



Bilosomes as a Potential Carrier to Enhance Cognitive Effects of *Bacopa monnieri* Extract on Oral Administration

V. Anoop Narayanan¹, Ankitha Sharma¹, Rajesh K. S.¹, Arunraj T. R.¹, Gururaj M. P.¹
Parasuraman S.², Anish John¹

¹Nitte (Deemed to be University), NGSM Institute of Pharmaceutical Sciences (NGSMIPS), Derlakatte, Mangalore, Karnataka, India

²Unit Head, Unit of Pharmacology, Faculty of Pharmacy, AIMST University, Bedong, Malaysia

Address for correspondence V. Anoop Narayanan, MPharm, PhD, Nitte (Deemed to be University), NGSM Institute of Pharmaceutical Sciences, Derlakatte, Mangalore 575018, Karnataka, India (e-mail: anoopvn84@gmail.com).

J Health Allied Sci^{NU} 2023;13:421–430.

Abstract

The Indian system of medicine, Ayurveda employs *Bacopa monnieri* extract (BME) for memory enhancement. This study attempts to prepare and test a more potent formulation by incorporating BME in nanovesicles. BME-loaded liposomes and bilosomes (bile salt-stabilized liposomes) were formulated using soy phosphatidylcholine. Liposomes and bilosomes had homogeneous size distribution and an average size of 285.7 nm and 84 nm, respectively, with satisfactory zeta potential. Spherical multilamellar bilosomes and unilamellar liposomes were observed under transmission electron microscope (TEM), with BME entrapment efficiency of 85% and 45%, respectively. During a 72 h interval, bilosomes and liposomes released 78% and 65% of the loaded BME, exhibiting a biphasic release, following the Higuchi model diffusion. Both liposomes and bilosomes were stable in simulated gastric and intestinal fluids. When tested on dementia-induced Swiss albino mouse models using the Y-maze apparatus, the bilosome-treated group showed significant cognition enhancement activity than those treated with liposomal vesicles. The better pharmacological effect shown by bilosomes may be attributed to better bioavailability, possibly augmented by higher entrapment efficiency, and improved vesicle integrity afforded by bile salts. Likewise, bilosomes were more stable than liposomes in simulated gastric and intestinal fluids. Taken together, innovative formulation techniques hold substantial promise for enhancing the ethnopharmacological claims of BME.

Keywords

- ▶ brain delivery
- ▶ *Bacopa monnieri*
- ▶ liposome
- ▶ bilosome
- ▶ nootropic
- ▶ cognitive enhancement

Introduction

A cognitive decline challenges the aging brain. In the context of a steadily growing geriatric population, it is essential to address this emerging public health problem with indigenous solutions.¹ It has been reported that 50 to 60% of those above 65 years of age suffer from neurodegen-

erative disorders that signal the primary signs of Alzheimer's disease (AD), namely, a decline of cognitive function or dementia.² AD development is associated with extracellular amyloid (A) plaques and intracellular neurofibrillary tangles, composed of a hyperphosphorylated protein in the cortical and limbic regions of the brain.³ As per

article published online
November 28, 2022

DOI <https://doi.org/10.1055/s-0042-1757969>.
ISSN 2582-4287.

© 2022. The Author(s).

This is an open access article published by Thieme under the terms of the Creative Commons Attribution License, permitting unrestricted use, distribution, and reproduction so long as the original work is properly cited. (<https://creativecommons.org/licenses/by/4.0/>)

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

reports, 55.2 million people are affected by dementia globally, and 60% are in low- and middle-income countries. Dementia cases may rise to 78 million by 2030 and 139 million by the end of 2050. Dementia associated with various neurological conditions remains the seventh leading cause of death worldwide. The World Health Organization (WHO) has called for urgent action in dementia management and risk reduction.⁴

Nootropic formulations are expected to address the above concerns. Nootropics are cognition enhancers, but a cognition enhancer is not necessarily a nootropic.⁵ A cognition enhancer improves memory and attention, whereas nootropics enhance mental abilities. Both learning and memory are variations in synaptic neural connectivity and psychological processes. In recent times, there has been a tremendous demand for herbal products to treat chronic ailments in developing countries and the West.⁶

Bacopa monnieri (BM), family Scrophulariaceae, is known as Brahmi in India and has been used since 500AD. In Ayurveda BM is known as “Medha Rasayana” to treat anxiety, impaired memory, epilepsy, and cognitive processes such as understanding, memory, and recall.⁷ *Bacopa monnieri* extract (BME) scavenges free radicals and protects against deoxyribonucleic acid (DNA) damage in a dose-dependent manner. BME is an antioxidant (half as potent as vitamin E) and exhibits anti-stress, immunomodulatory, anti-aging, cognition facilitating, and anti-inflammatory effects. The memory-enhancing property of BM is credited to its anti-lipid peroxidation property and has been prescribed for long-term therapy at low doses.⁸ The pharmacological activities of BM have been extensively studied and have been linked to the saponin constituents known as “bacosides.” Bacosides are a complex mixture of structurally related compounds such as jujubogenin or pseudojujubogenin glycosides.⁷ Alkaloids brahmine and herpestine have been isolated from Brahmi. Tetra cyclic triterpenoid saponin, Bacoside A, and B (crystalline mixture of several saponins) are the main bioactive constituents of the plant. Bacoside A is the most abundant of these. Other saponins include bacoside A1, A3, bacoside saponins A, B, C, D, E, and F. The plant constituents also include d-mannitol, hersaponin, betulic acid, and flavonoids.⁹

The phytoconstituents often fail to offer therapeutic benefits owing to their poor bioavailability caused by hepatic first-pass metabolism or incomplete absorption. The technological advancements in drug delivery can overcome these challenges enabling the phytonutrients to achieve therapeutic concentrations at the site of action effortlessly. A novel vesicular drug delivery system aims to deliver the active ingredient to the site of action at a rate determined by the body's need during treatment.¹⁰ Nanomedicine is likely to offer better therapeutic success than conventional drug delivery approaches.¹¹ Several studies on tumors and central nervous system (CNS) disorders such as Alzheimer's disease (AD) use nano-vesicular formulations.¹² The therapeutic index, solubility, stability, and rapid degradation of drug molecules have been improved using vesicular drug delivery systems.¹³ Liposomes

entrap the compounds, avoid fast removal or degradation from the systemic circulation, and facilitate their penetration through the blood–brain barrier (BBB) and distribution in the brain tissue, making liposomes an efficient drug delivery system for the brain.^{12,14} Liposomal delivery generally requires lower doses because of improved bioavailability and tissue distribution. Liposomes contain multiple components, the most important being phospholipids and cholesterol. Soy phosphatidylcholine (SPC) is the most widely used phospholipid in liposomal preparations.¹⁰

Liposomes mimic cell membranes by exhibiting excellent entrapment ability, safety, and biocompatibility. Although parenteral delivery of liposomes has been successful, oral delivery is thwarted by various factors such as gastrointestinal (GI) instability. Liposome stability and permeability can be significantly improved for oral administration by modifying the composition of the lipid bilayers or by adding polymers or ligands for targeting.¹⁵ Liposomes administered orally would be destroyed partially by gastric acid, leading to the release of the drug payload. Surviving liposomes would subsequently move from the stomach into the small intestine, where intestinal enzymes break down liposomes. Liposomes that survive this stage penetrate the mucus layers and contact intestinal epithelia. Even at this point, liposomes can still be destroyed. Only liposomes that survive the digestive process would be absorbed by M cells as integral vesicles to the lymphatic system.¹⁶

A variety of methods are employed to improve liposomal stability. Adding bile salts enhances lipid biomembranes' flexibility, allowing them to survive the adverse effects of bile acids in the GI tract. Imaging studies have shown that bilosomes that survive the GI environment can be absorbed as entire vesicles.¹⁷ Bilosomes are vesicular carriers used to protect proteins and peptides from degradation in the gastrointestinal tract (GIT) to a certain extent. Vesicles are developed by incorporating bile salts into the lipid bilayers, making them resistant to the disruption in GIT. As a result, the vesicle protects the entrapped agents from the harsh GI environment.¹⁸ In this novel drug delivery system, carriers such as deoxycholic acid are introduced into the membrane of liposomes.¹⁹ Bile salts improve the penetration of lipophilic drugs across biological membranes, thereby enhancing oral bioavailability.²⁰ Here we attempt to enhance the nootropic activity of BM through bilosomal delivery, facilitating value addition to the traditional knowledge system in Ayurveda.

Materials and Methods

Materials

The alcoholic extract of BME was a gift sample from Elixir Extracts Pvt. Ltd., Kochi, Kerala. Soya phosphatidylcholine (SPC or soy lecithin) was procured from Hi-Media Laboratories, Mumbai. Cholesterol was purchased from Merck, Mumbai. Sodium deoxycholate, pepsin (from porcine gastric mucosa), pancreatin (from the porcine pancreas) was purchased from Sigma–Aldrich. Chloroform was purchased from

Loba Chemie, Mumbai. All chemicals used were of analytical or pharmaceutical grade.

Froth Test for Saponins in the *Bacopa monnieri* Extract
BME (50 mg) was mixed with 5 mL of distilled water in a stoppered test tube, shaken well for ~5 minutes, and allowed to stand for 30 minutes. The honeycomb-like froth formation indicated the presence of saponins.²¹

Fourier Transform Infrared Spectroscopy Studies

The Fourier transform infrared spectroscopy (FT-IR) spectra of BME and extract-loaded nanoformulations were recorded in a wavelength range of 4000 to 500 cm^{-1} on a M-15, Bruker Alpha Fourier transforms infrared spectrophotometer, USA, employed with an ATR crystal. Transmittance peaks were identified and compared with the literature reports.

Formulation of Liposomes

Method 1: Solvent Evaporation Technique

To prepare liposomes (LS11, LS12, and LS13), equal volumes of BME, SPC, and various amounts of cholesterol were dissolved in 10 mL of ethanol in a beaker. ►Table 1 shows the ratio of materials used in the formulation. The mixture was added to 80 mL of distilled water under stirring at 15000 rpm on a magnetic stirrer (on a magnetic stirrer) maintained at 80°C. Stirring was continued for 15 minutes, maintaining the temperature to evaporate the ethanol. The mixture was allowed to cool and was sonicated for 10 minutes (50% amplitude) using a probe sonicator (Sonics, USA).²²

Method 2: Thin-film Hydration Method (LS2)

BME-loaded liposomes (LS21, LS22, and LS23) were prepared following a thin-film hydration process in a rotary flash evaporator. BME, SPC, and cholesterol were dissolved in 10 mL of chloroform in a round bottom flask; the quantities are represented in ►Table 1. A rotary evaporator was used to evaporate the organic solvent, maintained at 65°C under reduced pressure for 30 minutes. The resulting thin, dry film

was left overnight for complete evaporation of organic solvents and then hydrated using a phosphate buffer (pH 7.4) to obtain a crude dispersion of BME-loaded liposomes. Then crude dispersion was further sonicated for 10 minutes (50% amplitude) using a probe sonicator.²³

Formulation of Bilosomes by Thin-film Hydration Method

BME-loaded bilosomes (BS1, BS2, and BS3) were prepared using a rotary flash evaporator by a thin-film hydration process, slightly modifying the previous method. As given in ►Table 1, BME and SPC were dissolved in 10 mL of chloroform in a round bottom flask. The organic solution was evaporated at 65°C under reduced pressure for 30 minutes until a thin, dry film was formed in a rotary flash evaporator. It was left overnight to achieve complete evaporation of the organic solvent. Crude dispersion of BME-loaded bilosome was achieved by hydrating the thin, dry film with 10 mL of distilled water containing bile salt (sodium deoxycholate). The dispersion was then sonicated for 10 minutes using a probe sonicator.²⁴

Characterization of Liposomes and Bilosomes

Vesicle Size and Zeta Potential Analysis

The average vesicle size and size distribution (polydispersity index, PDI) of formulated liposomes and bilosomes were analyzed by dynamic light scattering (DLS) using Nano ZS, Malvern Instruments, UK. The surface charge of vesicles was measured in terms of zeta potential (Nano ZS, Malvern Instruments, UK). Only those formulations with desirable size distribution were considered for further characterization.

High-Resolution Optical Microscopy

On account of the acceptable size range, formulations LS23 and BS3 were taken for further studies. The vesicles formed were examined for their morphology under a high-power optical microscope (BIOVIS particle analyzer, India). A drop of the liposome/bilosome sample was sufficiently diluted

Table 1 Formulation chart of liposome and bilosome formulations showing the method of preparation and their composition

Formulation code	Formulation type	Method used	Ingredients			
			BME (mg)	SPC (mg)	Cholesterol (mg)	Bile salt(mg)
LS11	Liposomes	Solvent evaporation	100	100	–	–
LS12			100	100	15	–
LS13			100	100	30	–
LS21		Thin-film hydration	100	100	–	–
LS22			100	100	15	–
LS23			100	100	30	–
BS1	Bilosomes	Thin-film hydration	100	400	–	5
BS2			100	800	–	5
BS3			100	1600	–	10

with distilled water, smeared on a glass slide, protected with a coverslip, and inspected under microscopic magnification (100× objective), and the images were recorded.

Transmission Electron Microscopy of Formulations

The morphology and vesicular size of BME-loaded liposomes LS23 and bilosomes BS3 were further analyzed by high-resolution transmission electron microscopy (HRTEM) using JEM-2100 Electron Microscope, Jeol, Japan. After suitable dilution, a drop of the formulation was adsorbed on a copper grid, negatively stained with 1% phosphotungstic acid, and air-dried at room temperature for 10 minutes. The morphological appearance of the selected vesicles was recorded by TEM.²³

Extract Entrapment Efficiency of Vesicles

Flocculation was induced for LS23 and BS3 by adding potassium chloride solution (1 mL of 3M KCl) before centrifugation. The untrapped BME in the formulations was removed by ultracentrifugation (Beckman, USA) at 40,000 rpm for 1 hour at 4°C. The concentration of untrapped extract in the supernatant was analyzed by UV-Vis spectrophotometry at 321 nm (Spectroquant Prove 600, Merk, Germany).^{25,26}

The percentage entrapment efficiency was determined using the following equation

$$\% \text{ Extract entrapment efficiency} = \frac{\text{Amount of extract added} - \text{amount of free extract}}{\text{Amount of extract added}} \times 100$$

In Vitro Drug Release Study

Drug release from nanovesicles was studied using the dialysis method. Dialysis kits (Pur-A-Lyzer Maxi Dialysis Kit, Sigma-Aldrich, India) of 12 to 14 kDa molecular weight cut-off were used for this method. Dialysis kits were pre-soaked in distilled water for 10 minutes and later loaded with 3 mL of the formulations. Loaded dialysis kits were fixed on the floating rack and placed in a beaker containing 200 mL of phosphate-buffered saline (PBS; pH 7.4) as a release medium, maintained at 37 ± 0.5°C with continuous magnetic stirring at 50 rpm. Samples of 5 mL were withdrawn at different intervals of 0.5, 1, 2, 3, 4, 6, 7, 9, 24, 48, 69, 72 h, and replaced with the medium. The concentration was determined by UV-visible spectrophotometry by measuring the absorbance after suitable dilution.²⁷

In Vitro Gastrointestinal Stability of Nanovesicles

Simulated gastric fluid (SGF) was prepared by dissolving 2 g of NaCl and 7 mL of concentrated HCl in 1 L of deionized water, then adjusting the pH to 1.2. To mimic gastric digestion, pepsin (0.032 mg/mL) was added to the above solution. Simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g of dipotassium phosphate, 190 mL of 0.1 M sodium hydroxide, 3.32 g of sodium chloride, 8.76 g of calcium chloride, and 0.1 g of sodium deoxycholate (bile salt); in water. The solution was diluted to 1 L, and the pH was adjusted to 7.4. Pancreatin was added to the above solution at a 0.032 mg/mL concentration for intestinal digestion. The

liposomes and bilosomes were added to SGF and SIF in separate standard flasks, mixed well, and incubated in the water bath for 30 minutes at 37 ± 0.5°C. Samples were withdrawn after 1 minute and 30 minutes. Vesicle size, PDI, and zeta-potential of liposomes and bilosomes were determined during GI digestion.²⁸

In Vivo Cognitive Enhancement Activity Studies of Nanovesicles

Selection of Dose

To assess the nootropic activity of BME, the dose was fixed at 200 mg/kg, (1/10th of the previously reported maximum dose in acute toxicity studies, i.e., 2000 mg/kg). Formulations equivalent to the above amount of drug were used for evaluation.²⁹

Treatment Schedule and Evaluation of Nootropic Activity

Nootropic activity was evaluated with the help of a Y maze spontaneous alternation test using Male Swiss albino mice. The animals were housed and maintained in large, spacious polyacrylic cages at ambient room temperature with a 12-hour light/12-hour dark cycle. The animals were fed with water and a standard rat pellet diet *ad libitum*. The study was approved by the Institutional Animal Ethics Committee (IAEC) of NGSM Institute of Pharmaceutical Sciences with approval number NGSMIPS/IAEC/JUNE-2020/191.

Male Swiss albino mice (22 to 30 g) were randomly divided into five groups of five animals each.

- Group I: Scopolamine (0.5 mg/kg)
- Group II: Normal control (saline)
- Group III: Scopolamine + BME (200 mg/Kg)
- Group IV: Scopolamine + Liposome (LS23)
- Group V: Scopolamine + Bilosome (BS3)

The animals in Group I and II were administered orally once daily with vehicle (0.6% w/v sodium carboxymethyl cellulose [Na CMC], 10 mL/kg) for 7 days. Group III was administered with BME (200 mg/kg) orally once daily for 7 days. For seven days, groups IV and V were administered with the formulation LS23 and BS3 containing BME equivalent to 200 mg/kg. On the seventh day, after half an hour of BME administration, each group except Group II received scopolamine (0.5 mg/kg) intraperitoneally.³⁰ After 45 minutes of scopolamine administration on day 7, the nootropic activity was evaluated using the Y maze. To evaluate short-term memory, the percentage of trials in which the mice entered all three arms on consecutive choices alternatively (without returning to the previous arm) was recorded as an alternation.³¹

Statistical Analysis

The data for nootropic effect of BME, LS23 and BS3 formulations are expressed as mean ± standard error of the mean (SEM). The data obtained were subjected to a one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism software. *p*-Values less than 0.05 are considered statistically significant (indicated by **).

Results and Discussion

Identification of *Bacopa monnieri* Extract

The procured BME was brownish-green with a characteristic odor and powdery appearance. In the froth test, a characteristic honey-comb-like froth was formed, indicating the presence of saponin glycosides. Steroid saponin glycoside, such as bacoside A₃ in the extract, helps with memory enhancement.

Formulation of Liposomes and Bilosomes

BME-loaded liposomes and bilosomes were prepared as described in the methodology section. The BME was dissolved in ethanol to solubilize lipophilic active constituents, improving the entrapment. ►Table 1 depicts the formulation chart of liposomes and bilosomes, showing the preparation method and composition. Solvent evaporation yielded larger vesicles; therefore, the thin-film hydration method prepared the final formulations of liposomes and bilosomes. The difference between the formulation of liposomes and bilosomes was the incorporation of bile salt in the latter during their hydration process. Adding cholesterol modulates membrane permeability and elasticity, reducing vesicle size.³²

Characterization of Liposomes and Bilosomes

Vesicle Size, Zeta-potential, Morphology

We used Malvern Zetasizer, which employs the dynamic light scattering technique, to determine the vesicle size of the formulations. Results are shown in ►Table 2. Although liposomes prepared by solvent evaporation method are known for their high entrapment efficiency and ease of preparation, they yielded large vesicles even after sonication. Vesicle size and entrapment efficiency are directly related because increasing the vesicle size increase the volume of extract encapsulated. Additionally, increasing the amount of soy lecithin enhances the medium's lipophilicity, increasing the entrapment efficiency and vesicle size. Due to the large vesicle size, liposomes prepared by solvent evaporation were excluded from advanced characterization.

The amount of cholesterol affects the vesicle size and drug release from liposomes.³³ Cholesterol increases membrane

hydrophobicity, favoring the inclusion of hydrophobic molecules.³⁴ Furthermore, the increased vesicle size for SPC liposomes could be attributed to the packing parameter and phase transition temperature. Saturated phospholipids with shorter carbohydrate chains have lower packing parameters, favoring smaller vesicles' formation. On the contrary, phospholipids with monounsaturated carbohydrate chains have the highest packing parameter and lower phase transition temperature, helping the formation of larger liposomes.³⁵

Sodium deoxycholate or bile salts impart a negative charge to the vesicles, increasing the zeta potential. Smaller vesicles are observed in bilosomes with better zeta potential, possibly due to bile salt's presence. Also, the vesicle size is known to increase due to the repulsion between the bilayers in multilamellar bilosomes, which was not observed here.²⁴ A detailed comparison of vesicle size is given in ►Table 2.

The polydispersity index (PDI) is an essential measure of dispersion characteristics related to size uniformity. PDI values of more than 0.4 imply high heterogeneity, and those 0.2 or less indicate homogeneity. PDI of LS23 was found to be 0.5 ± 0.15 , and BS3 was found to be 0.386 ± 0.01 , showing moderate homogeneity in size distribution.³⁶

The zeta potential values for BME-loaded liposomes LS23 and bilosomes BS3 were satisfactory, with values of -16.8 and -15.1 mV, respectively, implying moderate stability of the formulations due to electrostatic repulsion. The incorporation of bile salts and cholesterol showed increased zeta potential. Formulations with lower zeta potential and high polydispersity were excluded from further evaluation.

►Fig. 1 and ►Fig. 2 show the high-resolution optical microscopy and TEM images of BME-loaded liposomes LS23 and BME-loaded bilosomes BS3. These studies confirmed that both systems have a well-defined spherical shape and proper structure. Vesicle size analysis proved that both formulations were good candidates for clinical applications, well within the optimum size requirements for good biological activity.

FT-IR Analysis

Infrared spectroscopy helps determine or predict any physicochemical interactions between different components in a

Table 2 Data for estimation of vesicle size, polydispersity index (PDI), zeta potential and extract Loading of vesicles

Formulation	Vesicle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Extract loading efficiency
LS11	1585.5 ± 2.0	0.608 ± 0.12	-8.45	–
LS12	1011 ± 0.9	0.51 ± 0.08	-11.4	–
LS13	899 ± 3.1	0.661 ± 0.11	-14.5	–
LS21	652.4 ± 2.2	0.630 ± 0.7	-7.8	–
LS22	533.2 ± 1.8	0.506 ± 0.21	-12.5	–
LS23	285.7 ± 3.0	0.5 ± 0.15	-16.8	45%
BS1	132 ± 2.2	0.722 ± 0.2	-9.2	–
BS2	149.6 ± 1.8	0.66 ± 0.08	-10.4	–
BS3	84.0 ± 0.7	0.386 ± 0.01	-15.1	85%

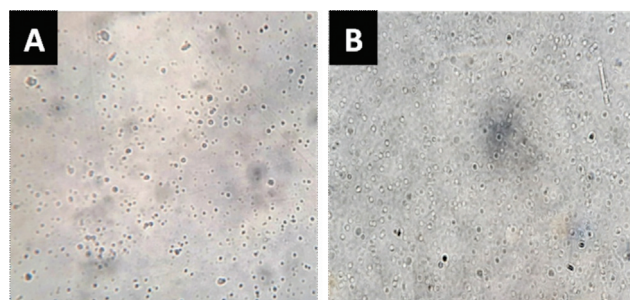


Fig. 1 Microscopic images showing vesicles under optical microscope (Magnification 1000 times). (A) Liposome LS23 and (B) Bilosomes BS3.

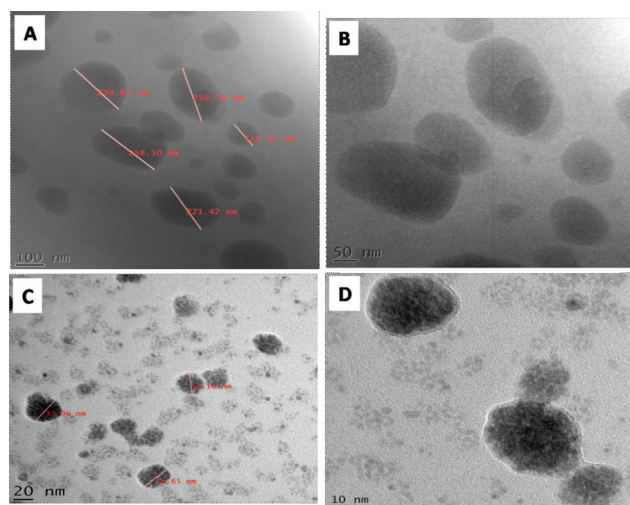


Fig. 2 TEM image (A) and (B) BME-loaded unilamellar liposome LS23, (C) and (D) BME-loaded bilosomes BS3 showing multi-lamellarity.

formulation. Therefore, infrared spectroscopy can be a tool to select chemically compatible excipients. The compatibility between soy lecithin, cholesterol, sodium deoxycholate, and BME was established using an FTIR spectrophotometer. Absorption peaks are shown in ►**Fig. 3A**. FTIR spectra obtained with the alcoholic BME were concordant with the literature available. The prominent peaks associated with BME, namely O-H bonded (3650–3600), C-H stretch (3000–2850), C=O (Ketone) (1725–1705), C=C (Alkene) (3319–3383), CH₃-R(Alkyl) (2944–2882) and C-O bond (furanosyl, pyranosyl) (1075–1018) were observed. The constituent peaks were present in both formulations (►**Fig. 3B** and **3C**), thereby ruling out the possibility of any chemical incompatibility between the extract and the lipids used.

Encapsulation Efficiency

Evaluating extract loading efficiency helps estimate the amount of BME entrapped in the vesicles. The encapsulation efficiency was determined to be 45% for LS23, and 85% for BS3. The phospholipid composition of the liposomal membrane influences the drug loading efficiency and drug release kinetics. The membrane's hydrophobicity, thickness, and compactness determine the drug loading efficiency and leakage of molecules entrapped in liposomes.³⁷ As far as BS3 is concerned, the encapsulated fraction of extract in-

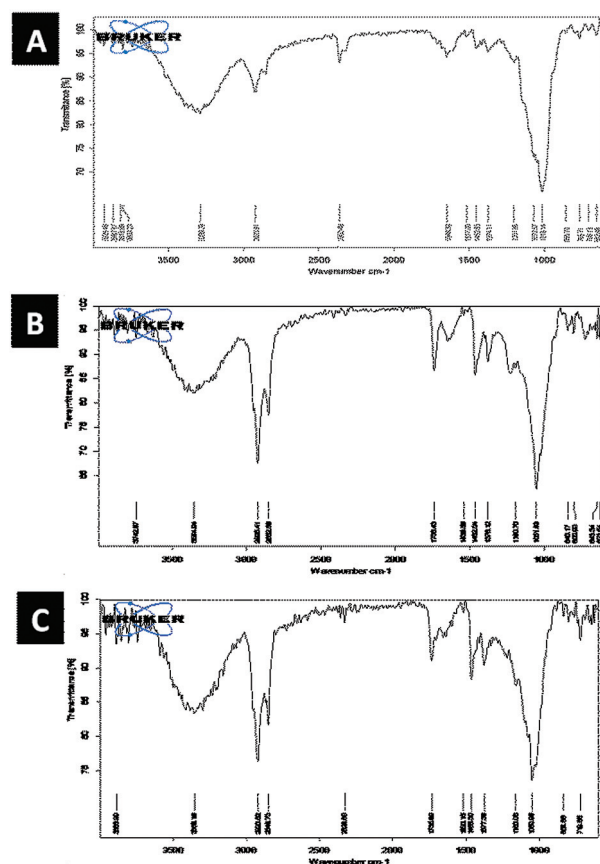


Fig. 3 FTIR spectrum shows (A) BME, (B) BME-loaded liposomes, (C) BME-loaded bilosomes.

creased proportionately with an increase in SPC concentration. It may be concluded that SPC concentration has a two-way effect on entrapment efficiency.³⁸

Encapsulation of extract was lower in LS23 without bile salt in the bilayers. Nevertheless, the entrapment efficiency of BS3 increased with increased SPC in the presence of bile salt. Reports state that the surface-active properties enable sodium deoxycholate to perturb the acyl chains of the lipid matrix and incorporate them into the surface of the membrane bilayer perpendicularly. Thus, sodium deoxycholate increases membrane flexibility, thereby improving the solubility of a highly lipophilic drug in the membrane. Thus, incorporating bile salts might facilitate BME penetration into the lipid bilayer.

Entrapment efficiency increased significantly with bile salts and high lipid content, implying that lipid and bile salts are the main factors influencing entrapment. The change in the vesicle size also affects the entrapment efficiency as a function of the sodium deoxycholate-to-PC molar ratio. Sodium deoxycholate in the exterior aqueous medium can decrease the surface tension of the vesicles, possibly resulting in a reduction in vesicle size.³⁹ Increased bile salt concentrations would result in the development of micelles in the dispersion medium, improving drug solubility in the medium and lowering percentage entrapment. Furthermore, high concentrations of bile salts have a fluidizing effect on lipid bilayers, resulting in the loss of the encapsulated medication. Raising the molar

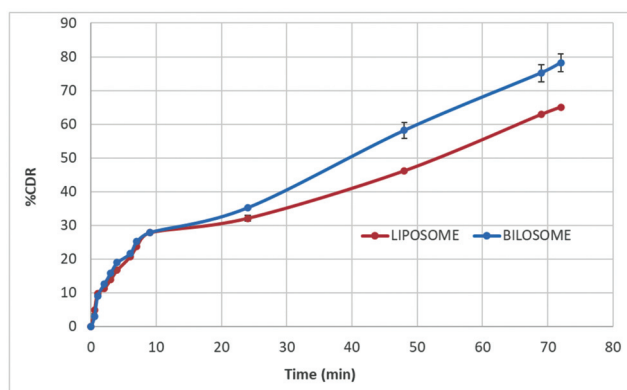


Fig. 4 In vitro drug release pattern of BM-loaded liposomes LS23 and BME loaded bilosomes BS3 (values are average of three trials).

ratio of SPC would increase the lipophilicity of the systems, increasing the amount of entrapped drugs.²⁴

In Vitro Drug Release Studies

–**Fig. 4** shows the in vitro drug release pattern of BME-loaded liposomes LS23 and bilosomes BS3. A biphasic release profile was observed in nanovesicular formulations, i.e., a rapid initial burst release followed by sustained release. The burst release of BME might be due to the detachment of surface-loaded extract and untrapped one. In contrast, the sustained release period could be due to BME release from the vesicular bilayer into the release medium over time.⁴⁰

In Vitro Release Kinetics

The release rates are shown in –**Table 3**. The optimized liposomal and bilosomal suspensions (LS23 and BS3) followed first-order release kinetics with a significant regression coefficient. The drug release process was analyzed using the exponential model Korsmeyer–Peppas and Higuchi. The mechanism of drug release followed the Higuchi model with a higher regression coefficient (R^2) than the Korsmeyer–Peppas model. The Higuchi model showed drug release by diffusion through the bilayers.⁴¹

In Vitro GI Stability of Liposomes and Bilosomes

Change in Vesicle Size and PDI of Nanovesicles during Gastric Digestion

–**Fig. 5 A** shows the stability of BME-loaded liposomes LS23 and bilosomes BS3 in the gastric fluid. In the absence of pepsin, the vesicle size of BME-loaded liposomes was 285 nm with a PDI of 0.5. After 1 minute of digestion with SGF, the

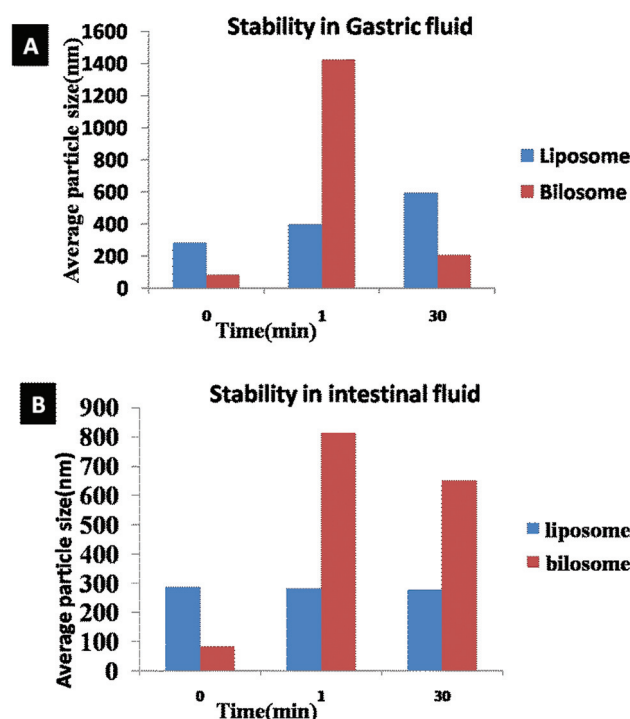


Fig. 5 (A) Stability of BME loaded liposomes LS23 and bilosomes BS3 in gastric fluid, (B) stability of BME-loaded liposomes and BME-loaded bilosomes in intestinal fluid.

vesicle size increased to 397 nm, further increasing to 595 nm after 30 min. PDI decreased to 0.40 after 1 minute of digestion and reduced to 0.24 after 30 minutes. In the absence of pepsin, the vesicle size of BME-loaded bilosomes was 84 nm with a PDI of 0.5. After 1 minute digestion with SGF, vesicle size increased to 1421 nm, which dropped to 210 nm after 30 min. PDI was 0.23 after 1 minute of digestion and did not decrease much after 30 minute. According to the findings, liposome’s increased size during digestion suggests vesicle aggregation or fusion. The significant drop in pH during the gastric phase, from neutral (pH 7.4 liposomal formulation) to severely acidic (pH 1.5); changes in pH alter the strength and extent of colloidal interaction between particles, permitting liposome coalescence.

Additionally, an osmotic effect due to the pH gradient across the phospholipid membrane (inside–outside the vesicles) could promote destabilization and fusion.²⁸ Studies to stabilize liposomes with bile salts and other surfactants reported an initial increase in the size followed by a decrease in the present study.⁴² The reduction in the vesicle size after 30 minutes of digestion may correspond to the detachment of

Table 3 In vitro drug release kinetics of BME-loaded liposome and bilosome formulations

Formulation code	Kinetic models								
	Zero-order		First-order		Higuchi		Korsmeyer–Peppas		
	R ²	K	R ²	K	R ²	K	R ²	n	K
LS23	0.929	0.759	0.966	–0.005	0.978	7.085	0.976	0.759	11.341
BS3	0.952	0.951	0.980	–0.008	0.985	8.913	0.950	0.951	11.44

loose aggregates formed initially, and this phenomenon rules out the possibility of a fusion of vesicles.

Change in Vesicle Size and PDI of Nanovesicles during Intestinal Digestion

► **Fig. 5B** illustrates the stability of BME-loaded liposomes LS23 and bilosomes BS3 in simulated intestinal fluid. In the absence of pancreatin, the vesicle size of BME-loaded liposomes was 285 nm with a PDI of 0.5. After 1 minute of digestion with SIF, the vesicular size decreased to 282 nm and 276 nm after 30 min. PDI decreased to 0.37 after 1 minute and further reduced to 0.33 after 30 minutes. In the absence of pancreatin, the vesicle size of BME-loaded bilosomes was 84 nm with a PDI of 0.38. After 1 minute of digestion with SIF, the vesicular size increased to 814.7 nm, then decreased to 651 nm after 30 minutes. PDI was 0.43 after 1 minute of digestion and 0.74 after 30 minutes. Previous studies showed that the pancreatic enzyme in SIF could modify the organized structure of assembled lipids or damage the liposomal membrane severely, indicating that the liposomes exhibited lower stability in SIF than in simulated gastric fluid.⁴³ The kinetic stability of the liposomal bilayer can be influenced by its composition and surface properties. Cholesterol, a principal constituent in many biological membranes, can increase bilayer thickness and membrane ordering and even reduce membrane permeability. Incorporating cholesterol has been assumed to provide physicochemical stabilization to liposomes.⁴⁴

In Vivo Cognitive Enhancement Activity Studies of Nanovesicles

BMs memory-enhancing and cognitive effects are believed to be mediated by bacoside A (bacoside A3, bacoside II, bacosaponin C, and bacoside X).⁴⁵ A recent report shows that BME inhibits selected human cytochrome P450 (CYP) drug metabolizing enzymes, in addition to altering the expression of rat hepatic and intestinal CYP drug metabolizing enzymes and intestinal P-glycoprotein.⁴⁶ Thus, it is conceivable that the bacoside constituents in BME may be metabolized in vivo to active forms before exerting their pharmacological effects. ► **Fig. 6A** and **6B** depicts the nootropic effect of BME-loaded liposomes and bilosomes on Swiss albino mice, compared with a formulation. Bilosomal delivery of BME significantly improved behavioral characteristics in dementia-induced mice than the extract, possibly because of the better absorption from GIT and better permeability via the BBB. ► **Table 4** provides the number of Y maze alternations reflecting the nootropic effect of BME and nanovesicles (LS23 and BS3). The bilosomes treated group showed more alternations in the Y-maze apparatus than the BME and liposomal vesicles. Better nootropic performance might be attributed to higher entrapment efficiency and, smaller bilosomes enhanced GIT penetration.

The protective effects of sodium deoxycholate on vesicle integrity might have improved BME's oral absorption and bioavailability.⁴⁷ Including negatively charged lipids changes the vesicle surface charge, affecting biodistribution, and clearance.⁴⁸

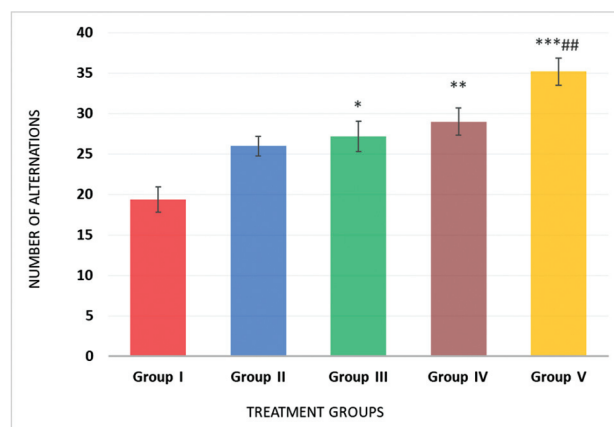


Fig. 6 Number of alternations of test and control groups. All values of mean ± SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compare with that of group I. ## $p < 0.01$ compare with that of Group II (repeated measure ANOVA followed by Tukey's *post hoc* test).

Table 4 Nootropic effect of BME, and nanovesicle formulation (LS23 and BS3) on Swiss albino mice

Groups	Number of alternations
Group I	19.4 ± 1.57
Group II	26.0 ± 1.23
Group III	27.2 ± 1.85*
Group IV	29.0 ± 1.67**
Group V	35.2 ± 1.66***##

Note: All values of mean ± SEM ($n = 5$). Group I: scopolamine treated, Group II: normal control, Group III: scopolamine + BME (200 mg/Kg) treated, Group IV: scopolamine + LS23 treated, Group V: scopolamine + BS treated.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$ compared with that of Group I.

$p < 0.01$ compared with that of Group II (repeated measure ANOVA followed by Tukey's *post hoc* test).

Scopolamine, a muscarinic antagonist, phosphorylates tau proteins leading to the formation of β -amyloid in the brain, resulting in memory impairment.⁴⁹ Studies have shown that ethanolic extract of BM could prevent β -amyloid-induced toxicity in cultured neurons by inhibiting acetylcholinesterase enzyme and lipid peroxidation.⁵⁰ In transgenic mice bearing human *PS-1* and *A β PP* mutations, long-term treatment of BME reduces β -amyloid in different brain regions, possibly the reason behind cognitive enhancement.⁵¹ In the animal model of dementia, Brahmi Ghrita (an ayurvedic preparation containing BM in ghee base) has shown a positive effect, possibly by reversing the depletion of acetylcholine, decreasing the choline acetyltransferase activity, and reducing muscarinic, cholinergic receptor binding in the frontal cortex and hippocampus, but also by alleviating cholinergic degeneration, lowering norepinephrine, and increasing 5-hydroxytryptamine levels in the hippocampus, hypothalamus, and cerebral cortex.^{1,52,53}

Conclusions

Due to suboptimal bioavailability, many phytoconstituents fail to validate ethnopharmacological claims in full measure. One cannot employ higher doses to overcome obstacles such as incomplete absorption, first-pass effect, etc. In this context, the present study has demonstrated the possibility of enhancing the pharmacological effects of BME by loading them in bilosomes. Various phytoconstituents with promising therapeutic or nutritional benefits can be formulated as vesicles. High-quality bioavailability evidence would entail using more advanced and direct techniques such as radiolabeling.

Funding/Acknowledgments

The authors are grateful to the Management of Nitte (Deemed to be a University) for the laboratory facilities and support for conducting this study. We acknowledge the kind help offered by Dr. Unnikrishnan M K, Professor, NGSIM Institute of Pharmaceutical Sciences, for the language corrections while writing the manuscript.

Conflict of Interest

None declared.

References

- Russo A, Borrelli F. *Bacopa monnieri*, a reputed nootropic plant: an overview. *Phytomedicine* 2005;12(04):305–317. Doi: 10.1016/j.phymed.2003.12.008
- Simpson T, Pase M, Stough C. *Bacopa monnieri* as an antioxidant therapy to reduce oxidative stress in the aging brain. *Evid Based Complement Alternat Med* 2015;2015:615384
- Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. *Int J Nanomedicine* 2019;14:5541–5554. Doi: 10.2147/IJN.S200490
- WHO. Global status report on the public health response to dementia. Geneva World Health Organization. Published online 2021:137. Accessed August 5, 2022, at: <https://www.who.int/multi-media/details/global-status-report-on-the-public-health-response-to-dementia>
- Bhattacharjee A, Shashidhara SC, Saha S. Nootropic activity of *Crataeva nurvala* Buch-Ham against scopolamine induced cognitive impairment. *EXCLI J* 2015;14:335–345
- Akanksha B, Avijit M, Seema G, Chakaraborthy GS. Screening of tephrosia purpurea for nootropic activity. *An Int Res J.* 2014;5(05):676–683
- Mondadori C, Ducret T, Borkowski J. The memory-enhancing effects of the piracetam-like nootropics are dependent on experimental parameters. *Behav Brain Res* 1989;33(01):79–82. Doi: 10.1016/S0166-4328(89)80020-8
- Kumar N, Abichandani LG, Thawani V, Gharpure KJ, Naidu MUR, Venkat Ramana G. Efficacy of standardized extract of *Bacopa monnieri* (Bacognize®) on cognitive functions of medical students: a six-week, randomized placebo-controlled trial. *Evid Based Complement Alternat Med* 2016;2016:4103423. Doi: 10.1155/2016/4103423
- Rajani M. *Bacopa monnieri*, a nootropic drug. *Bioact Mol Med Plants* 2008:175–195
- Raj A, Sarath Chandran C, Narayanan AV, et al. Lipid-based vesicles: a non-invasive tool for transdermal drug delivery. *J Pharm Innov* 2021;x:1–14
- Nayak PP, S N, Narayanan A, Badekila AK, Kini S. Nanomedicine in cancer clinics: are we there yet? *Curr Pathobiol Rep* 2021;9(02):43–55. Doi: 10.1007/s40139-021-00220-6
- v A, Cutinho LI, Mourya P, Maxwell A, Thomas G, Rajput BS. Approaches for encephalic drug delivery using nanomaterials: the current status. *Brain Res Bull* 2020;155:184–190. Doi: 10.1016/j.brainresbull.2019.11.017
- Grimaldi N, Andrade F, Segovia N, et al. Lipid-based nanovesicles for nanomedicine. *Chem Soc Rev* 2016;45(23):6520–6545. Doi: 10.1039/c6cs00409a
- Priprem A, Watanatorn J, Sutthiparinyanont S, Phachonpai W, Muchimapura S. Anxiety and cognitive effects of quercetin liposomes in rats. *Nanomedicine* 2008;4(01):70–78. Doi: 10.1016/j.nano.2007.12.001
- He H, Lu Y, Qi J, Zhu Q, Chen Z, Wu W. Adapting liposomes for oral drug delivery. *Acta Pharm Sin B* 2019;9(01):36–48. Doi: 10.1016/j.apsb.2018.06.005
- Taira MC, Chiamoni NS, Pecuch KM, Alonso-Romanowski S. Stability of liposomal formulations in physiological conditions for oral drug delivery. *Drug Deliv* 2004;11(02):123–128. Doi: 10.1080/10717540490280769
- Chonn A, Cullis PR. Recent advances in liposomal drug-delivery systems. *Curr Opin Biotechnol* 1995;6(06):698–708. Doi: 10.1016/0958-1669(95)80115-4
- Conacher M, Alexander J, Brewer JM. Oral immunisation with peptide and protein antigens by formulation in lipid vesicles incorporating bile salts (bilosomes). *Vaccine* 2001;19(20-22):2965–2974. Doi: 10.1016/S0264-410X(00)00537-5
- Kumar D, Sharma D, Singh G, Singh M, Rathore MS. Lipoidal soft hybrid biocarriers of supramolecular construction for drug delivery. *ISRN Pharm* 2012;2012:474830. Doi: 10.5402/2012/474830
- Aburahma MH. Bile salts-containing vesicles: promising pharmaceutical carriers for oral delivery of poorly water-soluble drugs and peptide/protein-based therapeutics or vaccines. *Drug Deliv* 2016;23(06):1847–1867. Doi: 10.3109/10717544.2014.976892
- Saesong T, Temkitthawon P, Nangngam P, Ingkaninan K. Pharmacognostic and physico-chemical investigations of the aerial part of *Bacopa monnieri* (L.). *Wettst* 2019;41:397–404
- Huang Z, Li X, Zhang T, et al. Progress involving new techniques for liposome preparation. *Asian J Pharm Sci* 2014;9(04):176–182. Doi: 10.1016/J.AJPS.2014.06.001
- Wang A, Ahmad A, Ullah S, Cheng L, Ke L, Yuan Q. A cheap and convenient method of liposome preparation using glass beads as a source of shear force. *AAPS PharmSciTech* 2017;18(08):3227–3235. Doi: 10.1208/s12249-017-0812-3
- Ahmed S, Kassem MA, Sayed S. Bilosomes as promising nanovesicular carriers for improved transdermal delivery: construction, in vitro optimization, ex vivo permeation and in vivo evaluation. *Int J Nanomedicine* 2020;15:9783–9798. Doi: 10.2147/IJN.S278688
- Han L, Zhan H, Sun X, Zhang ZR, Deng L. A density-changing centrifugation method for efficient separation of free drugs from drug-loaded particulate delivery systems. *AAPS J* 2019;21(03):33. Doi: 10.1208/s12248-019-0306-1
- Al-Mahallawi AM, Abdelbary AA, Aburahma MH. Investigating the potential of employing bilosomes as a novel vesicular carrier for transdermal delivery of tenoxicam. *Int J Pharm* 2015;485(1-2):329–340. Doi: 10.1016/j.ijpharm.2015.03.033
- Panwar P, Pandey B, Lakhera PC, Singh KP. Preparation, characterization, and in vitro release study of albendazole-encapsulated nanosize liposomes. *Int J Nanomedicine* 2010;5:101–108
- Vergara D, López O, Bustamante M, Shene C. An in vitro digestion study of encapsulated lactoferrin in rapeseed phospholipid-based liposomes. *Food Chem* 2020;321(March):126717. Doi: 10.1016/j.foodchem.2020.126717
- Sireeratawong S, Jaijoiy K, Khonsung P, Lertprasertsuk N, Ingkaninan K. Acute and chronic toxicities of *Bacopa monnieri* extract in Sprague-Dawley rats. *BMC Complement Altern Med* 2016;16:249
- Kwon SH, Lee HK, Kim JA, et al. Neuroprotective effects of chlorogenic acid on scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice. *Eur J*

- Pharmacol 2010;649(1-3):210–217. Doi: 10.1016/j.ejphar.2010.09.001
- 31 Kraeuter AK, Guest PC, Sarnyai Z, ; AK K. The Y-maze for assessment of spatial working and reference memory in mice. *Methods Mol Biol* 2019;1916:105–111. Doi: 10.1007/978-1-4939-8994-2_10
 - 32 Chong PLG, Zhu W, Venegas B. On the lateral structure of model membranes containing cholesterol. *Biochim Biophys Acta* 2009;1788(01):2–11. Doi: 10.1016/j.BBAMEM.2008.10.010
 - 33 Mohammed AR, Weston N, Coombes AGA, Fitzgerald M, Perrie Y. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *Int J Pharm* 2004;285(1-2):23–34. Doi: 10.1016/j.IJPHARM.2004.07.010
 - 34 Subczynski WK, Wisniewska A, Yin JJ, Hyde JS, Kusumi A. Hydrophobic barriers of lipid bilayer membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol. *Biochemistry* 1994;33(24):7670–7681. Doi: 10.1021/BI00190A022
 - 35 Zhao L, Temelli F, Curtis JM, Chen L. Preparation of liposomes using supercritical carbon dioxide technology: Effects of phospholipids and sterols. *Food Res Int* 2015;1(77):63–72
 - 36 Ardani HK, Imawan C, Handayani W, Djuhana D, Harmoko A, Fauzia V. Enhancement of the stability of silver nanoparticles synthesized using aqueous extract of *Diospyros discolor* Willd. Leaves using polyvinyl alcohol 2016;755:11001
 - 37 Alves AC, Magarkar A, Horta M, et al. Influence of doxorubicin on model cell membrane properties: insights from in vitro and in silico studies. *Sci Rep* 2017;7(01):6343
 - 38 Jahanfar S, Ghavami M, Khosravi-Darani K, Jahadi M, Mozafari MR. Entrapment of rosemary extract by liposomes formulated by Mozafari method: physicochemical characterization and optimization. *Heliyon* 2021;7(12):e08632. Doi: 10.1016/J.HELIYON.2021.E08632
 - 39 Sun J, Deng Y, Wang S, Cao J, Gao X, Dong X. Liposomes incorporating sodium deoxycholate for hexamethylmelamine (HMM) oral delivery: development, characterization, and in vivo evaluation. *Drug Deliv* 2010;17(03):164–170. Doi: 10.3109/10717541003667764
 - 40 Mohsen AM, Salama A, Kassem AA. Development of acetazolamide loaded bilosomes for improved ocular delivery: preparation, characterization and in vivo evaluation. *J Drug Deliv Sci Technol* 2020;59 (July):101910. Doi: 10.1016/j.jddst.2020.101910
 - 41 Jain A, Jain SK. In vitro release kinetics model fitting of liposomes: an insight. *Chem Phys Lipids* 2016;201:28–40. Doi: 10.1016/j.chemphyslip.2016.10.005
 - 42 López O, de la Maza A, Coderch L, López-Iglesias C, Wehrli E, Parra JL. Direct formation of mixed micelles in the solubilization of phospholipid liposomes by Triton X-100. *FEBS Lett* 1998;426(03): 314–318. Doi: 10.1016/S0014-5793(98)00363-9
 - 43 Liu W, Ye A, Liu W, Liu C, Singh H. Stability during in vitro digestion of lactoferrin-loaded liposomes prepared from milk fat globule membrane-derived phospholipids. *J Dairy Sci* 2013;96 (04):2061–2070. Doi: 10.3168/JDS.2012-6072
 - 44 Qin Y, Chen H, Zhang Q, et al. Liposome formulated with TAT-modified cholesterol for improving brain delivery and therapeutic efficacy on brain glioma in animals. *Int J Pharm* 2011;420(02): 304–312. Doi: 10.1016/J.IJPHARM.2011.09.008
 - 45 Ramasamy S, Chin SP, Sukumaran SD, Buckle MJC, Kiew LV, Chung LYC. In silico and in vitro analysis of bacoside A aglycones and its derivatives as the constituents responsible for the cognitive effects of *Bacopa monnieri*. *PLoS One* 2015;10(05):e0126565. Doi: 10.1371/JOURNAL.PONE.0126565
 - 46 Singh R, Panduri J, Kumar D, et al. Evaluation of memory enhancing clinically available standardized extract of *Bacopa monnieri* on P-glycoprotein and cytochrome P450 3A in Sprague-Dawley rats. *PLoS One* 2013;8(08):e72517. Doi: 10.1371/JOURNAL.PONE.0072517
 - 47 Arafat M, Kirchohofer C, Mikov M, Sarfraz M, Löbenberg R. Nano-sized liposomes containing bile salt: A vesicular nanocarrier for enhancing oral bioavailability of BCS class III drug. *J Pharm Sci* 2017;20(00):305–318. Doi: 10.18433/J3CK88
 - 48 Mayer LD, Tai LCL, Ko DSC, et al. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. *Cancer Res* 1989;49(21): 5922–5930
 - 49 Francis PT, Palmer AM, Snape M, Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatry* 1999;66(02):137–147. Doi: 10.1136/JNNP.66.2.137
 - 50 Limpeanchob N, Jaipan S, Rattanakaruna S, Phrompittayarat W, Ingkaninan K. Neuroprotective effect of *Bacopa monnieri* on beta-amyloid-induced cell death in primary cortical culture. *J Ethnopharmacol* 2008;120(01):112–117. Doi: 10.1016/j.jep.2008.07.039
 - 51 Holcomb LA, Dhanasekaran M, Hitt AR, Young KA, Riggs M, Manyam BV. *Bacopa monnieri* extract reduces amyloid levels in PSAPP mice. *J Alzheimers Dis* 2006;9(03):243–251. Doi: 10.3233/JAD-2006-9303
 - 52 Sara SJ. Noradrenergic-cholinergic interaction: its possible role in memory dysfunction associated with senile dementia. *Arch Gerontol Geriatr Suppl* 1989;1:99–108
 - 53 Saraf MK, Prabhakar S, Khanduja KL, Anand A. *Bacopa monnieri* attenuates scopolamine-induced impairment of spatial memory in mice. *Evid Based Complement Alternat Med* 2011;2011:236186. Doi: 10.1093/ECAM/NEQ038