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Development and *in vitro* characterization of embelin bilosomes for enhanced oral bioavailability

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ABSTRACT: The goal of this study was to formulate a nanotechnology-based system incorporating surfactants, cholesterol (CHL), and sodium deoxycholate (SDC) and optimization was done using central composite design of design-expert® software. The variables used were X1 (Surfactant; Span 80) and X2 (Bile salt; SDC). *In vitro*, release kinetics assessment of the drug revealed an increase in drug release of the drug. Transmission electron microscopy (TEM) exhibited a round shape of developed bilosomes with few having rough surfaces. Fourier Transform Infrared Spectroscopy (FT-IR) data exhibited no specific physiochemical interaction between active and additives. Differential scanning calorimetry (DSC) studies showed the molecular state and the indication of no interactions among the formulation ingredients. The mean vesicle size, polydispersity index, zeta potential, and entrapment efficiency (%) of optimized bilosome formulation were observed to be 211.1 nm, 0.513, 47.8 mV, and 99.664 % respectively. Overall, the obtained results confirmed that Embelin-loaded bilosome could be promising for oral drug delivery.

KEYWORDS: Embelin; bilosomes; nanocarriers; anti-microbial; entrapment efficiency; zeta potential; particle size

1. INTRODUCTION

Embelin (EMB) is a benzoquinone found in *Embelia ribes*, a medicinal plant also known as false black pepper. It belongs to the myrsinaceae family and holds medicinal potential. It is found in several regions of India, including the central and lower Himalayan regions [1]. Embelin is widely used in various Ayurvedic formulations and is mentioned in ancient Ayurvedic texts like the Sushrutha Samhitha, Ashtanga hridayam, and Charakh Samhita. In addition to being utilized in Ayurvedic formulas, E. ribes is also used in Tibetan (Byidanga), Unani (Baobarang), Siddha (Vaivilangam), etc. It has a variety of uses, including wound healing, anti-microbial, anti-inflammatory, and antioxidant effects. It has shown that Embelin exhibits anti-tumorigenic properties against different cancer types [2].

Innovative vesicular delivery system i.e. bilosomes, improves the medicinal drugs' payload and colloidal stability. It has a good ability to pass across cellular membranes and is made up of bile salt. Bile salt improves permeation and prevents bilosomes from degrading in the digestive system. Bile salts stabilize the vesicles, inhibit the effects of gastric acid, and have permeability enhancer properties. Bilosomes have a dual action: they enhance uptake by intestinal cells and inhibit enzymatic activity at the absorption site. Compared to liposomes and niosomes, bilosomes can entrap medicines, boost bioavailability, and maintain higher vesicle integrity; whereas it exhibits less gastrointestinal discomfort and little medication leakage [3].

This research article aims to develop an innovative carrier such as bilosomes, to improve the absorption of Embelin, which are resistant to disruption by digestive enzymes and allow increased drug absorption owing to higher tissue penetration, since the upper portion of the small intestine is the drug absorption site. Additionally, oral administration of bilosomes might enable a decrease in medication dosage [4]. The study aimed to develop bilosomal systems loaded with EMB to increase EMB's bioavailability by encouraging oral administration [5].

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The recent research has shown that bilosomes have gastrointestinal stability and they attain higher absorption by intestinal cells on account of their deformable structure.These properties of bilosomes are ideal. Hence this research aimed to formulate Embelin loaded bilosomes so that Embelin can reach the upper portion of the small intestine which is the main absorption site of the drug. Therefore the bioavailability of Embelin is enhanced and an innovative carrier system is developed.

2. RESULTS AND DISCUSSION

2.1. Analysis of Factorial Design

Factorial designs were used to analyze factors affecting delivery system characteristics, based on exploratory trials. Surfactant type (Span 80) (X1) and bile salt concentration (SDC) (%, w/v) (X2) were determined as key independent variables. Predicted R2 values closely matched adjusted R2 (Table 1), confirming model validity. The impact of X1 and X2 on vesicle properties (PS, PDI, ZP) was graphically represented (Figures 1-3), with results summarized in Table 2.

Table 1. The output data from the central composite design analysis of investigated Embelin bilosomal systems was examined

Investigated responses	Source (Model)	R^2	R^2 Adjusted	R^2 Predicted	Sequential p-value	Remark
Particle size (PS) (nm)	**Linear**	**0.37**	**0.24**	**0.20**	**0.097**	**Suggested**
	2FI	0.51	0.35	0.24	0.139	
	Quadratic	0.51	0.21	1.31	0.813	
Polydispersity index (PDI)	Linear	0.12	0.04	0.47	0.50	
	2FI	**0.2549**	**0.0066**	**0.58**	**0.248**	**Suggested**
	Quadratic	0.35	0.10	1.42	0.50	
Zeta potential (ZP) (mV)	**Linear**	**0.1760**	**0.0112**	**0.6083**	**0.3798**	**Suggested**
	2FI	0.18	0.08	1.40	0.79	
	Quadratic	0.27	0.21	3.00	0.002	

Note: X1 surfactant type, X2 sodium deoxycholate concentration (% w/v)

Span 80 (sorbitan monooleate) as a surfactant was chosen due to its ability to stabilize the bilosome formulation by lowering the interfacial tension between the bilayer components and the aqueous phase. Span 80 is preferred over Span 60 in this study due to its superior ability to create smaller, more uniformly distributed vesicles with higher drug entrapment efficiency and adequate zeta potential. This leads to a more stable and effective bilosome formulation for the oral delivery of Embelin. Span 80's ability to reduce interfacial tension and its hydrophobic nature make it an ideal choice for enhancing the bioavailability of hydrophobic drugs in nanocarrier systems

2.2. Optimization

Thirteen experimental runs were conducted based on a central composite design, including five center points with identical composition, as depicted in Table 2. The experimental data obtained were input into software for optimization. The PS, ZP, and PDI of EMB-BIL formulations ranged from 211.1 to 1833 nm, 36.0 to 59.0 mV, and 0.1045 to 1.251, respectively. The impact of independent variables on dependent variables was analyzed graphically through 3D response surface plots (Figure 1a-c), enabling the simultaneous expression of the effects of multiple variables on one response. Various experimental models were fitted to the results of each dependent variable to determine the best-fit model. The linear model exhibited the best fit, as indicated by the highest regression value (R^2) compared to other models [17].

2.3. Effect of Span 80 and Sodium deoxycholate on Particle size

The vesicle size of all prepared EMB-BIL formulations ranged from 211.1 to 1833 nm (Table 2). The one-factor graph, 3D response surface graph, and contour graph (Figure 1a-c) illustrated the impact of independent variables on the size. Surfactant concentration exhibited a negative effect on particle size, as an increase in surfactant concentration reduced the interfacial tension between cholesterol and the aqueous phase, resulting in size reduction. Similarly, bile salt concentration also demonstrated a negative effect on vesicle size, attributed to decreased surface tension and increased flexibility of BIL with increasing bile salt concentration. However, at high concentrations, bile salt tended to form aggregates [18, 19]. The predicted R^2

of 0.2 reasonably aligns with the adjusted R^2 of 0.24, indicating that the model provides an adequate signal (Table 1). The graphical presentation (Figure 1 (a, b, c)) illustrated the actual and predicted values.

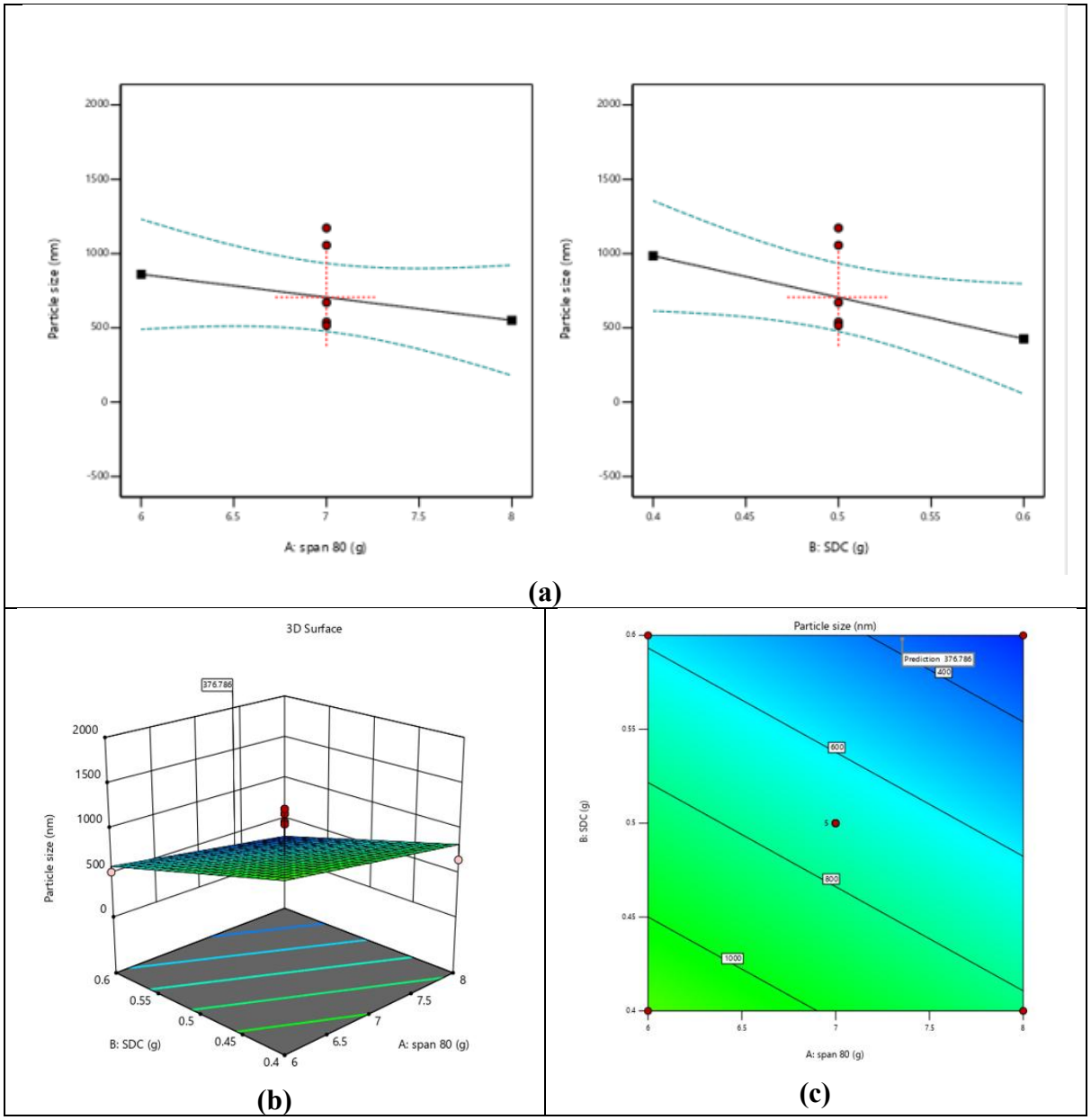

Figure 1. (a) One-factor graph; (b) 3D response surface graph, and (c) countor graph showing the effect of an independent variable over the particle size

2.4. Effect of Span 80 and sodium deoxycholate on Zeta potential

The ZP value indicates particle charges and reflects dispersion stability. Ideally, a ZP value around ±30 mV ensures sufficient charge repulsion among particles for system stability. The anionic charges observed in our systems are attributed to the inclusion of SDC in the vesicular constructs [20]. The obtained ZP values ranged from 36.0 mV to 59.0 mV, indicating that most systems possess adequate charges to prevent vesicle aggregation. Surfactant concentration significantly influenced ZP value whereas SDC concentration does not affect ZP value. The surfactant showed a negative effect on zeta potential. The zeta potential of particles increases with an increase in cationic surfactants as they form a diffused layer around the particles, whereas the ZP decreases with non-ionic surfactants as they tend to get adsorbs at the particle-liquid interface [21]. This suggested that with an increase in the non-ionic surfactant concentration i.e. span 80, used in BIL formulation, resulted in decreased zeta potential value (Figure 2 a-c).

Table 2. The experimental runs and corresponding response variable values obtained for the prepared Embelin bilosomes were represented in a design matrix

Run	Factor 1 (X1): Span 80	Factor 2 (X2): SDC (mg)	Response 1 Particle size (PS) (nm)	Response 2 Zeta Potential (ZP)(mV)	Response 3 Polydispersity index (PDI)
O1	7	600	622.4	36.9	0.561
O2	7	500	539.1	47.2	0.2974
O3	8.4	400	1833	52.4	1.251
O4	7	400	655.5	36	0.1465
O5	6	641	211.1	47.8	0.513
O6	8	358	716.4	56.2	0.2539
O7	6	500	417.1	59	0.1623
O8	7	500	1172	49.6	1.051
O9	7	500	670.6	54.3	0.2576
O10	7	500	1056	43.3	1.029
O11	8	500	1153	55.0	1.107
O12	7	600	453.7	44.4	0.1045
O13	6	500	515.6	56.8	0.2215

2.5. Effect of Span 80 and Sodium deoxycholate on Polydispersity index

A polydispersity index (PDI) of zero indicates optimal and consistent particle distribution, while a PDI of 1 signifies wide polydispersity and heterogeneous systems. Among the vesicular systems, seven exhibited PDI values less than 0.5, indicating limited size distribution and good uniformity, while two systems had PDI values around 0.5. The factors influencing PDI were consistent with those affecting particle size (PS). Surfactant concentration demonstrated a negative effect on PDI, with increasing surfactant concentration leading to decreased PDI values. Similarly, SDC also contributed to decreasing PDI values, albeit to a lesser extent.

2.6. Effect of formulation variables on entrapment efficiency percentage

EE% reflects the ability of vesicles to encapsulate EMB. EE% ranged from 99.42% to 99.77%, with higher values observed in Span® 80-based bilosomal systems at specific surfactant and SDC concentrations. Surfactant type (X1) significantly impacted EE%, with higher concentrations of Span 80 leading to increased entrapment efficiency [5]. The increase in Span 80 concentration rendered bilosomes more hydrophobic, reducing EMB leakage into the outer aqueous medium and thus enhancing entrapment efficiency. SDC concentration (X2) did not notably affect EE% [22].

2.7. Transmission Electron Microscopy

TEM image of the optimized bilosomal formulation, O5 (Figure 4), displayed spherical vesicles, some of which exhibited a rough surface. This indicated that the optimized batch maintained a typical bilayer structure characteristic of vesicular nanosystems. Additionally, TEM revealed vesicles within the nanometric range (under 100 nm), confirming the formulation as a nanobased formulation.

2.8. Solid-State Characterization of EMB-Loaded Bilosomes

2.8.1. Differential scanning calorimetry (DSC)

DSC thermogram of pure Embelin and optimized batch O5 were analyzed and depicted in Figure 5. The DSC thermogram of the optimized batch showed an endothermal peak at 152.3 Cel-54.95mW and pure Embelin showed an endothermal peak at 147.3Cel-9.06mW respectively within the temperature range from 25°C to 250°C. The optimized batch showed an endothermal 152.3Cel-54.95mW peak compared with the pure drug peak at 147.3Cel-9.06mW (Figure 5). This change was found in the physical mixture (Figure 5). These results possibly indicated that there was no interaction among the excipients in the physical mixture.

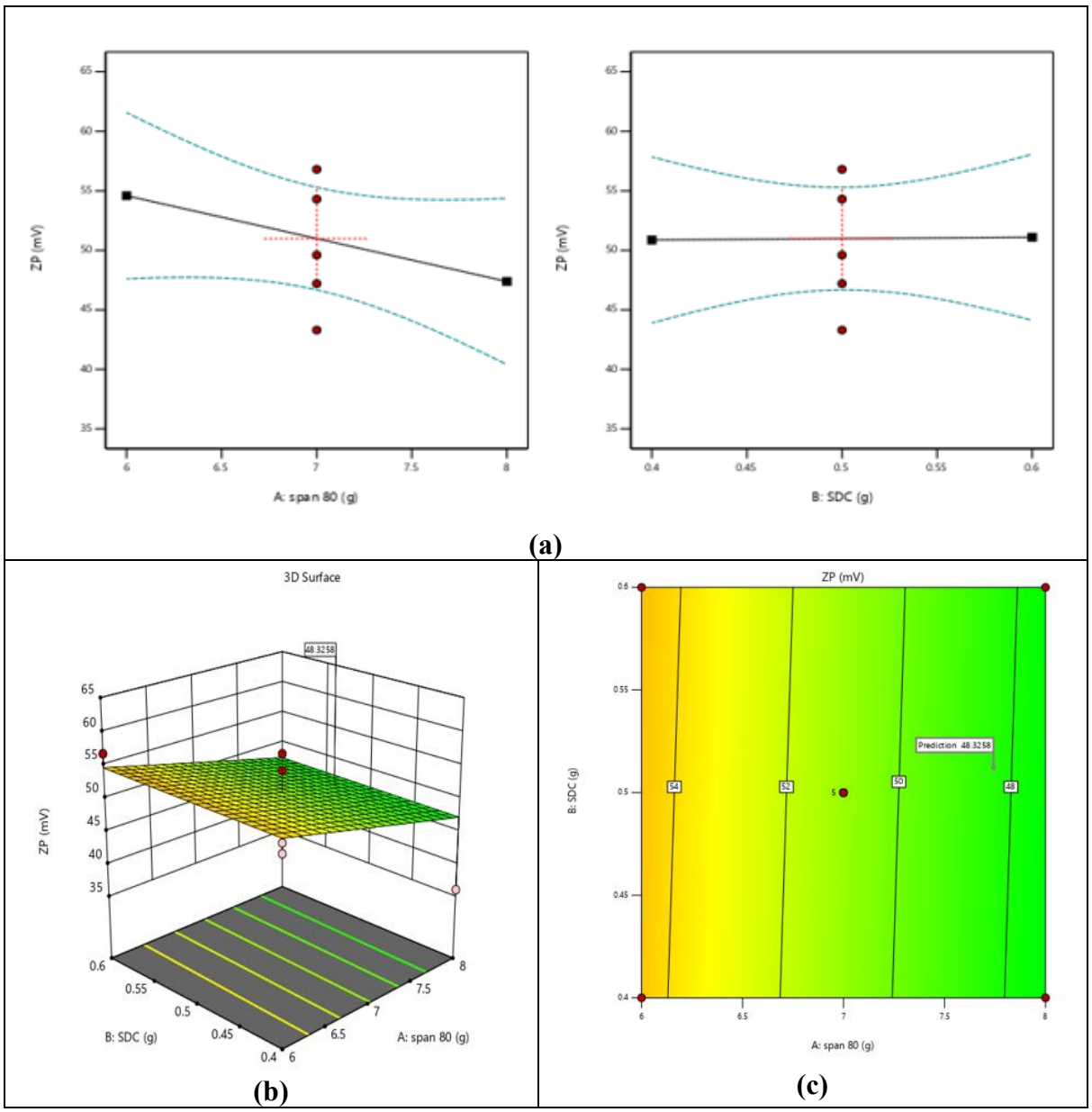

Figure 2. (a) One factor graph; (b) 3D response surface graph, and (c) countor graph showing effect of independent variable over the Zeta potential

2.8.2. Fourier Transform Infrared Spectroscopy (FTIR)

Embelin-loaded optimized Batch (O5) showed no specific physiochemical interaction when compared with the FTIR spectra of pure Embelin (Figure 6). It was noted that all significant peaks corresponding to the functional group of the drug were evident in the physical mixture. No significant variance was detected in the wave number (cm-1) of the drug, but only the drug intensity in the optimized formulation is less, as the total concentration of the drug added was less and also broadening effect was observed [23].

2.9. In Vitro Drug Release Study

The observations revealed that the in vitro release of an optimized batch of bilosomal system (O5) (represented by a blue line in Figure 7.) exhibited nearly 81% of the release of Embelin in nearly 240 min; whereas, in pure Embelin solution where no formulation strategy was exhibited, the release of Embelin was hardly 7% in 240 min. This reveals the tremendous release of Embelin, a poorly soluble component when it was converted into a nanobased formulation with the help of span 80 as a surfactant and SDC as a bile salt.

Thus this represents 12 times enhancement in the drug release when Embelin is been converted into a nano-based formulation [24-27].

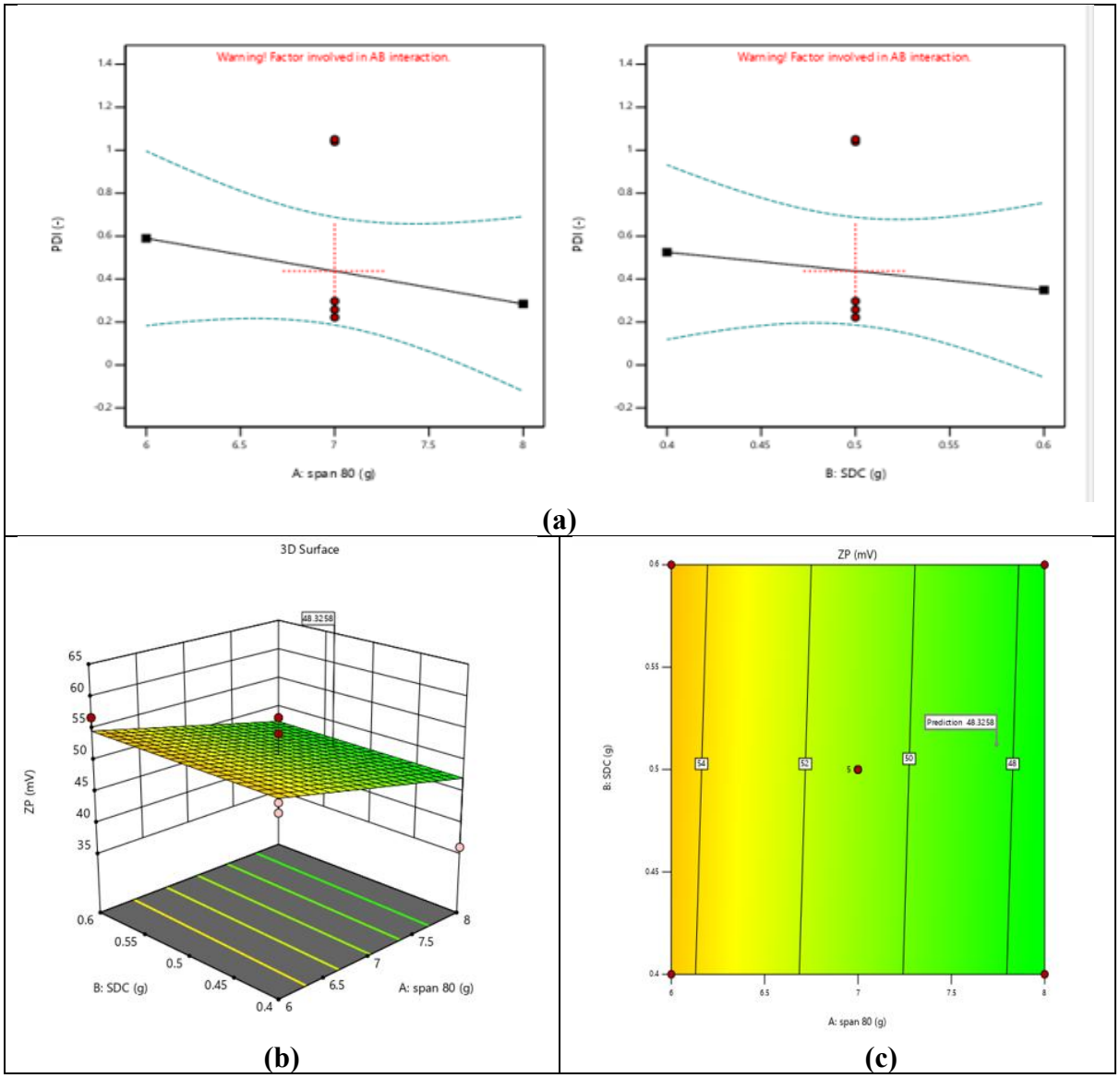

Figure 3. (a) One factor graph; (b) 3D response surface graph, and (c) countor graph showing effect of independent variable over the Polydispersity index

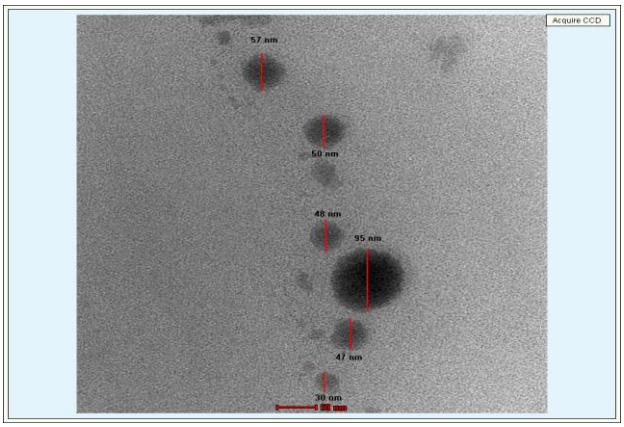

Figure.4. Transmission electron microscope's micrographs of EMB-loaded bilosomes

3. CONCLUSION

In this study, the ability of nano-based systems to efficiently enhance the *in-vitro* release of EMB was studied. Using design-expert® software central composite design was employed for optimization of formulation variables X1 (Surfactant; Span 80) and X2 (Bile salt; SDC). The nano-based systems revealed that all vesicles had spherical shape with a typical bilayer structure. Good EE% along with high *in-vitro* drug release over time was observed when compared with EMB solution. The FTIR studies indicated no specific physicochemical interaction between pure EMB, placebo formulation and optimized batch of EMB loaded nano formulation. This led to inclusion of EMB loaded nano formulation in the field of pharmaceutical drug delivery for effective release of EMB.

Substances like surfactants or bile salts can temporarily disrupt the intestinal epithelium, allowing better absorption of the drug which eventually increases oral bioavailability. The study focused on developing and evaluating Embelin-loaded bilosomes to enhance oral bioavailability. By employing factorial design and optimization using central composite design, the researchers investigated the effects of surfactant (Span 80) and bile salt conc. (SDC) on bilosome characteristics. The formulated bilosomes exhibited properties like spherical shape, typical bilayer structure, high entrapment efficiency, and enhanced in vitro drug release. Characterization studies using TEM, DSC, and FTIR provided additional insights. Based on *in vitro* study data, research work emphasizes good release which can be co-related with further enhanced bioavailability. While the study can explicitly mention improvements in oral bioavailability through *in vivo* study and clinical trials, it suggests potential for future research or clinical trials to explore this aspect.

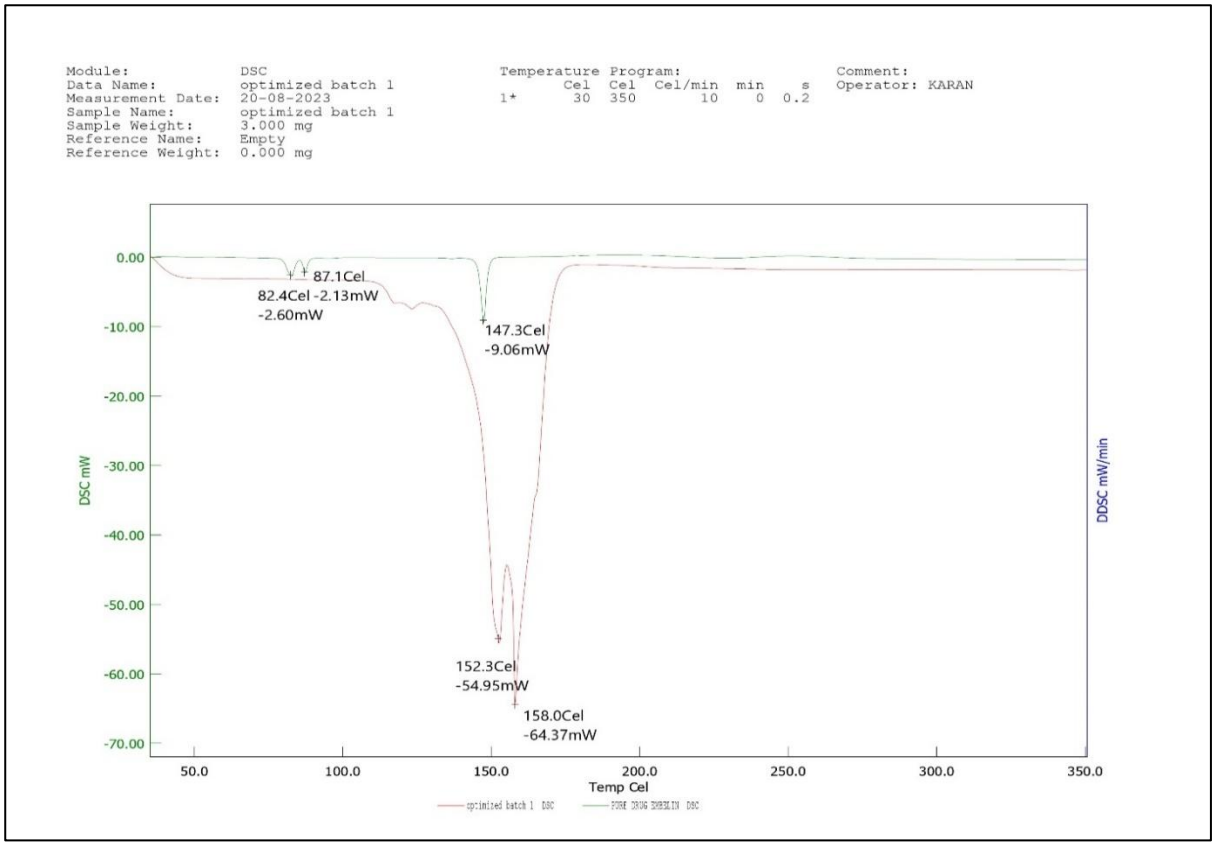

Figure 5. DSC thermograms of A) pure Embelin and B) Embelin-loaded optimized Batch (O5)

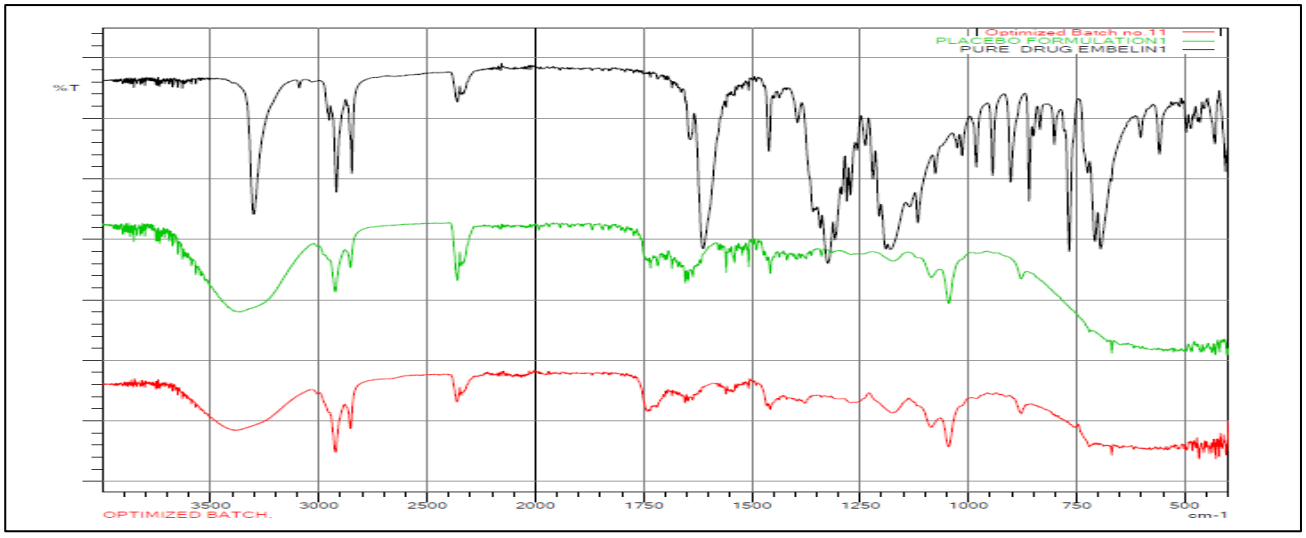

Figure 6. FTIR spectra of (A) pure drug Embelin; (B) placebo formulation, and (C) Embelin-loaded optimized Batch (O5)

4. MATERIALS AND METHODS

4.1. Materials

Embelin is isolated in the research lab at Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai, and crude drug (Vidanga) is purchased from Yucca Enterprises, Wadala (E), Mumbai. Sorbitan monooleate and cholesterol LR were purchased from Fine Chemical Industries, Mumbai, India. Sodium deoxycholate was acquired from Loba Chemie Pvt. Ltd., Cuffe Parade, Mumbai. Sorbitan monostearate was acquired from S D Fine Chem Ltd, Worli, Mumbai, and ethyl alcohol was purchased from Gogia and Company, Mumbai.

4.2. Methods

4.2.1. Preparation of Embelin-loaded bilosomes

Bilosomes loaded with EMB were formulated through the thin-film hydration technique with sorbitan monostearate, cholesterol (if present), and sodium deoxycholate (SDC) [6]. The examined variables comprised the type of surfactant (Span 60® or Span 80®), the molar ratio of surfactant to cholesterol (7:0, 7:1, and 7:3), and the concentration of SDC (ranging from 0.4% to 5% w/v). Variables investigated included surfactant type (span 60® or span 80®), surfactant/cholesterol molar ratio (7:0, 7:1, and 7:3), and SDC concentration (0.4 to 5% w/v). Ethyl alcohol was used to dissolve Embelin, surfactant, and cholesterol (if present), followed by evaporation under reduced pressure to form a film [7]. Hydration with SDC solution yielded EMB-loaded bilosomes, which were sonicated to achieve uniform dispersion. The prepared bilosomes were stored at 4°C until further use (Tables 2 & 3).

5. CHARACTERIZATION OF EMBELIN-LOADED BILOSOMES

5.1. Evaluation of Polydispersity Index, Particle Size, and Zeta Potential

Photon correlation spectroscopy (MICROTRAC Nanotrac Wave II, USA) was employed to ascertain the average particle size (PS) of bilosomes, which assesses changes in scattered light intensity caused by particle motion [8]. Each formulation was diluted tenfold with deionized water before analysis.

5.2. Determination of Embelin Entrapment Efficiency Percentage

The % EMB entrapped in bilosome was verified by assessing the free EMB. A few milliliter of developed bilosome was spun at room temperature and 10,000 rpm for 1 hour [9-11]. The clear liquid at the top was separated, and its UV absorbance was assessed at λ_{max} 282 nm (SHIMADZU UV1800, software UVPROBE 2.5; Kyoto, Japan). Embelin EE % was computed accordingly.

$$\text{EMB EE\%} = (a\text{-}b/a) \times 100$$

Where, a : Total amount of Embelin

b : Amount of Free Embelin

Table 3. Preparation of Embelin-loaded Bilosomes

Code	X1 Surfactant type	X2 Span: CHL molar ratio	X3 SDC conc. (%w/v)	Particle size (nm)	Zeta Potential (mV)	PDI
B1	Span 60	7:0	2.5	636.8	43.2	0.306
B2	Span 60	7:0	5	810.3	26.9	0.694
B3	Span 60	7:1	2.5	2757	39.3	0.578
B4	Span 60	7:1	5	707	29.6	0.1339
B5	Span 60	7:3	2.5	2635	37.1	0.409
B6	Span 60	7:3	5	2323	45.5	2.273
B7	Span 80	7:0	2.5	1073	7	0.0315
B8	Span 80	7:0	5	394.1	50	0.1077
B9	Span 80	7:1	2.5	1390	40.9	1.637
B10	Span 80	7:1	5	755.5	73.2	0.621
B11	Span 80	7:3	2.5	1258	39.3	1.865
B12	Span 80	7:3	5	1385	49.5	1.587

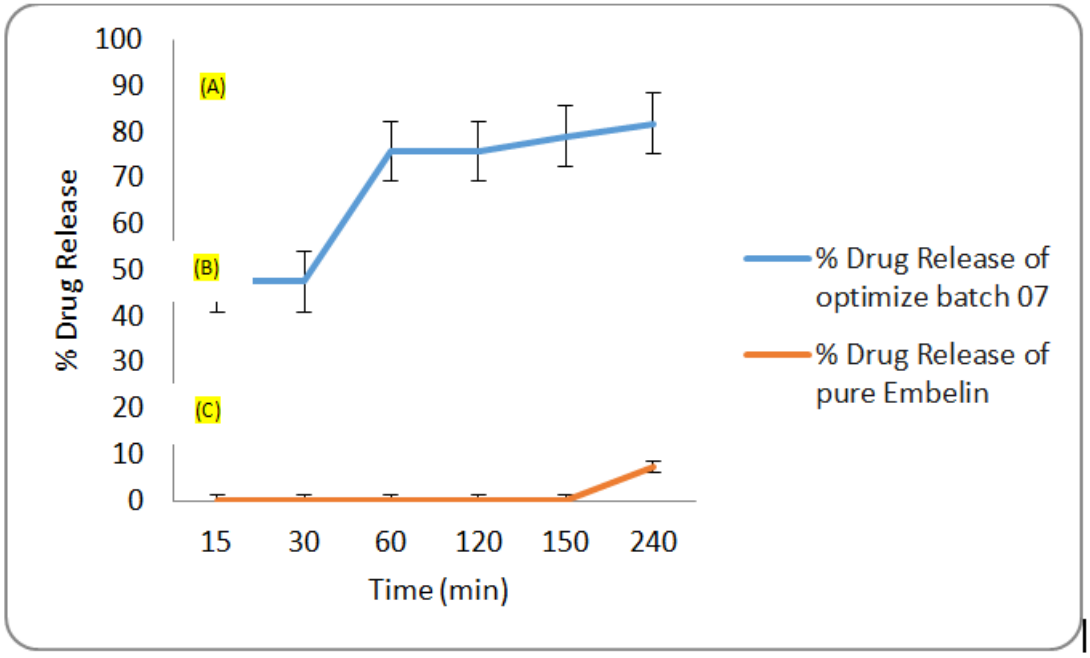

Figure 7. *In vitro* drug release study of Embelin-loaded optimized Batch (O5), and pure drug Embelin

5.3. Transmission Electron Microscopy (TEM)

The structural features of a typical EMB-loaded optimized bilosome were analyzed using TEM (Tecnai 12 120KV; San Diego, California). A diluted dispersion of the chosen bilosomal system was placed on a copper lattice, negatively tinted with 1% phosphotungstic acid (w/v), and dried in ambient conditions at 25°C for 10 min and then subjected to analysis [12, 13].

5.4. Solid-State Characterization of EMB-Loaded Bilosomes

5.4.1. Differential scanning calorimetry (DSC)

The thermal behavior of pure EMB and the refined bilosomal formulation, which includes a physical blend of EMB with additional bilosome components like SDC and Span® 80, were evaluated using DSC (HITACHI DSC 7020, software NEXTA; Tokyo, Japan). Samples of 3 mg were accurately measured and deposited into aluminum pans. The thermal span for heating extended from 30 to 350°C at a sampling frequency of 10°C/min [14].

5.4.2. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR data sets of pure EMB were analyzed, along with the optimized EMB-loaded bilosomal system i.e. O5 bilosome and placebo bilosome were recorded on FTIR Spectrometer (SHIMADZU FTIR 8400 S, software LABSOLUTIONS; Kyoto, Japan) [15, 16].

5.5. In Vitro Drug Release Study

The drug liberation mechanism was explored through UV-Vis spectroscopy employing a dialysis technique. A dialysis bag sourced from Himedia, with a diameter of 17.5mm molecular weight 12,000 to 14,000 Da, and an aperture of 2.4nm, was submerged in a buffer solution of pH 6.8 before initiating the study. Consequently, 500 mL of the dissolving medium was utilized to guarantee the drug's solubilization for a full day to preserve the sink condition. The dialysis sac that had been pre-treated was filled with Embelin bilosomal optimized batch (O5), equivalent to 5 mg of Embelin, and then placed in 500 mL of freshly prepared phosphate buffer at 37 ± 1 °C. The digital magnetic stirrer's rpm was adjusted to 100, and aliquots containing 5 ml were taken out at prearranged intervals of time (15, 30, 60, 120, 180, and 240 minutes). Another pre-treated dialysis sac was filled with a pure solution of Embelin equivalent to 5 mg content for comparison purposes. To keep the sink condition in place, an equivalent amount of phosphate buffer was substituted. Finally, a UV-VIS spectrophotometer set to 282 nm was used to analyze the aliquots and quantify the amount of Embelin present. Comparisons were made between the pure Embelin and optimized batch of EMB-loaded bilosome and the release pattern was studied in (Figure 7) [16].

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Conflict of interest statement: According to the authors, there are no conflicts of interest related to the publication of this manuscript.

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