was observed at 1.64–1.24 ppm. However, $^1\text{H-NMR}$ of EPC showing signals at 0.985, 1.450 and 2.99 δ (ppm) indicated the presence of methyl group protons of aliphatic side chain, methylene protons of aliphatic side chain and methylene protons attached to $-\text{C}(=\text{O})-\text{C}-$, respectively, which are the characteristic of nonpolar portion of phosphatidylcholine molecule. While the shift value at 5.131 δ (ppm) was due to second methylene group proton near N atom ($\text{CH}_2\text{CH}_2\text{N}$) of choline and signals at the 3.635 δ (ppm) was due to the proton of methyl group linked to N-atom of choline which showed significant broadening due to their involvement in complexation. Likewise, the signals of compound at 7.550 and 8.508 δ (ppm) were also involved in complexation.

Above results suggested that phenyl hydroxyl moiety of drug was complexed with choline part of phosphatidylcholine (Fig. 7).

Differential scanning calorimetry studies

Differential scanning calorimetry (DSC) is a fast and reliable method to screen drug-excipient compatibility and provides distinct information about the possible interactions. In DSC, an interaction is concluded by elimination of endothermic peak(s), appearance of new peak(s), and change in peak shape and its onset, peak temperature/melting point and relative peak area or enthalpy. Figure 8 shows the DSC thermograms of pure embelin (a), pure phospholipid (b), physical mixture of embelin and phospholipid (c), & EPC (d). The thermogram of embelin showed a single peak with an onset of 147 °C. Thermogram of phospholipids exhibit a flat thermogram. Physical mixture of embelin and phospholipids shows one sharp peak at 147 °C, at the same endotherm of the embelin. The thermogram of the complex (EPC) exhibits a single peak which differs from the peak of embelin. It is evident that the original peaks of embelin disappear in the thermogram of complex. This showed that embelin may interact with phospholipids and the interaction is hydrophobic in nature. There is also some contribution of hydrogen bonding apart from hydrophobic interaction in the embelin–phospholipids interaction. The $-\text{OH}$ groups of the phenol rings of embelin are involved in hydrogen bonding whereas the aromatic rings could be involved in hydrophobic interaction. The interaction of embelin with the polar part of phospholipids molecules make the long hydrocarbon tail of phospholipids to turn freely and ‘envelop’ the polar head of phospholipids containing the embelin molecule.

XRD studies

The XRD of embelin–phospholipid complex showed that the sharp peaks of embelin representing the crystalline nature of the embelin were disappeared and the broad peak similar to phospholipid was seen. The result confirmed the formation of phospholipid complex and it suggested that the embelin in phospholipid complex was either in amorphous form or molecularly dispersed. Unlike liposomes, bonding between drug and the phospholipids in development of embelin–phospholipid complex, might have resulted into the significant change of its X-ray diffraction pattern (Fig. 9).

In vitro study

The EPC showed better dissolution profile than the free embelin. Unlike the free embelin (which showed a total of

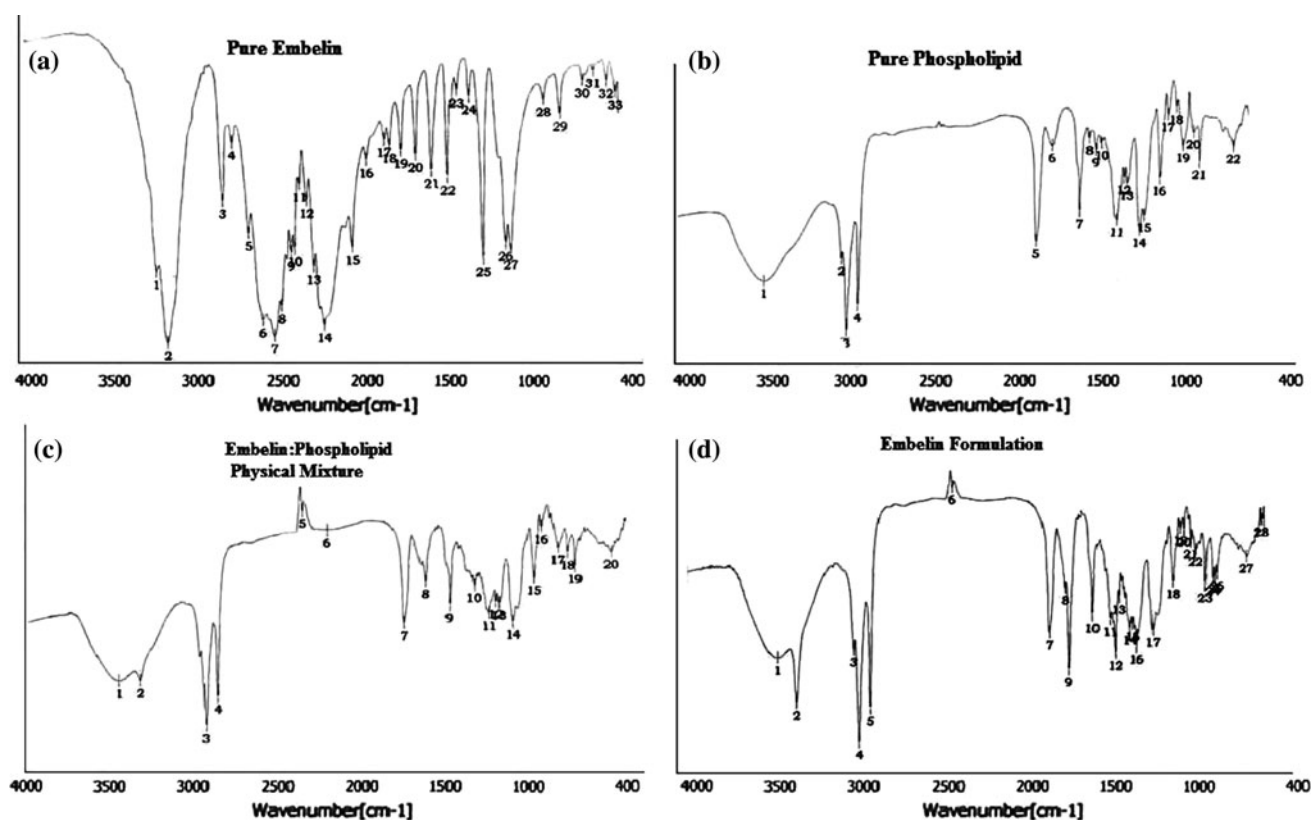
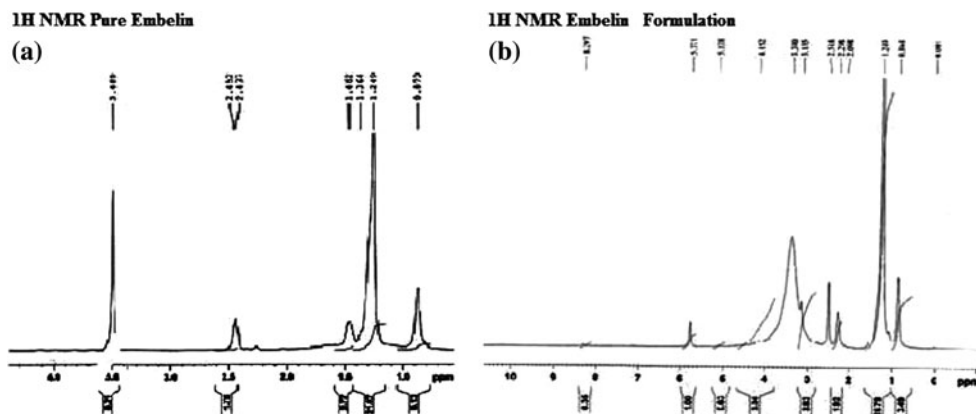


Fig. 6 FTIR spectra of a pure embelin b pure phospholipid c physical mixture d EPC

Fig. 7 ¹H NMR Spectra of a Pure embelin b EPC



only 19% drug release at the end of 120 min), EPC (1:3) showed 99.80% release at the end of 120 min of dissolution study in distilled water(Fig. 10). On the other hand, formulations at various ratios of embelin, phospholipids were showing significantly less embelin release as compared to EPC (1:3). The physical mixture also showed less embelin release as compared to EPC (1:3) justifying the effect of process on the embelin behaviour from crystalline to amorphous. Phospholipids being an amphiphilic surfactant increased the solubility of the embelin by wetting and dispersion phenomena, therefore the dissolution profile of the complex was found to be improved [31].

Conclusion

In the present study, we prepared embelin–phospholipid complex by a simple and reproducible method. The physicochemical investigations showed that embelin formed a complex with phospholipids. The complex EPC has enhanced aqueous and *n*-octanol solubility, compared with embelin and the physical mixture. FTIR, DSC and XRD curves of phospholipids complex showed that embelin and phospholipids form complex due to formation of bonds like hydrogen bonding or Vander Waals force. Release profile shows the percent release of embelin from EPC was much

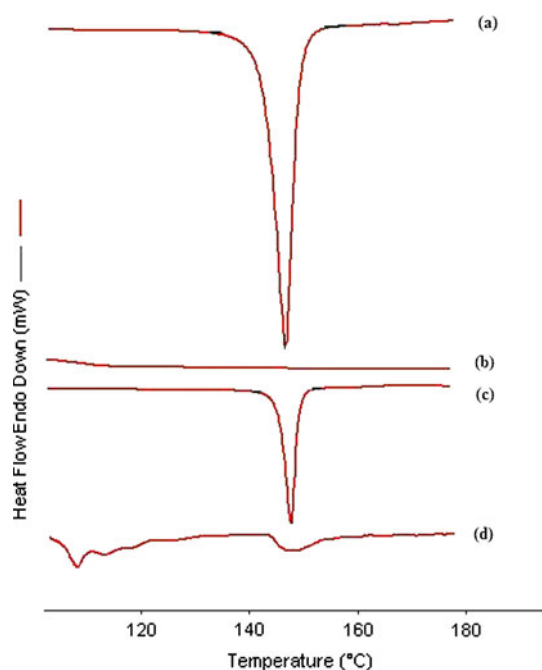


Fig. 8 DSC thermograms of pure embelin (a), pure phospholipid (b), physical mixture of embelin and phospholipid (c), & EPC (d)

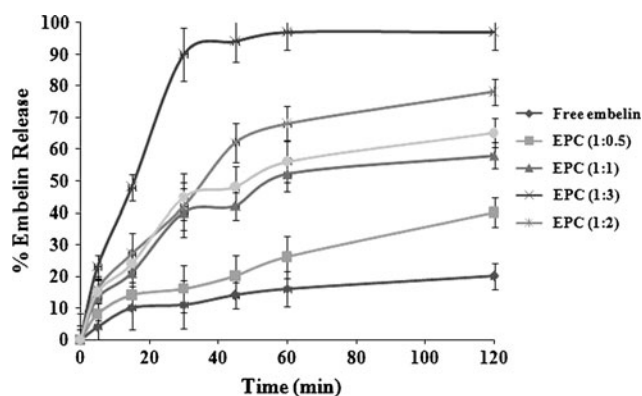


Fig. 10 In-vitro release profile of free Embelin, EPC and physical mixture (error bar; $n = 3$)

higher than the pure embelin. Our studies illustrated the potential of embelin–phospholipid complex as a novel drug delivery tool for effective and improved delivery of poorly soluble phytoconstituents like embelin. Yet, novel delivery strategy of phospholipid complexation offer significant promise and is worthy of further exploration in attempts to enhance the medicinal value, and application of this interesting molecule from Mother Nature.

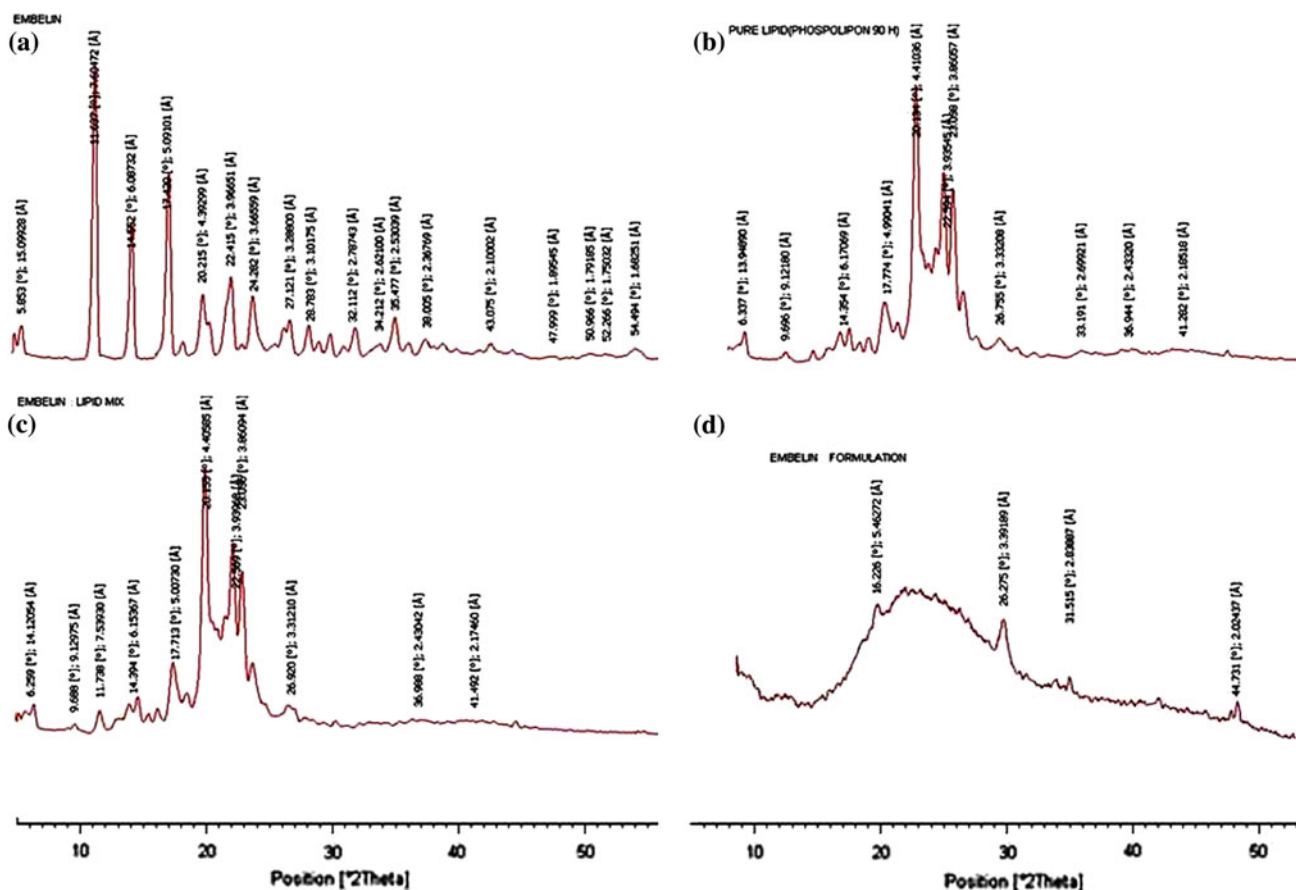


Fig. 9 XRD diffractograms of pure embelin (a), pure phospholipid (b), physical mixture (c), & EPC (d)

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