

A NOVEL PHYTO FORMULATION OF BACOPA PHOSPHOLIPID COMPLEX SPECTRAL CHARACTERIZATION AND ITS PHARMACOKINETIC STUDIES

Abstract

A novel phyto formulation, *Bacopa* phospholipid complex (BPC) was developed, its physicochemical properties and pharmacokinetic studies were evaluated in albino rats for its improved absorption and bioavailability, and compared with *Bacopa monniera* extract (*Bm*). The current study comprises the analytical parameters such as Phase contrast microscope, SEM, TEM, AFM, FTIR, XRD, Zeta potential and TGA/DTA were used to characterize the BPC. The structure of BPC was assed as spherical under microscopic view, asymmetrical and spheroid shape vesicles under SEM, in which *Bm* was embedded in the phospholipids. TEM images depict the formation of vesicular structure and strong physical entanglement of *Bm* and phospholipids, 2D & 3D surface morphology was observed in AFM. The FT-IR spectroscopy showed the characteristic peaks of functional groups of bioactive compounds that are present in BPC. Likewise, the crystalline nature of the *Bm* and BPC was demonstrated by XRD. The particle size of the BPC was evaluated as 350.6 nm on average and zeta potential value was -36.6 mV which endorse the stability of the complexation. Further, the thermal behavior of BPC was observed through TGA/DTA. In pharmacokinetic studies, higher serum concentrations of Bacoside -A3 (1.08 mg/mL), Bacopaside II (5.59 µg/mL), Jujubogenin (1.8 µg/mL), Bacopasaponin - C (1.18 µg/mL) and Bacopaside – I (2.4 µg/mL) were observed and compared with *Bm*. Hence, BPC has shown enhancement

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of absorption and bioavailability compared to *Bm* in rats. Therefore, the *Bacopa* phospholipid complex (BPC) is beneficial as a functionalized material for analytical purposes as well pharmaceutical ingredient.

Keywords: *Bacopa* Phospholipid Complex (BPC); TEM; AFM; Pharmacokinetic Studies.

I. INTRODUCTION

Besides the traditional use of herbal medicines, extensive research has been conducted to establish a pharmacological basis of action and drug delivery, aiming to provide scientific insights [1]. Notably, the bioavailability of phytoconstituents is often limited, leading to reduced efficacy and inadequate systemic delivery [2]. Additionally, water solubility plays a pivotal role in modulating the delivery characteristics of phytoconstituents, thereby affecting crucial pharmacokinetic properties such as absorption, diffusion, release rate, and overall drug delivery [3]. Nevertheless, currently various approaches such as solid dispersions [4], pro-drug strategies [5], cyclodextrin complexation, and phospholipid complexation [6,7], solvent deposition [8] have been suggested to overcome this problem. Among these, the phospholipid complexation was an advanced and prominent replica for enhanced bioavailability, drug-loading capacity, and controlled drug release with significant clinical advantages [9-11]. Due to the amphiphilic nature of the phospholipids, choline binds to the phytoconstituents which are further surrounded by the hydrophobic part of the phospholipid *i.e.* phosphatidyl portion. Thus, the phospholipids are appropriate to practice as excipients for hydrophobic drugs/phytoconstituents [12]. The phospholipid complex not only enhances the bioavailability, but also influences the aqueous solubility, phytoconstituents/drug uptake and release, and provides protection against drug destruction in the oral cavity [13]. *Bacopa monniera* (*Bm*), is an ayurvedic medicine used to treat various ailments including insomnia, epilepsy, and anxiety, is endorsed as a mild sedative and memory booster [14]. Moreover, various studies have shown that Brahmi (common name of *Bm*) plays a substantial role in the prevention/treatment of neurological diseases and enrichment of cognitive conditions [15]. Further, a recent study disclosed that the ethanolic extract of *Bm* contains five dammarane saponin glycosides *viz.* Bacoside A3, Bacopaside-II, 3-*O*-[α -L-arabinofuranosyl (1 \rightarrow 2)- β -D-glucopyranosyl-(1 \rightarrow 3)]- α -L-arabinopyranosyl] jujubogenin, Bacopasaponin C and Bacopaside I [14].

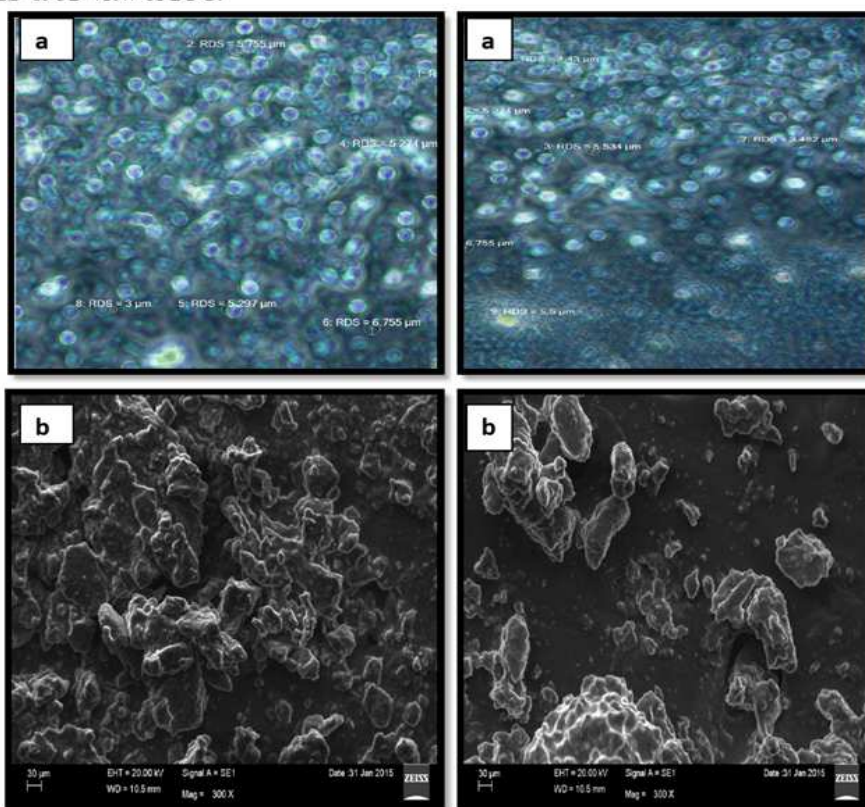
Equally, the evaluation of novel bioactive constituents such as liposomes, polymeric nanoparticles, phytosomes have significance in the development of Phyto-formulations. Nevertheless, the Phytoconstituents complexed with phospholipids maintained enhanced solubility, stability, bioavailability, absorption, and permeation of phytoconstituents through safeguard(s) destruction of physical and chemical assets [16]. Furthermost oral-drugs (lipophobic) cannot pass across lipid-rich biological membranes due to their polar nature and large molecular size. Therefore, attempts have been made to develop phytosome delivery system to overcome this problem and enhance bioavailability [17]. To this aspect, phospholipids are mainly employed to make phytosomes by considering their biocompatible together phyto active constituents. Likewise, earlier studies on phytosome technology have demonstrated that the number of plant extracts such as grape seeds, maidenhair tree, crataegus, green tea, milk thistle, and ginseng heightened the therapeutic efficacy and oral absorption [18]. Another study [19] concluded that the silybin– phospholipids complex has antioxidant potential and, more protective potential of the liver than its pure molecule. The curcumin phospholipid complex has also been evaluated in terms of hepatoprotection against carbon tetrachloride toxicity, which has shown a marked increase in bioavailability, absorption, and therapeutic efficacy in mice through pharmacokinetic studies [2]. Therefore, the present investigation aims to formulate the bacopa phospholipid complex (BPC) and to evaluate its physicochemical and pharmacokinetic properties.

II. METHODOLOGY

- 1. Preparation of *Bacopa Monniera* (*Bm*) Extract:** *Bacopa monniera* (*Bm*) plants were collected from Thummala Gunta fields, Tirumala hill ranges, Tirupati, Andhra Pradesh, India and was identification and authentication of *Bm* has done by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India (Voucher No.1213). The plant material underwent a series of steps for preparation: it was dried, ground into a powder, and extracted using ethanol solvents with a Soxhlet apparatus as mentioned in [20].

Fig:1 Morphology of *Bacopa* phospholipid complex

(a)Phase Contrast Microscopic Images of BPC shows spherical shapes ranges from 2 to 6 μm with a magnification of 400X (b) Scanning Electron Micrographs (SEM) with different magnifications (100X&300X) (c) Electron micrographs (TEM) shows particle size distribution (20 to 32 μm) with magnifications 300X (d) Atomic Force Microscopy 2D & 3D view of BPC.



- 2. Formulation of Bacopa-Phospholipid Complex (BPC):** In the formulation of the Bacopa-phospholipid complex (BPC), we followed a methodology similar to that described by [21].

III. CHARACTERIZATION TECHNIQUES

- 1. Microscopic View of the Complex:** The morphological observation of BPC was done by a Phase contrast microscope. The suspension of BPC was made with water and a drop of BPC was placed on a micro-slide, covered with a cover slip, and then observed under the microscope.

2. **Scanning Electron Microscopy (SEM):** The morphology of BPC was observed by using a scanning electron microscope (Zeiss EVO 50).
3. **Transmission Electron Microscopy (TEM):** BPC was analyzed through an electron microscope (Hitachi H-7500 operated at an accelerating voltage at 200KV) by suspending in phosphate buffer (pH7.4) through sonication with 10 min.
4. **Atomic Force Microscopy (AFM):** 2D and 3D topography of BPC was measured through atomic force microscopy (AFM- Solver Next, NT-MDT, Russia).
5. **UV and FT-IR Spectroscopy:** The optical properties of BPC were measured by UV–Vis spectrophotometer (Genesys 10S UV–Vis spectrophotometer) and FTIR spectrophotometer (Bruker Alfa –T model 109974) to detect the characteristic peaks/functional groups of the sample.

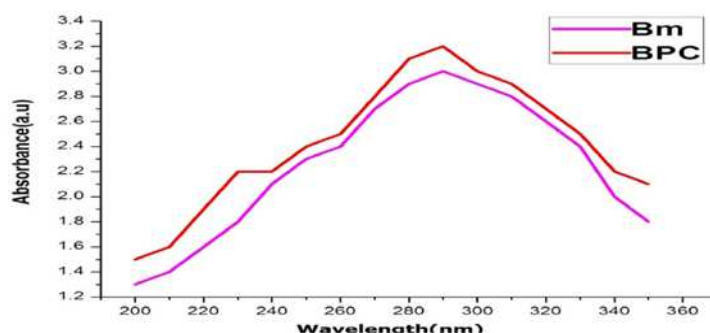
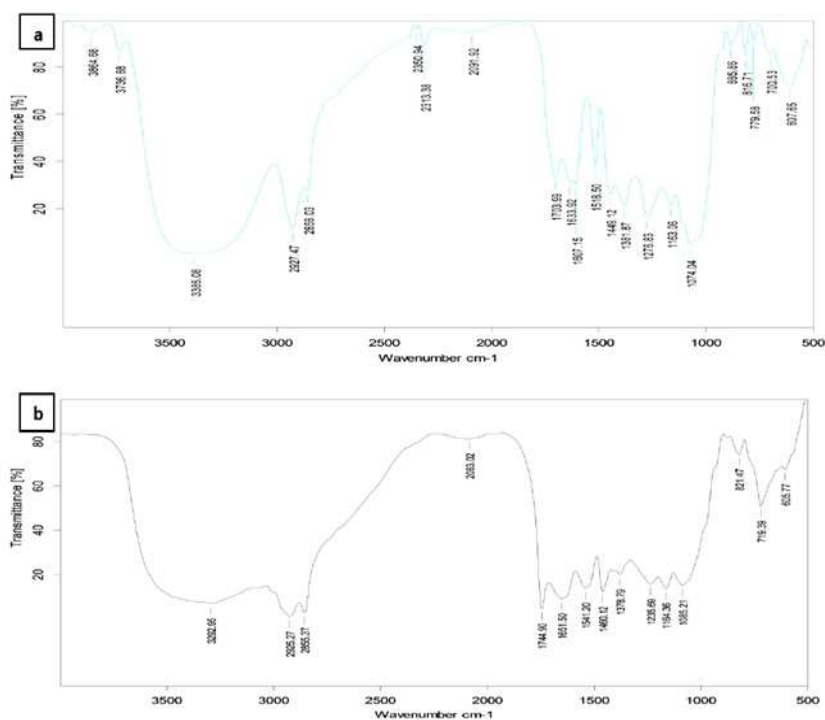
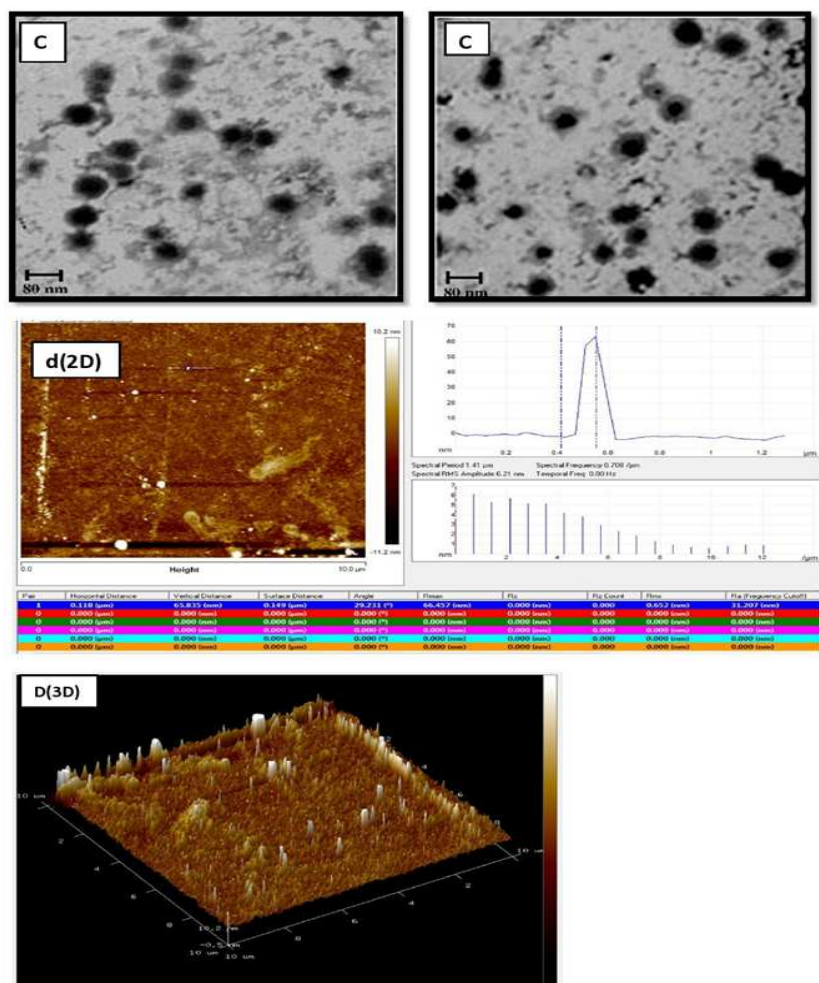
Fig: 2 UV spectra of *Bm* and BPC

Fig:3 FTIR Spectra of (a)Bm and (b)BPC





- Measurement of Particle Size:** 100 μL of BPC was diluted with PBS (pH 7.4) and the diameter of the BPC vesicle was determined by a particle size analyzer (Horibo scientific nanopartica SZ100 Z).
- Measurement of Zeta Potential:** Zeta potential (ZP) was used to discern the physical stability of the sample. Greater stability of the particles is attained through superior electrostatic repulsion between the particles. The Zeta potential of the BPC suspension was measured by using a Zeta size analyzer (Horibo scientific nanopartica SZ100 Z). 1 mL of the BPC was diluted to 10 mL with double distilled water, in this 5mL sample was transferred to a cuvette and the zeta potential was measured.
- X-ray Diffraction Analysis:** X-ray diffraction patterns of BPC and *Bm* were measured by using Bruker Axs- D8 Discover Powder X-ray diffractometer (Germany). The X-ray generator was maintained at 40 KV and 40 mA of tube current, Ka lines of copper is used as the radiation source. The scanning angle ranged from 1 to 60°C of 2θ in step scan mode (step width 0.4 /min).
- Thermal Gravimetric Analysis (TGA)/Differential Thermal Analysis (DTA):** Detection and measurements like thermal effects, loss of solvent, and decomposition of BPC and *Bm* were measured by using TA-Instruments, SDT Q600.

10. Entrapment Efficiency: BPC was formulated with different molar ratios batches 1-5 with 0.5, 1.0, 1.5, and 2.0 M egg lecithin, respectively. *Bm* entrapment efficiency in BPC was analyzed by centrifugation. From all batches, 100 mg of BPC was dispersed in 50 ml of distilled water and centrifuged at 20,000 rpm in a refrigerated centrifuge. The collected clear solution was filtered to measure the particle-free *Bm* concentration after suitable dilution with fresh phosphate buffer saline. The absorbance was read at 266 nm in a UV spectrophotometer to calculate the entrapment efficiency using the following formula.

$$\text{Entrapment Efficiency} = \frac{\text{Weight of the } Bm \text{ incorporated in the BPC}}{\text{Weight of the } Bm \text{ initially taken}} \times 100$$

Weight of the *Bm* initially taken

IV. DISSOLUTION STUDY (IN VITRO RELEASE)

The dialysis bag diffusion technique was used to measure the *in vitro* release of BPC [22]. The study was performed in a media of phosphate buffer (pH 5.5) and methanol (75:25). 2 mg of BPC was suspended in distilled water, transferred to a cellulose dialysis bag, and sealed on both sides. The dialysis bag contained BPC and media were shifted to the receptor unit and the temperature of the receptor unit was maintained at $32 \pm 2^\circ\text{C}$ in a rotatory water bath shaker (100 rpm). To avoid evaporation of the dissolution medium, the receptor unit was covered with aluminum foil. Total release time is 0 to 12 h with 1 h interval. For every 1 h, 2 mL of sample was collected from the receptor unit and replaced with the same amount of phosphate buffer and methanol media. After completion of time intervals (0-12 h), the collected samples were read in a spectrophotometer at 200 to 400 nm.

$$\text{BPC released (\%)} = \frac{\text{Amount of BPC released}}{\text{Initial amount BPC}} \times 100$$

V. IN VIVO PHARMACOKINETICS STUDIES

Rats were randomized into two groups, each contain six rats age of 12 months. Ethical committee approved this study by CPCSEA/IAEC (1677/PO/Re/S/2012/CPCSEA/IAEC-37dt.6/6/16). The experimental animals received human care based on the Guidelines of the National Institutes of Health and Animal Care. Animals were procured from the Sri Venkateswara Traders, Bengaluru, Karnataka. Animals were kept for fasting for overnight before serum collection and allowed to drink water *ad libitum*. Both *Bm* and BPC 40 mg/kg b.w suspension was made with distilled water and Tween-20 (1% v/v) administered orally. After that, blood samples were collected from retro-orbital plexus into tubes with heparin at time intervals of 0, 1, 2, 3, 4, 5 h.

VI. PREPARATION OF SERUM SAMPLES

The collected blood samples were centrifuged at 3000 rpm for 10 min and serum was isolated separately. 10 mL of aliquots were transferred to 10 mL volumetric flask and final

volume was made with methanol, after that turbid solution was centrifuged at 5000 rpm for 10 min in 15 mL volumetric centrifuge tubes. After centrifugation supernatant was collected and measured to find the bacosides content through HPLC.

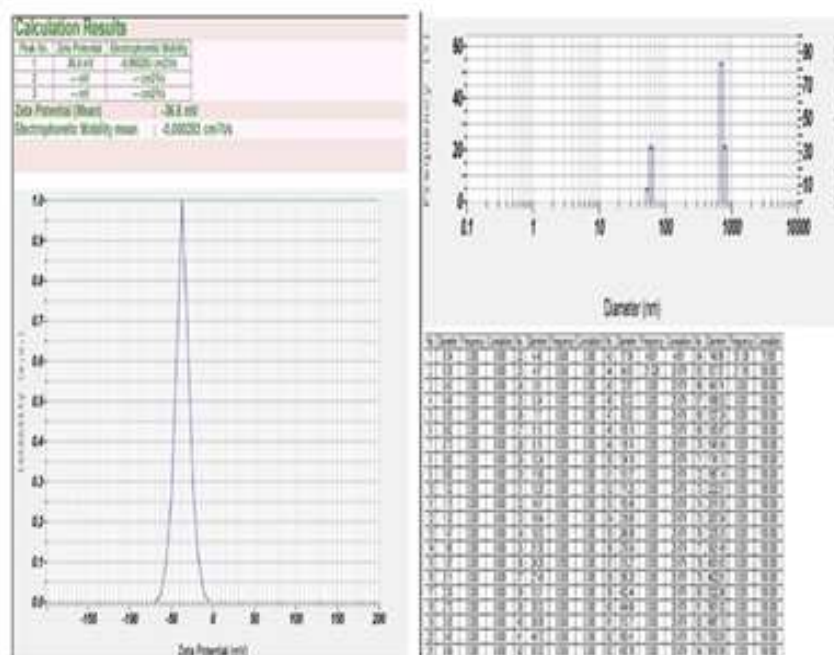
- 1. HPLC Analysis:** The bacoside concentration in serum was measured by HPLC (Shimadzu LC-2010, Japan). The stationary phase contains Hypersil ODS, C-18 250mm X 4.6 mm, 5 mM was kept at 25°C. Mobile phase consists of 0.2% phosphoric acid: acetonitrile: methanol (60:30:10) pH 3 adjusted with NaOH. The flow rate was 1.5 mL/min and effluent was monitored at 205 nm and peaks observed were noted down [23].
- 2. Statistical Analysis:** Data expressed as mean \pm SEM and difference between groups were considered significant at $p < 0.05$ using one-way ANOVA (Analysis of Variance).

VII. RESULTS AND DISCUSSION

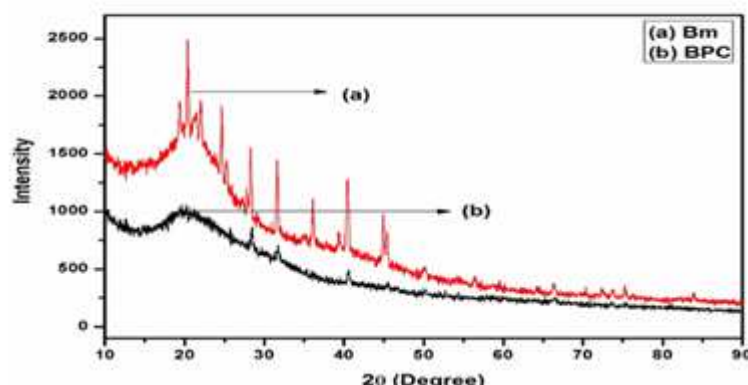
- 1. Morphological Features of BPC by Phase Contrast Microscope:** The surface morphology of BPC was studied by a phase contrast microscope. The microscopic view of BPC indicated the presence of vesicles that are spherical in shape. In these vesicles, phospholipids and *Bm* were intercalated in the lipid layer Fig 1(a).
- 2. Scanning Electron Microscopy (SEM):** The SEM Fig. 1(b) showed that the *Bm* was associated with phospholipid, in the formation of a complex with irregular vesicles. This may be due to the hydrogen bonding between the *Bm* constituents and phospholipids [24]. This is in consonance with the validation that a pure molecule of curcumin hydrogenated soy phosphatidylcholine lipid layer in the curcumin phospholipid complex. The morphology of the curcumin phospholipid complex is spherical and self-closed structure, suggesting that phospholipids carry the amphipathic nature of water dispersion associated with phospholipids [25]. In the same mode, *Bm* is also equally scattered in the phospholipids and forms asymmetrical spheroid shape vesicles during the complexation [18].
- 3. Transmission Electron Microscopy (TEM):** Further, the TEM image of BPC reveals that many spherical to spheroid shape vesicles are arranged as a very thin layer surrounding the uniform particles, which validates the *Bm*-enriched phospholipid complex as presented in Fig 1(c). The vesicles have strong physical entanglement of *Bm* and phospholipids, and the polar bodies of phospholipids combined with *Bm* that gives strong aggregation for BPC.
- 4. Atomic Force Microscopy (AFM):** AFM measurements show the BPC surface topography, which is indifferent spherical or ellipsoidal Fig. 1(d) heights in a heap. These piles were elevated suddenly from the level of the surface and accomplished renowned heights compared to the adjacent side heaps. The average size of the BPC was 10 μ m with spectral RMS in 6.21 nm. This complexation of *Bm* and phospholipids didn't change the original amphipathic nature and morphology. *Bm* and BPC absorption peaks were observed at 294 nm and are consistent with the previous report [26].

- 5. UV and FT-IR Spectroscopic Analysis:** The UV spectra of *Bm* and BPC are shown in Fig. 2, with absorption peaks observed at 296 nm. FT-IR spectrum Fig. 3(a&b) showed the presence of functional groups both in *Bm* and BPC. Likewise, the major absorption peaks of *Bm* showed at 3386 cm^{-1} assigned to primary, secondary amines N-H stretch, 2927 cm^{-1} integrated for alkane C-H stretch, 2091 cm^{-1} assigned to alkynes (terminal), and 1703 cm^{-1} corresponds to C=O stretch of carbonyl derivatives. While the BPC showed prominent peaks at 3292 cm^{-1} (N-H stretch) signifies primary, secondary amines, 2925 cm^{-1} (C-H stretch) corresponds to alkane, and 1744 & 1651 cm^{-1} (C=O stretch) corresponds to esters/acid and aldehyde functionalities, and 1235 cm^{-1} represents C-N/C-O/C-C deformations of aliphatic amines/ethers. Further, the FT-IR spectra of BPC showed broadening, sharp and articulate peaks of various bio-active molecules. The FT-IR broad bands demonstrated that the interaction between *Bm* and phospholipids has a strong steric hydrogen bonding (OH) which accommodates the formulation of BPC. As well, the shifting of bands from higher frequency to lower frequency indicates the new bonding(s) between phospholipids and *Bm* constituents. Moreover, the BPC showed additional bands that signify the choline groups N-(CH₃)₃ presence in phospholipids. [17].
- 6. Particle Size and Zeta Potential:** Zeta potential was used to estimate the firmness of formulations and the present study also includes the particle size and zeta potential measurements of BPC. It was observed that BPC has an average particle size of 552.5 nm, and the zeta potential value was -36.6 mV, indicating the stability of the formulation as graphically exemplified in Fig. 4.

Fig:4 Particle size and Zeta potential of BPC

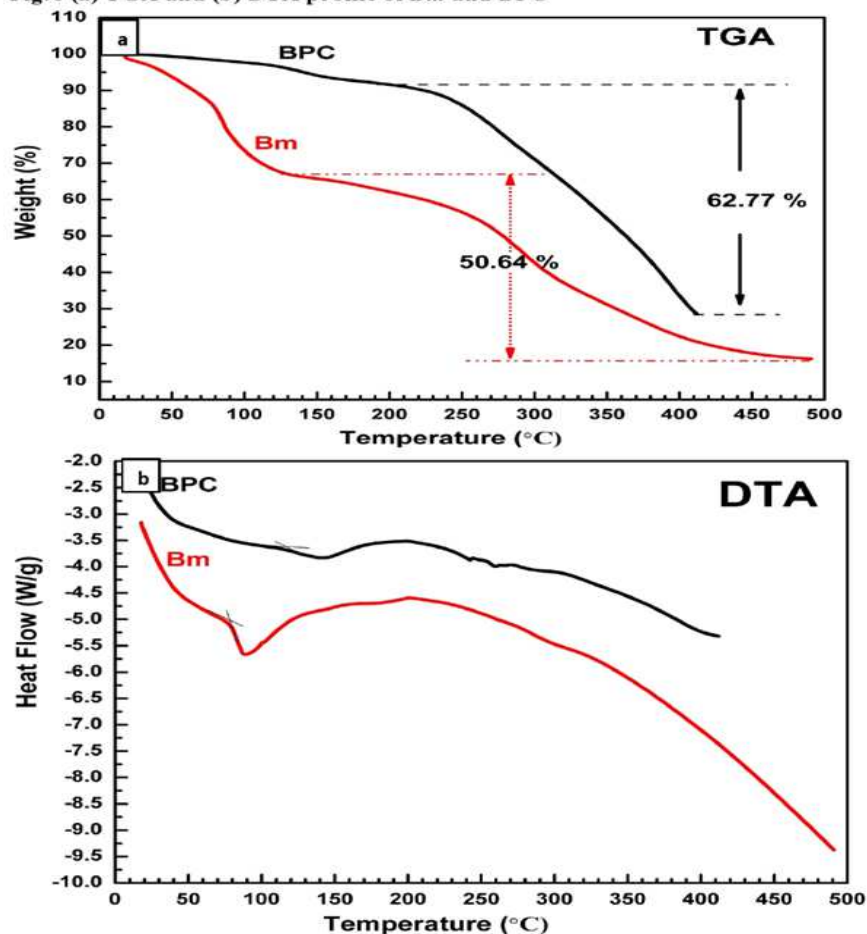


7. **X-ray Diffraction (XRD) Analysis:** The XRD profile in Fig. 5 shows intense crystalline peaks of *Bm* extract that indicate higher crystallinity. However, in the case of BPC, peaks are relatively less intense than *Bm* due to the crystalline diffraction of phospholipids during BPC formation [27]. As well, the previous reports of the phospholipid complex of diclofenac and naringenin, puerarin, insulin, and salmon calcitonin also supported the current study [28].

Fig:5 XRD of (a) *Bm* and (b) BPC

8. **Thermal Analysis (TGA/DTA):** Thermogravimetry (TG) and differential thermal analysis (DTA) are the most common analyzing thermal tools used for the assessment of quality of the plant drugs [29]. TG curve of *Bm* in Fig. 6 showed the weight loss (50.64%) that gradually decreased from 131.21°C to 490.84°C due to the degradation of bounded water molecules. Similarly, the BPC of the TG curve shows a weight loss of 62.77% from 204.98°C to 411.79°C. Because of the abundance of hydrogen molecules in BPC more weight loss (62.77%) compared to the *Bm*. Furthermore, differential thermal analysis (DTA) is a suitable, authentic, and fast method to find the interaction between drugs and excipients. Since interactions are defined by the expulsion of endothermic peaks, the abrupt raise of new peaks, and a number of changes like temperature, and enthalpy also affect the peak [2]. The thermogram (DTA) in Fig.7 demonstrates that the endothermic peak at 76.88°C and exothermic peak at 194.67°C for *Bm*. Though, for the BPC, the endothermic peak was observed at 150°C and the exothermic peak at 200.96 °C, together with additional tiny endothermic peaks at 240°C and 270°C. This may be due to the hot motion of the phospholipid polar head groups and the phase transition(s). Accordingly, it was apparent that the *Bm* peaks were melted away in the thermogram of BPC.

Further, the phase transition temperature of BPC was a higher temperature than *Bm*, which ensures the complexation of BPC [30-32], and the fact that both *Bm* and phospholipids are bound by hydrogen bonds or Van der Waals force.

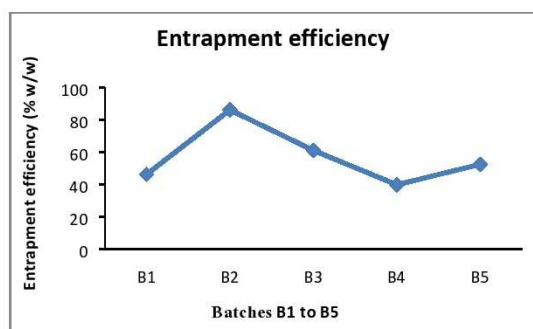
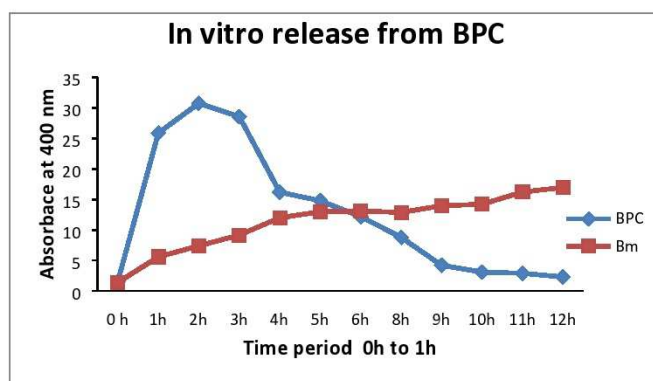
Fig:6 (a) TGA and (b) DTA profile of *Bm* and BPC

9. Entrapment Efficiency: The entrapment efficiency of *Bm* was calculated with different formulations as shown in the various batches listed below in Table 1. The maximum entrapment efficiency of 86.04 % was observed in B2 and the minimum of 39.80% was in B4 as depicted in Fig. 7. Consequently, batch B2 was used for the physicochemical and pharmacokinetic analysis.

Table 1: An Assessment of Entrapment Efficiency of Various Batches of *Bm* used in the Current Study

S.No	Batch No.	Entrapment Efficiency (w/w %) \pm SD
I	B1	46.2 \pm 0.4
II	B2	86.04 \pm 0.7
III	B3	61.02 \pm 0.9
IV	B4	39.8 \pm 0.29
V	B5	52.46 \pm 0.6

Fig:7 Entrapment efficiency of BPC

Fig:8 *In vitro* release of BPC

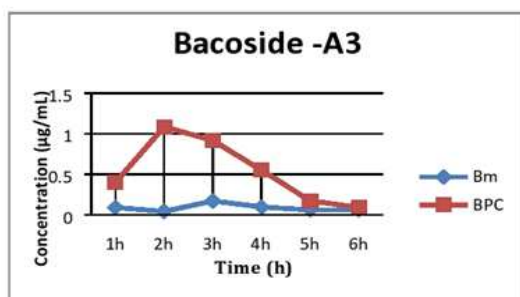
10. Dissolution Study (In Vitro Release): The *in vitro* release study was performed with the dialysis membrane diffusion technique for 12 h and the results were shown in Fig. 8. Throughout the process, the BPC was released by diffusion mechanism from the dialysis membrane. The *Bm* has OH ions, which can form hydrogen bonds between *Bm* and phospholipid molecules, and thus BPC was easily adsorbed. BPC was released within 2h, which may be due to the penetration of buffer into the BPC molecules by pores dissolving from the surface under favorable conditions like pH of the buffer, temperature, lipid concentration, and high solubility. The instantaneous *in vitro* release of BPC within 2 h may be due to well-constructed interactions with phospholipids and *Bm* containing phyto-constituents.

11. In Vivo Studies: A postulate that the pharmacokinetic studies of oral administration of BPC in rat serum indicates the presence of Bacoside A3, Bacopaside II, Jujubogenin, Bacopasaponin C and Bacopaside I. BPC was found to be rapidly absorbed in 1-2 h without presenting any absorption lag time with quality of bacosides and peak concentrations of Bacoside -A3 (1.08 $\mu\text{g/mL}$), Bacopaside II (5.59 $\mu\text{g/mL}$), Jujubogenin (1.8 $\mu\text{g/mL}$), Bacopasaponin -C (1.18 $\mu\text{g/mL}$) and Bacopaside - I (2.4 $\mu\text{g/mL}$). The *Bm* peak concentration of Bacoside -A3(0.17 $\mu\text{g/mL}$), Bacopaside II (1.86 $\mu\text{g/mL}$), Jujubogenin (0.15 $\mu\text{g/mL}$), and Bacopasaponin -C (1.18 $\mu\text{g/mL}$) and Bacopaside I (2.4 $\mu\text{g/mL}$) showed less bioavailability within 2h as labeled in Fig:9(a-e) that presenting more than 3h absorption time. Thus, the pharmacokinetic studies reveal the C_{max} and T_{max} of five components

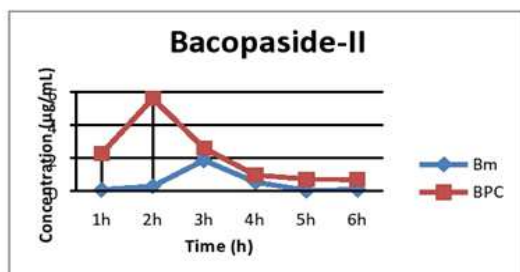
i.e. Bacoside A3, Bacopaside II, Jujubogenin, Bacopasaponin C, and Bacopaside I. The complexation of *Bm* with phospholipids shows physicochemical entanglement to improve its effective half-life in bioavailability and absorption compared with uncomplexed *Bm*, suggesting that the formulations can improve absorption and bioavailability in serum, tissue, and metabolism of *Bm*. Further, classified studies have shown that oral administration of lipid-based formulations of phyto constituents/drugs exhibits greater therapeutic potential than the original phyto constituents/drugs. In the same manner, the results of the current study are also in good agreement with the literature [17] because the serum peak concentration increased after the formulation of BPC compared to the *Bm*, which exhibited good bioavailability and absorption.

Fig: 9 The mean serum concentration (C_{max})–time(T_{max}) curves of *Bm* and BPC in rats after oral administration (n=6, Mean \pm SD).

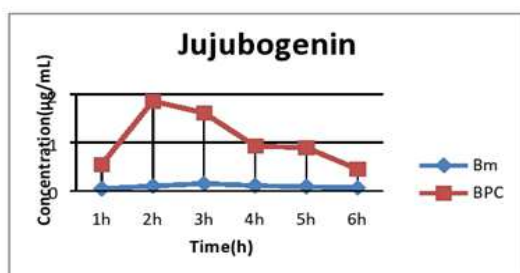
9 (a) Bacoside A3 concentration of *Bm* and BPC in serum of rats

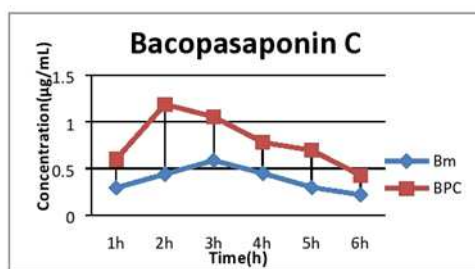
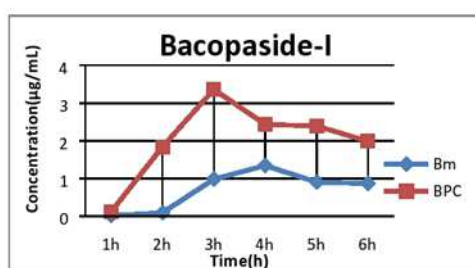


9(b) Bacopaside II concentration of *Bm* and BPC in serum of rats



9(c) Jujubogenin concentration of *Bm* and BPC in serum of rats



9(d) Bacopasaponin C concentration of *Bm* and BPC in serum of rats9(e) Bacopaside I concentration of *Bm* and BPC in serum of rats

VIII. CONCLUSIONS

BPC is formulated with *Bm* extract and phospholipids to enhance the absorption and bioactive principle with better therapeutic strategies. The morphology and structural studies such as the size and shape of the BPC were determined through various analytical parameters like phase contrast microscope, SEM, TEM, and AFM. Further, the BPC was optimized and characterized by UV–Vis spectroscopy by integrating the absorption peak observed at 296 nm. FT-IR investigations clearly depicted the presence of functional groups in the *Bm* and BPC. In addition, the validation of complex formulation was achieved by witnessing the shifting of *Bm* higher wave number to lower wave number in BPC due to the merging of *Bm* and phospholipids. Moreover, the Zeta potential value -36.6 mV demonstrated higher stability, and the XRD studies indicated the amorphous nature of BPC. Likewise, thermal studies also show the stability of BPC at 400°C . Moreover, the *in vitro* release studies indicate that novel BPC could be a better way for the immediate release of *Bm*. In pharmacokinetic studies, the serum peak concentration was increased after the formulation of BPC compared to the *Bm*. Therefore, from the perceived results that the BPC has been found as a potential for bacoside delivery and has been found to be a novel technology for drug delivery and disease therapy. Thus, the current study endorsed the complexation of plant extracts like BPC for pharmacological therapies as a substitute for its individuals after measuring its toxic parameters.

IX. ACKNOWLEDGMENTS

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