



Gemini Surfactant-Lipid-Based Nanovesicles (“GeminiSomes”) for Oral Delivery of Silybin: A Promising Strategy for Hepatoprotective Therapy

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Abstract

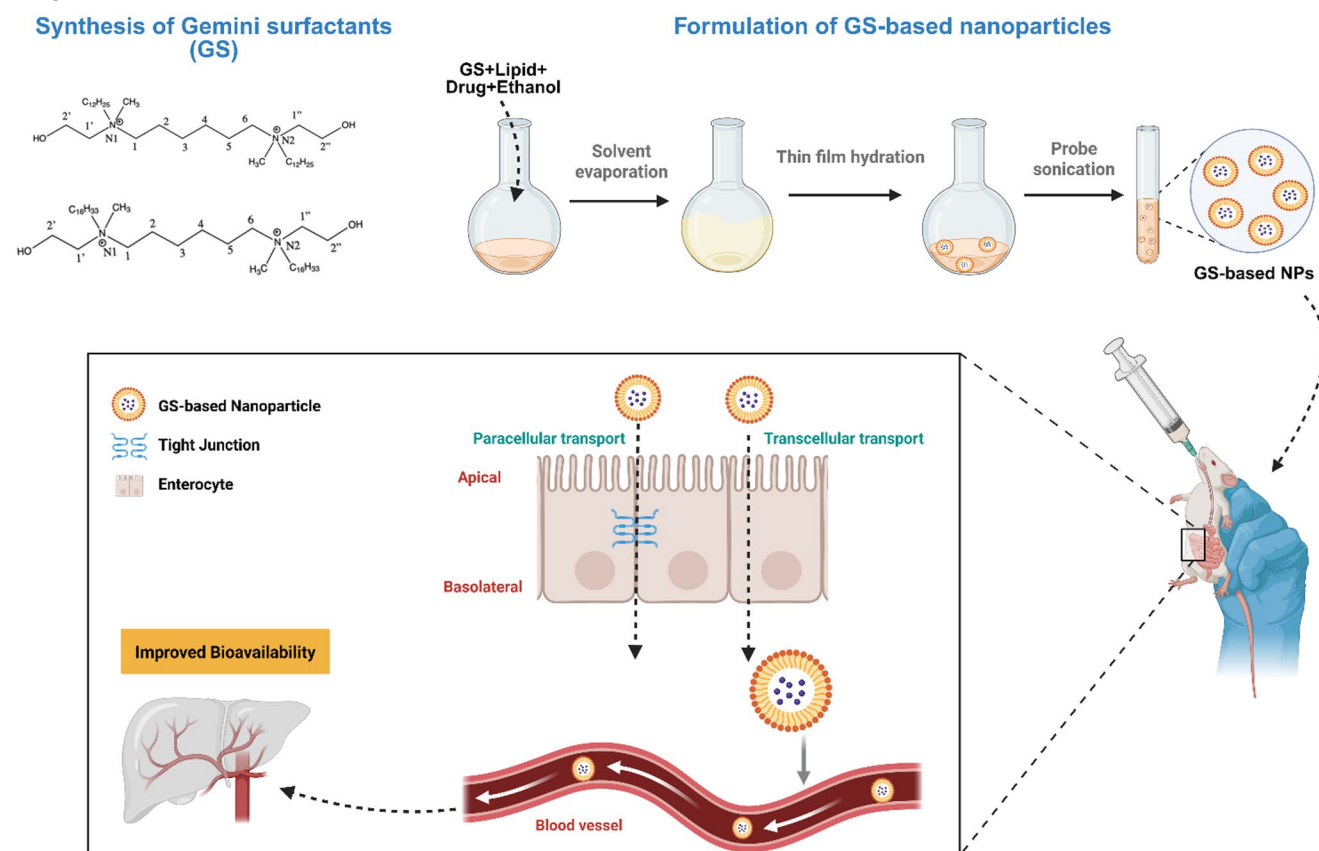
The rising global burden of hepatitis underscores the urgent need for effective liver-targeted therapies. Silybin, a natural flavanolignan from *Silybum marianum*, is well recognized for its hepatoprotective and antiviral potential but suffers from poor oral bioavailability due to low solubility and rapid metabolism. To address this, we developed a novel surfactant-based nanocarrier system to unlock the therapeutic potential of orally administered silybin. The optimized formulation, comprising biocompatible lipids and cationic surfactants, produced stable nanovesicles (121 nm) with high encapsulation efficiency and biphasic drug release. Caco-2 cell transport studies demonstrated a 4.8-fold enhancement in permeability, while pharmacokinetic analysis revealed a 5.3-fold increase in oral bioavailability. In vivo, the nanoformulation significantly mitigated hepatotoxicity in D-galactosamine- induced hepatitis model, reducing serum ALT, AST, and pro-inflammatory cytokines, while restoring liver architecture. These outcomes were further supported by histological evidence of tissue regeneration. This work introduces a clinically viable nanodelivery strategy that overcomes key biopharmaceutical challenges of silybin, offering a promising translational platform for improving oral hepatitis therapy through enhanced absorption and hepatoprotective efficacy.

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Graphical Abstract



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1 Introduction

Liver diseases are a growing global health concern, causing an estimated two million deaths each year [1]. These include conditions such as chronic liver disease, cirrhosis, viral hepatitis, and liver cancer, which are often progressive and difficult to manage. Among them, viral hepatitis has become the second leading infectious cause of death worldwide, with annual deaths rising from 1.1 million in 2019 to 1.3 million in 2022 [2]. The liver's essential role in detoxification and metabolic regulation makes it particularly vulnerable to damage from both internal factors (like oxidative stress and metabolic by-products) and external insults (such as alcohol, drugs, and infections) [3]. If not treated promptly, liver injury can lead to severe outcomes, including cirrhosis or hepatocellular carcinoma, due to impaired regeneration and chronic inflammation [4].

Herbal medicines are increasingly explored for hepatotherapy due to their perceived safety, affordability, and minimal side effects. Their hepatoprotective effects are mainly

linked to antioxidant activity, inhibition of lipid peroxidation, and modulation of inflammatory pathways [5]. Among them, flavonoids such as silybin have demonstrated notable therapeutic potential. Silybin (SIL), the principal active constituent of *Silybum marianum*, has long been used for liver disorders [6, 7]. However, its clinical efficacy is limited by poor aqueous solubility ($\sim 12.9 \mu\text{g/mL}$), low membrane permeability ($\sim 0.64 \mu\text{g/mL}$), and limited gastrointestinal absorption (23–47%), leading to very low oral bioavailability ($\sim 0.95\%$) [8–12].

Although the oral route remains the most patient-compliant and preferred route of administration, improving SIL's oral bioavailability remains a critical challenge [12, 13]. Recent advances in nanoformulation strategies have further highlighted the therapeutic potential of *Silybum marianum*-derived compounds, demonstrating that nanocarrier-based delivery systems can significantly enhance the hepatoprotective and anticancer efficacy of silybin by improving its solubility, stability, and cellular uptake [14–16]. Several nanocarriers, including solid lipid nanoparticles, liposomes,

polymeric micelles, and cyclodextrin inclusion complexes, have demonstrated improved pharmacokinetic profiles [17–19]. However, many of these systems still face challenges such as low drug loading efficiency, poor long-term stability, and the use of excipients with questionable safety profiles.

Surfactant-based nanocarriers, particularly those employing non-ionic or zwitterionic surfactants, have shown potential in enhancing membrane permeation and improving colloidal stability. However, their application is often limited by concentration-dependent cytotoxicity and formulation complexity [20].

In contrast, Gemini surfactants (GS) represent a structurally advanced class of dimeric amphiphiles composed of two hydrophobic tails and two hydrophilic head groups linked by a spacer [21]. This architecture confers significantly lower critical micelle concentrations (CMC), enhanced surface activity, superior micellar stability, and greater structural tunability compared with conventional single-chain surfactants [22–24]. For example, a cationic Gemini surfactant such as 12-2-12 has been reported to exhibit significantly lower CMC compared with its corresponding conventional single-chain analogue, dodecyltrimethylammonium bromide (DTAB) [25]. These physicochemical advantages can facilitate improved solubilization and stabilization of hydrophobic drugs, making them attractive candidates for drug delivery applications.

Gemini-conventional surfactant mixed systems have been reported to significantly influence micellization behaviour and enhance the solubilization of hydrophobic compounds such as phenanthrene under identical conditions, compared with conventional surfactant systems [26], indicating that interactions between Gemini and conventional surfactants can substantially modify self-assembly and functional properties of surfactant systems. In addition, incorporation of a cleavable cationic Gemini surfactant (16-E1-16) into a Pluronic P123-based system has been shown to alter micellization and gelation behaviour, resulting in faster ibuprofen release compared with both pure P123 and pure 16-E1-16 micelles [27]. GS are also capable of forming diverse self-assembled nanostructures, including bilayers, micelles, and vesicles, and have been successfully applied in nucleic acid delivery and stabilization of lipid-based systems [22–29]. For example, long-chain cationic Gemini surfactants have been incorporated into liposomal formulations to enhance drug retention and stability in atorvastatin-loaded systems, highlighting their utility as functional excipients in nanocarrier design [30]. Moreover, cationic GS such as 12-3-12 have been reported to enhance gastrointestinal bioadhesion and improve the

oral bioavailability of hydrophobic compounds, such as coenzyme Q10 nanocrystals, by promoting membrane interaction and improving solubilization [31]. These attributes make GS a promising candidate for oral delivery applications.

In the present study, two structurally similar GS variants were synthesized by varying hydrophobic tail length while maintaining a constant spacer group. These GS molecules were employed to formulate stable lipid-based nanovesicles (GeminiSomes) for oral delivery of SIL. The aim was to enhance SIL's intestinal permeability and address its biopharmaceutical limitations. The green, cost-effective synthesis, high drug loading capacity, and potential for improved bioavailability with reduced toxicity make GS-based nanovesicles a promising translational platform for oral delivery of poorly soluble therapeutics like SIL.

2 Materials and Methods

2.1 Chemicals

Silybin was procured from TCI Chemicals Pvt. Ltd., Hyderabad, India. 1-bromododecane, 1-bromohexadecane, 2-methylaminoethanol, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 5,5'-dithiobis-(2-nitrobenzoic acid) were procured from Sigma Aldrich, St Louis, MO, USA. Lipoid S 100 was supplied as a gift sample from Lipoid, Ludwigshafen, Germany. All other chemicals and solvents used were of analytical grade.

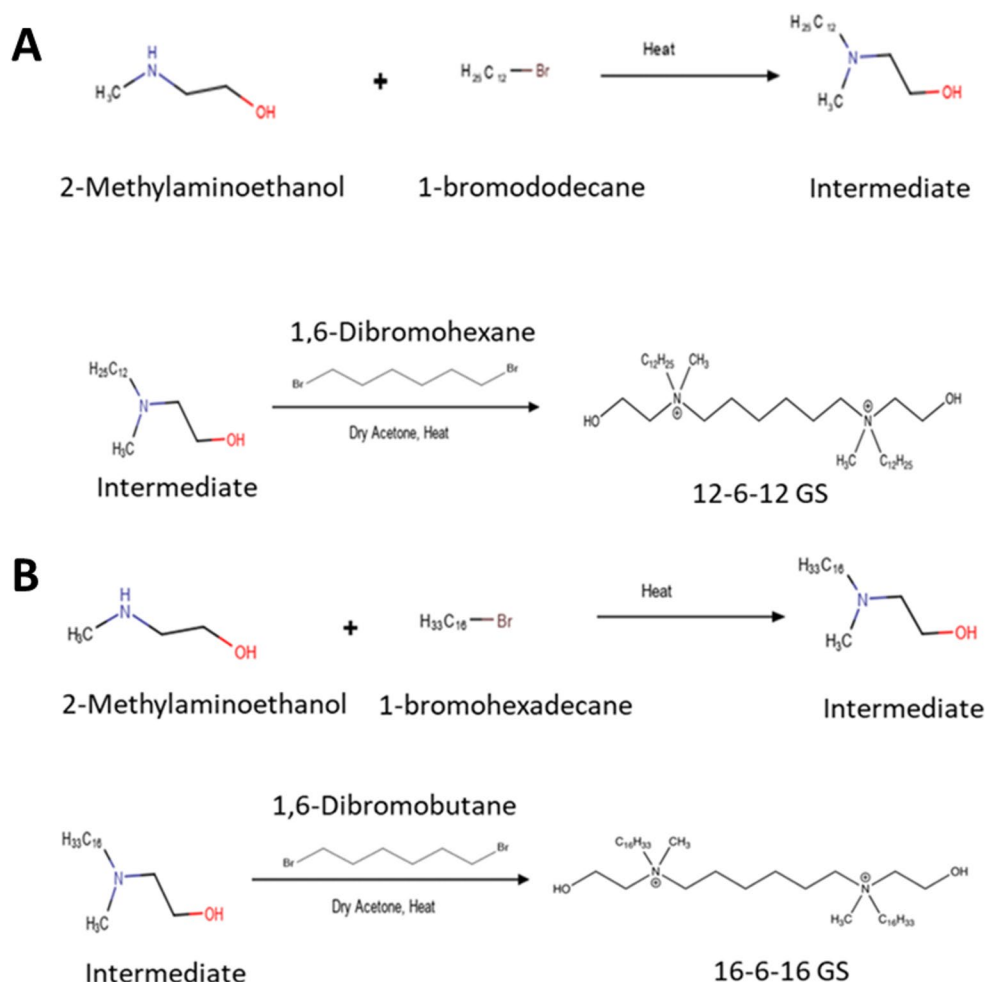
2.2 Cell lines and Culture Conditions

Human liver carcinoma (HepG2) and colorectal adenocarcinoma (Caco-2) cell lines were obtained from the National Center for Cell Science, Pune, India and were cultured in DMEM media with 1% antibiotic-antimycotic solution and 10% FBS at 37 °C and maintained in a CO₂ incubator with 5% CO₂ and 18–20% O₂.

2.3 Synthesis of Gemini Surfactants

In this study, two Gemini surfactants were synthesized: N1,N6-didodecyl-N1,N6-bis(2-hydroxyethyl)-N1,N6-dimethylhexane-1,6-diaminium and N1,N6-dihexadecyl-N1,N6-bis(2-hydroxyethyl)-N1,N6-dimethylhexane-1,6-diaminium (Fig. 1). The synthesis proceeded in two steps. First, 2-methylaminoethanol was reacted with either 1-bromododecane or 1-bromohexadecane at 90 °C for 12 h. The

Fig. 1 Reaction scheme for the synthesis of the Gemini surfactants (A) 12-6-12 GS. (B) 16-6-16GS



excess reagent was removed by vacuum distillation, and the organic layer was extracted using dichloromethane, washed with sodium hydroxide and brine, dried over sodium sulfate, filtered, and evaporated to yield the intermediate. In the second step, the intermediate was dissolved in anhydrous acetone and reacted with either 1,4-dibromobutane or 1,6-dibromohexane at 60 °C for 70 h. The mixture was then cooled and refrigerated for 12 h before evaporating the solvent. The resulting crude solid was washed with petroleum ether, filtered, and recrystallized from methanol. The final compounds were characterized and confirmed by mass spectrometry and H-NMR.

2.4 Formulation of SIL Loaded GS Nanovesicles

The GS surfactants were used in combination with lipids and cholesterol to formulate gemini surfactant-based nanovesicles (GeminiSomes). These nanovesicles were produced through a process involving thin film

evaporation, followed by size reduction using sonication. Briefly, 60% w/w of GS (12-6-12GS or 16-6-16 GS), 30% w/w of Lipoid S 100 and 10% of cholesterol were appropriately weighed and dissolved in 10 ml ethanol. The drug (SIL; 25% w/w) was added to the mixture and stirred until the solution became clear. A uniform thin dried film was obtained after vacuum evaporation using a rotary evaporator (IKA, RV 10) under reduced pressure. The film was hydrated for 1 h using phosphate buffer pH 7.4, and the dispersion was sonicated using a probe sonicator (PRO650, LABMAN, Chennai, Tamil Nadu, India) at a reduced temperature (using ice bath) to decrease the size of the nanovesicles. The untrapped drug was separated by centrifugation (22000 rpm for 1 h), and the pellet was collected. The fluorescent labelled nanovesicles were prepared in a similar manner as described above, where a 50 µg/mL Fluorescein isothiocyanate (FITC) solution in PB pH 7.4 was used during the rehydration [32]. The purified nanovesicles free from the untrapped

Table 1 Optimization of G16 surfactant nanovesicles using Box Behnken Design

Formulation code	Surfactant to lipid ratio	Sonication time	Cholesterol (%)
1	0.636	3	5
2	2.6	3	5
3	0.636	9	5
4	2.6	9	5
5	0.636	6	2
6	2.6	6	2
7	0.636	6	8
8	2.6	6	8
9	1.618	3	2
10	1.618	9	2
11	1.618	3	8
12	1.618	9	8
13	1.618	6	5
14	1.618	6	5
15	1.618	6	5

drug were lyophilized using 5% trehalose and stored at 4°C until further use.

2.5 Optimization of the GS16 Nanovesicles

Three significant factors were chosen based on the preliminary trials Table S1; Supplementary Information and optimized using Box Behnken Design (BBD). The BBD is a statistical experimental design that consists of three levels and three factors. This design involves a set of points that are located at the midpoint of each edge of a multidimensional cube, as well as replicated central point's designing the area of interest. The goal was to estimate the values of key variables that would result in maximum entrapment efficiency (%EE), minimum particle size, and PDI. The fifteen experiments presented in Table 1 were conducted using the Design Expert software (Version 9.0.3.1; Stat Ease, Minneapolis, USA). The measured parameters were particle size, PDI, zeta potential and entrapment efficiency. The regression coefficient was computed to determine the association between the input and output variables [33]. The validation of the model was carried out, where the model projected the parameters based on the desirability value. The % relative error was calculated using the equation given below. DOE optimization was performed only for GS-16. The optimized parameters obtained for GS-16 were later applied to GS-12, which was not independently optimized.

$$\% \text{Relative error} = \frac{\text{Predicted value} - \text{Observed value}}{\text{Predicted value}} \times 100$$

2.6 Characterization of SIL-Loaded GS Nanovesicles

2.6.1 Solid State Characterization

The solid-state characterization of the pure drug (SIL), GS16, GS-16-OPT-L, GS-12-OPT-L, LS100, GS16-physical mixture (GS16-PM), GS12-physical mixture (GS12-PM), was carried out using the procedure outlined in Sect. 1.1; supplementary information. The samples were subjected to FTIR, DSC and XRD.

2.6.2 Particle Size, Polydispersity Index and Zeta Potential

The particle size, PDI, and ZP were measured using a Zeta-sizer (Malvern Instruments, Malvern, UK). The particle size and PDI were determined at 25 °C using a disposable polystyrene cuvette. Prior to estimation, the sample was diluted 10-fold with MilliQ water. The ZP of the GeminiSomes was determined using the Zetasizer, where the electrophoretic mobility of the particle under electric field was determined. These parameters were measured for all the GeminiSomes [34]. Detailed procedure employed for determining entrapment efficiency and drug loading is given in Sect. 1.3; Supplementary Information.

2.6.3 Morphological Analysis

Structural morphology was further characterized by TEM (FEI Tecnai G2 Spirit Bio-Twin, The Netherlands). A drop of the diluted formulation was placed on a carbon-coated copper grid, dried under an infrared lamp, and imaged using an OSIS Veleta CCD camera [35].

2.7 In vitro Dissolution Study

The in vitro dissolution test on pure SIL, GS-16-OPT and GS-12-OPT was conducted in triplicate. The results were then presented as the cumulative amount of SIL released at each time point. Each sample was weighed to the equivalent of 2 mg of SIL and added to the dissolution medium. The drug release was evaluated using three different buffers, HCl buffer with a pH of 1.2 for a duration of 2 h, and PB with pH values of 6.8 and 7.4 for 24 h each. The samples were added to 200 mL of the dissolution media containing Tween 80 (0.5% w/v) to maintain sink conditions. This medium was held in a shaking incubator set at 150 rpm and 37 °C. Samples (2 mL) were collected and replaced with fresh buffer at specified intervals for both HCl buffer (0.25, 0.5, 0.75, 1 and 2 h) and PB pH 6.8 and 7.4 (0.25, 0.5, 1, 2, 4, 8, 12, and 24 h). The samples were filtered using a

0.22 µm syringe filter and diluted with the mobile phase. The concentration of SIL released at every time point was estimated using HPLC.

2.8 Stability Studies

Stability studies were performed on GS-16-OPT and GS-12-OPT formulations in both dispersion and lyophilized form. The samples were stored in an amber-type 1 glass vial hermetically sealed with a rubber stopper and were kept at a temperature of $4 \pm 2^\circ\text{C}$ for a duration of 90 days. The particle size, PDI, ZP and %EE were measured at 30, 60, and 90 days.

2.9 In Vitro Hepatoprotective Activity Against D-Galactosamine

The in vitro cytotoxicity of SIL, GS-16-OPT, GS-12-OPT, GS-16-OPT-P, and GS-12-OPT-P formulations were evaluated using the MTT assay. Caco-2 and HepG2 cell lines were used to investigate both intestinal and hepatic cytocompatibility. Briefly, cells were seeded in 96-well plates and incubated for 24 h. Following various treatments, the MTT reagent was added and incubated for 4 h, after which the formazan crystals were solubilized and absorbance was measured at 570 nm using a microplate reader [36]. % cell viability was calculated using the formula given below.

$$\% \text{Cell viability} = \frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of untreated cells}} \times 100$$

In vitro hepatoprotective activity was assessed in HepG2 cells using a D-galactosamine (D-Gal)-induced hepatotoxicity model [37]. A 200 µL suspension of HepG2 cells (20,000 cells/well) was seeded in 96-well plates and allowed to adhere for 24 h. The culture medium was then discarded, and hepatotoxicity was induced by incubating the cells with 25 mM D-Gal for 12 h. After induction, the medium was removed and cells were treated with test samples (SIL, GS-16-OPT, and GS-12-OPT at 50 µg/mL) for an additional 24 h. Following treatment, the MTT assay was performed as described above to assess cell viability and hepatoprotection.

2.10 Cellular Uptake Studies

Cellular internalization of FITC-labelled GS-16-OPT and GS-12-OPT nanovesicles was evaluated in Caco-2 cells using flow cytometry (BD FACSCalibur™, BD Biosciences,

USA). Cells were seeded in 6-well plates at a density of 2×10^5 cells/mL and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The medium was then replaced with fresh medium containing FITC-labeled formulations (50 µg/mL), followed by 30 min incubation in the dark. After treatment, cells were washed twice with PBS, detached using 0.25% trypsin-EDTA, and transferred into polystyrene tubes. Following centrifugation at $300 \times g$ for 5 min, the supernatant was discarded, and cells were resuspended in PBS. Fluorescence intensity was measured using a flow cytometer (excitation: 488 nm; emission: 535 nm) to quantify cellular uptake [38].

2.11 Permeability Studies in Caco-2 Cell Monolayer

The transepithelial electrical resistance values of Caco-2 monolayers of all wells were measured using the Millicell ER system before and after the experiments to measure the integrity of epithelial monolayers. Caco-2 cell monolayers with values above $300 \pm 15 \Omega \text{ cm}^2$ were selected for the study. The test samples (i.e., plain SIL, SIL-16-OPT, SIL-12-OPT (50 µg/mL; 500 µL)) were introduced to the donor compartment, and HBBS (1500 µL) was added to the receiver compartments. 200 µL was withdrawn from the receiver compartment at 0, 30, 60, 120 and 240 min, and the same volume was replenished with HBBS. The content of SIL in the above samples was analyzed by HPLC, and the apparent permeability coefficient (P_{app} : cm/s) from apical to basal or basal to apical was calculated using the equation given below.

$$P_{app} = dQ/dt \times 1/AC_0$$

where dQ/dt , A , C_0 is the steady state flux, surface area (cm^2) and initial concentration of drug used, respectively [39]. Additionally, *Ex vivo* intestinal permeation studies were performed, procedure is described in Sect. 1.4; Supplementary information.

2.12 Pharmacokinetics Studies

The in vivo pharmacokinetics studies for GS-16-OPT, GS-12-OPT, and marketed product (Silybon®) was conducted in male Wistar rats and compared with the pure SIL. The animals were assigned randomly to either the control or treatment groups, subjected to overnight fasting before the start of the experiment. The animals were segregated into 4 groups and orally administered pure SIL, GS-16-OPT, GS-12-OPT, or Silybon®, each at a dose of 200 mg/kg. At specific times, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h, the blood

was withdrawn from the tail vein of the rats and collected in centrifuge tubes containing 10% EDTA. The plasma was separated under centrifugation at 10,000 rpm for 10 min at 4 °C and stored for further processing at −20 °C. The plasma samples were processed using liquid-liquid extraction and the unknown concentration of the drug was assessed using the HPLC. All parameters were kept like those of the analytical method except for mobile phase composition (15 mM KH₂PO₄ buffer pH 3.8; 48:52) and injection volume (80 µL) and internal standard (naringenin). PK Solutions software (PK Solutions 2.0™) was used to assess the pharmacokinetic parameters [34].

2.13 In Vivo Hepatoprotective Studies

Hepatoprotective activity was evaluated in male Wistar rats ($n = 6$), divided into six groups. The control group received no treatment. Hepatotoxicity was induced in the positive control group via intraperitoneal injection of D-galactosamine (D-Gal, 400 mg/kg). The treatment groups received either SIL, GS-16-OPT, GS-12-OPT, or the marketed formulation Silybon® at an oral dose of 100 mg/kg/day for 7 days, following D-Gal administration (400 mg/kg), to assess their protective effects against liver injury. The control group received an equivalent volume of olive oil instead of D-Gal. 24 h post D-Gal administration, blood samples were collected via retro-orbital puncture. Samples were centrifuged at 4 °C for 5 min at 5000 rpm to separate the serum, which was stored at −20 °C for subsequent biochemical and anti-inflammatory analyses [40] (Sect. 1.5; Supplementary Information).

2.14 Statistical Analysis

Design Expert (Version 9.0.3.1; Stat Ease, Minneapolis, USA) was used to carry out the statistical assessment for formulation optimization. The PK Solutions 2.0 program was used to ascertain the pharmacokinetic parameters. Graph Pad Prism version 8.0.1 (San Diego, California, USA) was used to evaluate the data statistically. A ' p ' value of less than 0.05 was deemed statistically significant.

3 Results and Discussion

3.1 Synthesis and Characterization of GS

Two Gemini surfactants were synthesised and structurally confirmed prior to formulation development.

3.1.1 CMC Determination

Electrical conductivity is a commonly employed method to investigate the micellization behaviour of ionic surfactants in both aqueous and non-aqueous environments. Plotting specific conductivity versus concentration allows for the general determination of the critical micellization concentration (CMC) values of ionic surfactants. The CMC was determined from the conductimetry and was found to be 1.41 mM and 2.28 mM for 12-6-12 GS and 16-6-16 GS respectively as shown in Fig. 2C. Interestingly, 12-6-12 GS exhibited a lower CMC than 16-6-16 GS, indicating more favourable micellization despite its shorter hydrophobic chain.

Although an increase in alkyl chain length typically reduces CMC due to enhanced hydrophobic interactions, Gemini surfactant micellization is also strongly influenced by spacer architecture, headgroup electrostatic interactions, and molecular packing constraints. The combination of extended hydrophobic chains with a fixed spacer length in 16-6-16 may introduce steric and electrostatic effects that increase the free energy of micellization, thereby resulting in a comparatively higher CMC. These results suggest that micellization in Gemini surfactants is governed by a balance of hydrophobic interactions and interfacial electrostatic/steric effects rather than chain length alone.

3.1.2 Mass Spectrometry

The molecular weights of the synthesized surfactants were found to be 569.9 g/mol (12-6-12 GS) and 681.7 g/mol (16-6-16 GS) as per the mass spectrum shown in Fig. 2B. Mass spectra confirmed successful synthesis with close agreement between theoretical and experimental molecular weights, indicating high structural purity.

3.1.3 Proton NMR

Distinct chemical shift patterns corresponding to alkyl chains, spacer protons, and headgroup regions confirmed the structures of both surfactants (12-6-12 GS and 16-6-16 GS) (Fig. 2D; Table 2).

3.1.4 FTIR spectroscopy

The FTIR spectra of the synthesized GS (12-6-12 GS and 16-6-16 GS) were determined using the FTIR (Bruker, Alpha II) and the graphs are shown in Fig. 2E. For the 16-6-16 GS/12-6-12 GS surfactants, the peaks observed at

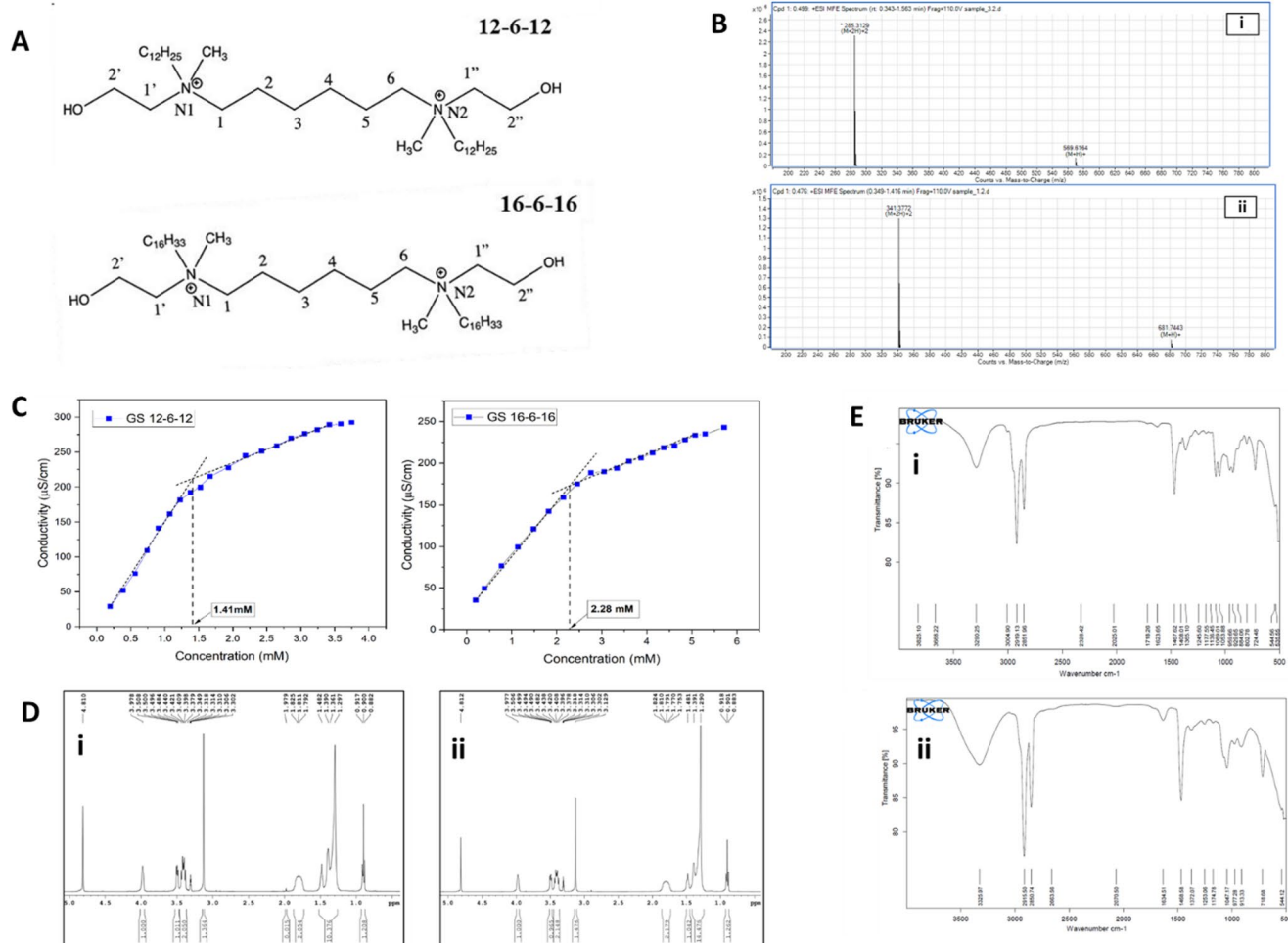


Fig. 2 (A) Structure of 12-6-12 and 16-6-16 Gemini surfactants; (B) Mass spectra of (i) 12-6-12 GS and (ii) 16-6-16 GS; (C) Conductivity of 12-6-12 and 16-6-16 GS; (D) NMR spectra (i) 12-6-12 GS

and (ii) 16-6-16 GS; (E) FTIR spectroscopy of Gemini surfactants (i) 12-6-12 GS and (ii) 16-6-16 GS

2915.5/2919.13 cm^{-1} and 2850.74/2851.96 cm^{-1} are associated with the C-H stretch, and a split peak is due to the presence of long hydrocarbon chains (C12 and C16 side chains of 12-6-12 GS and 16-6-16 GS, respectively). The broad peak at 3325.97/3290.25 cm^{-1} is due to the OH groups present as 2-hydroxyethyl groups in the 16-6-16 GS and 12-6-12 GS. The 1468.58/1467.62 cm^{-1} intense peak is due to the C-N stretching vibrations. These spectral features collectively confirm the structural integrity of the synthesized Gemini surfactants, supporting their suitability for nanovesicle formation.

The current study involved the preparation of nanovesicles using the synthesized surfactants followed by its optimization using the Design of Experiment approach. This study involved the use of lipids and cholesterol along with the surfactant to form a nanovesicles that can improve the

encapsulation of the drug. The process of formulating the nanovesicles was systematically performed by initially performing preliminary trials (Sect. 1.2 and Table S1; Supplementary Information), followed by optimization of the most critical formulation and process parameters that influence the characteristics of the nanovesicles. GS 16-6-16 was selected for optimization due to its superior preliminary performance, including higher entrapment efficiency (EE), smaller and more uniform particle size, and higher zeta potential (ZP), suggesting enhanced colloidal stability. The improved performance of GS-16 can be attributed to its longer C16 hydrophobic chains, which enhance hydrophobic interactions within the vesicular bilayer, promote tighter molecular packing, and improve structural integrity compared to the shorter-chain GS-12.

Table 2 δ -value of respective types of H atoms for 12-6-12 GS and 16-6-16 GS

12-6-12 GS			
Type of H	Integral	Splitting	δ -value
H, -CH ₃ of dodecyl	1.208	Triplet	0.882– 0.917.882.917
40H, -CH ₂ - of dodecyl	10.376	multiplet	1.297– 1.482.297.482
6H, N ¹ ,N ² -CH ₃	1.364	Singlet	3.140
4H, 1',1''-CH ₂	1.011+2.050	multiplet	3.302– 3.508.302.508
4H, 2',2''-CH ₂			
8H, N ¹ ,N ² -CH ₂			
2H, -OH	2H, -OH	singlet	3.978
4H, 2,3,4,5-CH ₂	2.054	multiplet	1.792– 1.825.792.825
16-6-16 GS			
Type of H	Integral	Splitting	δ -value
6H, -CH ₃ of hexadecyl	1.262	Triplet	0.883– 0.918.883.918
56H, -CH ₂ - of hexadecyl	14.476	Multiplet	1.290– 1.391.290.391
6H, N ¹ ,N ² -CH ₃	1.476	Singlet	3.129
8H, N ¹ ,N ² -CH ₂ 4H, 1',1''-CH ₂ 4H, 2',2''-CH ₂	2.148+0.965	Multiplet	3.378– 3.506.378.506
2H, -OH	1.000	Singlet	3.977
4H, 2,3,4,5-CH ₂	2.179	Multiplet	1.753– 1.824.753.824
6H, -CH ₃ of hexadecyl	1.262	Triplet	0.883– 0.918.883.918

3.2 Optimization of the 16-6-16 GS Gemini surfactant nanovesicles

A Box-Behnken Design (BBD) was employed with surfactant-to-lipid ratio (S/L), sonication time, and pulse “on” time as independent variables. Dependent variables included particle size, PDI, ZP, and %EE (Table S4; Supplementary information). ANOVA confirmed statistical significance of the generated models. The quadratic models obtained from the BBD are presented in Table S5; Supplementary information.

The statistical validation demonstrates that vesicle characteristics are governed by a synergistic interplay between formulation composition and mechanical energy input, emphasizing the necessity of systematic optimization rather than empirical formulation.

3.2.1 Effect of the independent variables on the particle size

The particle size of 16-6-16 GS nanovesicles (Batches G1-G15) ranged from 112.36 to 211.2 nm (Table S2). ANOVA results (Table S4) confirmed that the linear model was statistically significant ($p < 0.0001$, $F = 24.96$), with

good predictive capability ($R^2 = 0.87$ and adjusted $R^2 = 0.83$). The lack-of-fit was non-significant ($p = 0.2435$), indicating model adequacy and reliability.

Particle size decreased significantly with increasing S/L ratio, sonication time, and pulse “on” time, as evidenced in the perturbation, contour, and 3D response surface plots (Figure S2 A-C). The reduction in particle size with increasing sonication time and pulse duration can be attributed to acoustic cavitation, where the formation and collapse of microbubbles generate intense shear forces that break down larger vesicles into smaller ones [41]. Prolonged pulse “on” time further enhances cavitation intensity, thereby promoting more efficient size reduction [42].

Among all variables, the S/L ratio was the most influential factor. Higher surfactant content led to smaller vesicles due to increased interfacial charge density from dual cationic head groups, which enhanced electrostatic repulsion and prevented aggregation. Additionally, C16 hydrophobic chains promoted tighter bilayer packing, contributing to further size reduction [43]. This synergistic electrostatic-hydrophobic effect resulted in stable, compact nanovesicles. These findings are consistent with DLVO theory, where increased surface charge improves colloidal stability [42, 44], whereas higher lipid content reduces electrostatic stabilization and promotes vesicle growth [33].

3.2.2 Effect of the Independent Variables on the %EE

The entrapment efficiency (%EE) of 16-6-16 GS nanovesicles ranged from 36.37% to 69.59% (Table S2). The quadratic model was statistically significant ($p = 0.0002$, $F = 56.50$) with a non-significant lack of fit ($p = 0.6737$), confirming model adequacy. The model exhibited excellent fit with high correlation coefficients ($R^2 = 0.9903$; adjusted $R^2 = 0.9146$; predicted $R^2 = 0.9727$) (Table S3 and S5).

As shown in Figure S3, %EE increased with increasing S/L ratio. This improvement can be attributed to enhanced solubilization of the lipophilic drug within the hydrophobic domains of the vesicular bilayer, facilitated by the long alkyl chains and low HLB nature of the Gemini surfactant [45, 46]. Increased surfactant content also promotes the formation of a greater number of vesicular assemblies, thereby increasing the available hydrophobic compartments for drug encapsulation [47].

In contrast, higher sonication time and pulse “on” time reduced %EE, likely due to structural perturbation of vesicles and consequent drug leakage under excessive energy input [48]. Similar trends have been reported in previous studies, where optimized sonication enhances dispersion and entrapment, whereas over-processing leads to destabilization and reduced %EE [49].

3.2.3 Effect of the Independent Variables on Zeta Potential

The zeta potential (ZP) of 16-6-16 GS nanovesicles ranged from 20.1 to 36.4 mV (Table S2). The quadratic model showed statistical significance ($p = 0.0003$, $F = 15.53$) (Table S3 and S6) with a linear response pattern, indicating good predictability of the design space. ZP increased with increasing S/L ratio, as shown in Figure S4. This is attributed to the cationic nature of the Gemini surfactant, which enhances surface charge density at the vesicle interface, thereby improving electrostatic repulsion and colloidal stability. Sonication time and pulse “on” time showed negligible influence on ZP, indicating that surface charge is primarily composition-dependent rather than process-dependent in this system. The observed ZP values (20–40 mV) fall within the range considered suitable for electrostatically stable colloidal systems [50].

3.2.4 Effect of the Independent Variables on the PDI

The PDI values ranged from 0.138 to 0.392 (Table S2), indicating acceptable to moderate polydispersity across formulations. PDI was significantly influenced by the independent variables, showing an inverse relationship with S/L ratio, sonication time, and pulse “on” time. The quadratic model was highly significant ($p < 0.0001$, $F = 111.90$) with excellent fit ($R^2 = 0.9951$; adjusted $R^2 = 0.9862$) and a non-significant lack of fit ($p = 0.0789$) (Table S7).

The reduction in PDI with increasing S/L ratio can be attributed to enhanced interfacial charge density, which improves electrostatic stabilization and reduces aggregation (Figure S5). Increased surfactant concentration also lowers interfacial tension, promoting the formation of more uniform vesicles. Previous studies have reported that Gemini surfactants with dual hydrophobic chains facilitate tighter lipid anchoring, leading to improved size homogeneity [51].

3.2.5 Model Validation

Optimization using the numerical desirability function yielded an optimal formulation with a desirability value of 0.878 (Table S8). The predicted and experimental values showed good agreement (Table 3).

Table 3 The predicted, observed responses and % relative error for 16-6-16 GS nanovesicles

Response	Predicted	Observed	% Relative error
Particle size	121.78	121.4±0.22	0.31
Entrapment (%)	66.37	62.07±2.46	6.47
ZP	33.35	30.16±3.11	9.56
PDI	0.230	0.212±0.07	7.82

3.3 Optimization of the 12-6-12 GS Gemini Surfactant Nanovesicles

The formulation strategy for 12-6-12 GS nanovesicles (GS12) was based on optimization outcomes from GS16 systems, with variation in S/L ratios (0.125–2.6.125.6; batches F39-F46). As shown in Figure S6 and Table S1, increasing the S/L ratio resulted in a decrease in particle size and PDI, accompanied by an increase in zeta potential and entrapment efficiency. This trend is attributed to enhanced surface charge density and improved colloidal stabilization provided by the cationic Gemini surfactant. Maximum %EE (56.8%) was achieved at an S/L ratio of 2.6 (F46), beyond which vesicle integrity was compromised, indicating an optimal balance between surfactant concentration and structural stability.

3.4 Characterization of SIL-Loaded GS

3.4.1 Morphological Analysis

TEM analysis (Fig. 3A-B) confirmed the formation of discrete, spherical vesicles without aggregation, indicating good colloidal stability. Particle sizes observed by were consistent with DLS measurements [52]. TEM observations, together with the thin-film hydration method and lipid-surfactant-cholesterol composition, confirm the formation of lipid-surfactant hybrid nanovesicles rather than solid nanoparticles.

3.5 In Vitro Dissolution Studies

The in vitro dissolution of SIL was evaluated in 0.1 N HCl, phosphate buffer pH 6.8, and pH 7.4 to simulate gastric, intestinal, and systemic conditions. Pure SIL and the marketed formulation (Silybon® 140 mg, Micro Labs, India) exhibited poor dissolution across all media, confirming the inherently low aqueous solubility of SIL. In contrast, both GS-16-OPT and GS-12-OPT nanovesicles demonstrated significantly enhanced dissolution profiles compared to free drug and marketed formulation (Fig. 3C).

At pH 1.2, GS-16-OPT and GS-12-OPT released $49.39\% \pm 3.84\%$ and $57.98\% \pm 2.90\%$ of drug within 2 h, respectively, compared to $23.79\% \pm 1.39\%$ (pure SIL) and $25.66\% \pm 3.84\%$ (Silybon®), corresponding to an approximately 2.0–2.4.0.4-fold increase, reflecting enhanced solubility due to micelle formation and nanovesicle-mediated solubilization [53]. At pH 6.8, cumulative release reached $96.14\% \pm 2.14\%$ (GS-16-OPT) and $88.37\% \pm 2.28\%$ (GS-12-OPT) at 24 h, whereas at pH 7.4, $88.43\% \pm 3.80\%$ and $90.95\% \pm 1.12\%$ release was observed, respectively.

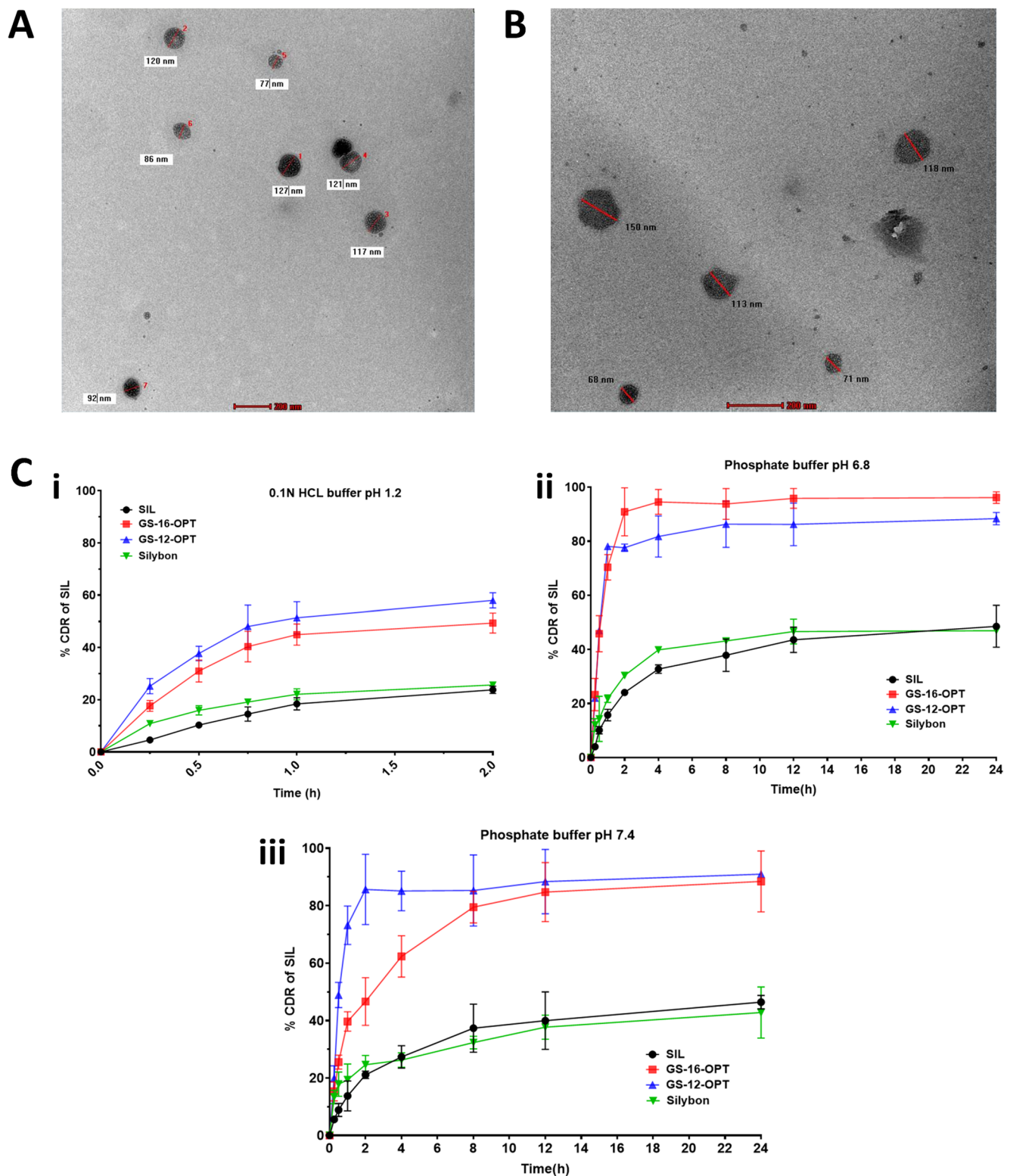


Fig. 3 Transmission electron microscopy of (A) GS-16-OPT and (B) GS-12-OPT. (C) In vitro dissolution study of (i) plain SIL, GS-16-OPT, GS-12-OPT and Silybon (marketed product) in 0.1 N HCl buffer, (ii) phosphate buffer pH 6.8 and (iii) phosphate buffer pH 7.4

Overall, GS-16-OPT exhibited slightly higher release at intestinal pH compared to GS-12-OPT.

The release profiles of both formulations exhibited a biphasic pattern, characterized by an initial rapid release phase followed by a sustained release phase. The initial burst release may be attributed to surface-associated drug and rapid micellar solubilization at the vesicle interface, whereas the sustained phase is governed by diffusion of drug from the lipid-surfactant vesicular matrix [54]. The enhanced dissolution behavior can be attributed to improved drug solubilization, reduced particle size, and formation of lipid-surfactant hybrid nanostructures, which collectively increase the effective surface area and improve wetting of SIL in aqueous media.

3.6 Stability Studies

Stability studies were performed under controlled conditions at 4 ± 2 °C over a 90-day period. However, due to instability in the GeminiSomes dispersion evidenced by phase separation observed on day 30, the study for the dispersed form was discontinued, and further evaluation focused on the lyophilized formulation. The lyophilized lipid-surfactant hybrid nanovesicles demonstrated minimal changes in composition and maintained stability throughout the 90-day period at 4 ± 2 °C (Table S10). Key quality attributes, including particle size, PDI, zeta potential, and entrapment efficiency, showed a maximum variation of only 5% from the initial values. Based on these findings, storage of the formulation in its lyophilized state in tightly sealed glass vials under refrigerated conditions (4 ± 2 °C) is recommended.

3.7 In Vitro Hepatoprotective Activity Against D-Galactosamine

The in vitro hepatoprotective activity of the optimized Gemini surfactant (GS-16-OPT and GS-12-OPT) was evaluated in HepG2 cells and compared with SIL [55]. Exposure to 25 mM D-galactosamine led to ~ 75% loss in cell viability of HepG2 cells. Treatment with 50 µg/mL SIL restored viability to ~ 68%, whereas GS-16-OPT and GS-12-OPT increased viability to 83% and 88%, respectively (Fig. 4A), demonstrating enhanced hepatoprotective effects. This improved protection is likely due to enhanced solubility of SIL, facilitated cellular internalization, and stabilization within the nanovesicular structure, which together mitigate D-Gal-induced cytotoxicity.

Fig. 4 In vitro assessment of silybin and GS-OPT formulations. (A) In vitro hepatoprotective activity of silybin, GS-16-OPT and GS-12-OPT against D-Galactosamine in HepG2 cells using MTT assay; (B) Cell uptake studies of GS-16-OPT and GS-12-OPT against SIL in Caco-2 cells using FACS; (C) FACS plots of SIL, GS-16-OPT and GS-12-OPT in Caco-2 cells; (D) Permeability of SIL, GS-16-OPT and GS-12-OPT in Caco-2 cells (Apical to Basolateral). **: $p < 0.01$, ***: $p < 0.001$; (E-F) Ex vivo permeation study (E) Time vs. permeation of SIL in µg/cm² of SIL, GS-16-OPT, GS-12-OPT and Silybin. (F) Apparent permeability (cm/s) of SIL, GS-16-OPT, GS-12-OPT and Silybin; (G) In vivo pharmacokinetic profile of pure SIL, GS-16-OPT, GS-12-OPT, and Silybin

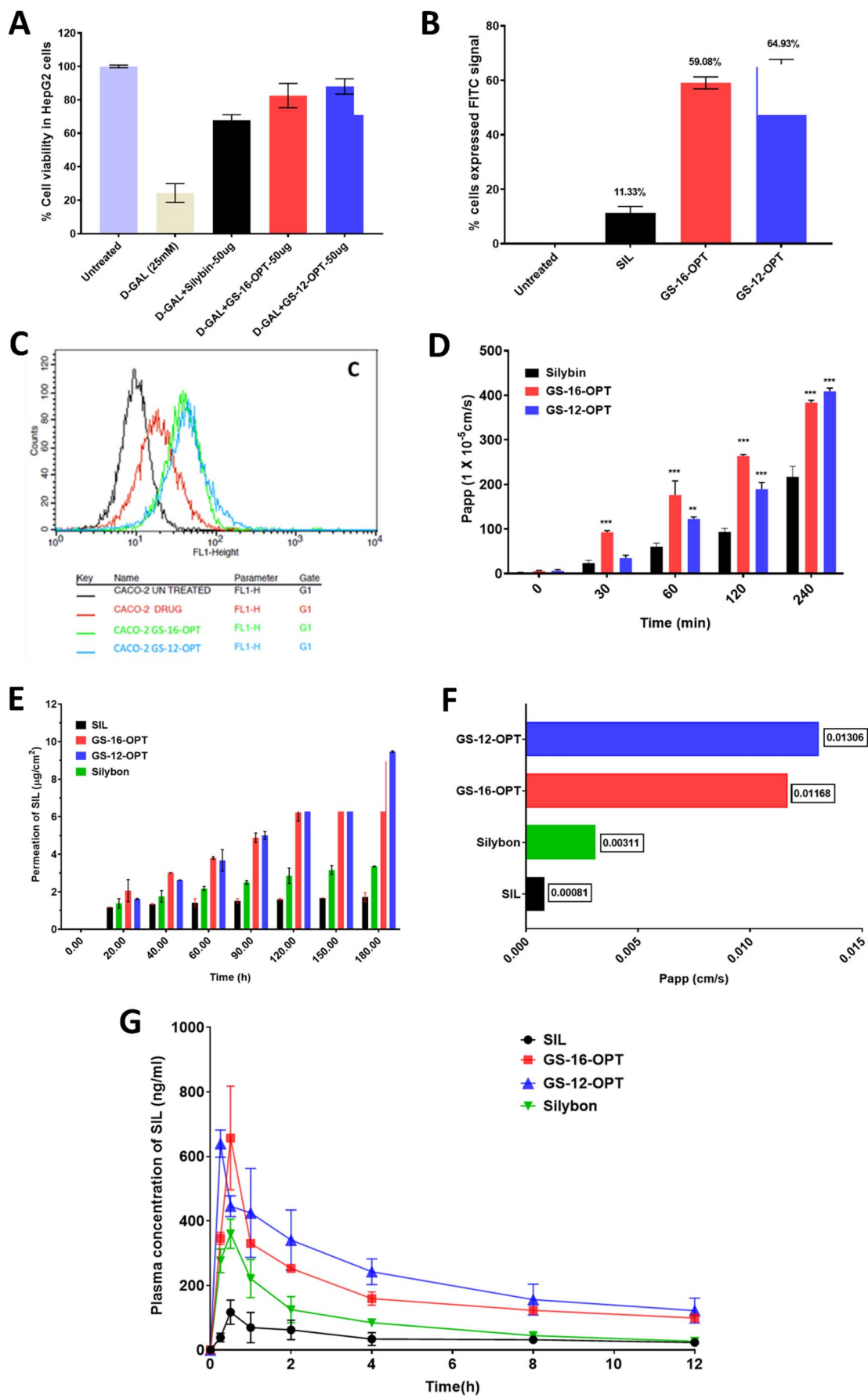
3.8 Cellular Uptake Study

The cell uptake studies were performed on Caco-2 cells using FITC-labelled SIL-loaded formulations. The percentage uptake for the plain SIL was found to be $11.33\% \pm 2.34\%$, while GS-16-OPT and GS-12-OPT showed $59.08\% \pm 2.21\%$ and $64.93\% \pm 2.77\%$, respectively (Fig. 4B). The overlay FACS plots for all the samples are shown in Fig. 4C. The findings demonstrate that GeminiSomes had a ~6-fold greater cellular uptake in Caco-2 cells than the pure SIL.

The enhancement in cellular uptake may be due to their cationic nature, which likely promotes membrane interaction, and the presence of lipid components, which may facilitate endocytosis-mediated internalization [56]. Thus, enhanced uptake is primarily attributed to improved membrane interaction and endocytic uptake pathways. Further mechanistic studies would be necessary to fully elucidate uptake pathways.

3.9 Permeability Studies in Caco-2 Cell Monolayer

The permeability of GS-16-OPT and GS-12-OPT nanovesicles was evaluated using Caco-2 cell monolayers, an in vitro intestinal barrier model. Initial TEER values ranged from 350 to 500 Ω·cm² and showed only a 5–10% decrease after 4 h of nanovesicle exposure, indicating preserved monolayer integrity [57]. Since tight junctions (3–10 Å) restrict particle transport and TEER remained stable, paracellular transport was likely minimal. Instead, improved transcellular transport was observed, as shown in Fig. 4D, where drug permeability across Caco-2 cells significantly increased ($p < 0.0001$) with GS-16-OPT and GS-12-OPT (50 µg/ml) compared to free drug. Apparent permeability (P_{app}) rose 1.5–2.5-fold, confirming enhanced transport. This demonstrates improved transcellular transport facilitated by the cationic surfactant-lipid matrix. This may be due to Gemini surfactants forming a stable lipid-surfactant assembly, enhancing membrane interaction and increasing membrane fluidity [58]. Compared to monomeric surfactants, Gemini surfactants may provide stronger interfacial organization, contributing to improved permeability.



3.10 Ex Vivo Permeation Study

Permeation of SIL, GS-16-OPT, GS-12-OPT, and Silybon[®] was assessed using a non-everted rat ileum model, a cost-effective and reproducible method for evaluating intestinal permeability [59]. As shown in Fig. 4E, GS-16-OPT and GS-12-OPT achieved significantly higher permeation of SIL (P_{app} : 0.011 and 0.013 cm/s respectively) compared to plain SIL and Silybon[®] (P_{app} : 0.0008 cm/s and 0.003 cm/s respectively). The enhancement ratios were 14.33 and 15.99, respectively (Fig. 4F). Improved permeation may result from increased membrane fluidity and transcellular transport [60], lipid-mucus interactions [61], and enhanced solubility and diffusion due to the surfactant-mediated solubility enhancement [34].

3.11 In Vivo Pharmacokinetics Study

Silybin, exhibits good in vitro activity but has poor oral bioavailability due to limited aqueous/lipid solubility, high molecular weight, and rapid Phase II metabolism with biliary and urinary elimination [34, 62]. To overcome these limitations, SIL was encapsulated in GeminiSomes (GS-16-OPT and GS-12-OPT) and evaluated for in vivo pharmacokinetics.

As shown in Fig. 4G and Table 4, GS-12-OPT and GS-16-OPT significantly increased systemic drug exposure compared to plain SIL and Silybon.

GS-16-OPT exhibited a higher C_{max} (731.6 ± 135.48 ng/mL) than GS-12-OPT (639.83 ± 42.39 ng/mL), indicating faster initial absorption. This is likely due to the longer C16 alkyl chains in GS-16-OPT, which enhance hydrophobic interactions within the vesicle bilayer, improve packing, and facilitate more rapid drug release at the absorption site. Conversely, GS-12-OPT demonstrated slightly higher total systemic exposure (AUC_{0-24} : 2752.63 ± 218.42 h·ng/mL vs. 2190.05 ± 96.82 h·ng/mL for GS-16-OPT), suggesting that subtle differences in alkyl chain length and micellization behaviour influence the overall in vivo pharmacokinetic profile.

Compared to pure SIL (C_{max} : 149.0 ± 37.58 ng/mL), both formulations showed marked enhancement, corresponding to 4.9-fold and 4.29-fold increases for GS-16-OPT and GS-12-OPT, respectively, and 2.03-fold and 1.77-fold increases over Silybon. These findings indicate that while GS-16-OPT favors a higher peak plasma concentration, GS-12-OPT achieves slightly greater total systemic exposure, reflecting a balance between rapid absorption and sustained systemic availability governed by Gemini surfactant structure.

The enhanced oral absorption may be attributed to reduced particle size, improved aqueous solubility, electrostatic mucoadhesion, and enhanced gastrointestinal retention [31]. Lipid incorporation likely facilitated lymphatic uptake via chylomicron formation, partially bypassing hepatic first-pass metabolism [38]. Furthermore, the Gemini surfactants may transiently enhance membrane permeability without inducing cytotoxicity, as supported by *in vitro* Caco-2 and HepG2 cell assays.

3.12 In Vivo Hepatoprotective Studies

Preclinical in vivo efficacy was evaluated in Wistar rats using the D-galactosamine (D-Gal)-induced hepatotoxicity model, which closely mimics human viral hepatitis by triggering liver necrosis, inflammation, and regeneration [63]. D-Gal depletes uridine pools, disrupts RNA and protein synthesis, and induces oxidative stress by generating free radicals [64]. It also activates mast cells and Kupffer cells, increasing histamine and TNF- α levels, thereby exacerbating hepatocellular damage through oxidative and inflammatory pathways [65].

3.12.1 Estimation of Biochemical Parameters

The introduction of D-Gal induces an alteration in the permeability of the cellular membrane in the liver. The change leads to the extracellular release of enzymes from hepatocytes, leading to elevated blood enzyme concentrations. Enzymes such as alanine aminotransferase (ALT),

Table 4 Pharmacokinetics parameters

Parameters	SIL	GS-16-OPT	GS-12-OPT	Silybon
C_{max} (ng/mL)	149.0 ± 37.58	$731.6 \pm 135.48^*$	$639.83 \pm 42.39^*$	360.33 ± 62.79
T_{max}	0.5 ± 0.00	0.5 ± 0.00	0.3 ± 0.00	0.5 ± 0.00
K_{el} (1/h)	0.07 ± 0.01	0.048 ± 0.011	0.062 ± 0.011	0.133 ± 0.06
$T_{1/2}$ (h)	10.19 ± 2.51	14.78 ± 3.53	11.34 ± 2.37	7.49 ± 6.72
AUC_{0-t} (h·ng/mL)	573.83 ± 183.52	$2190.05 \pm 96.82^{\#}$	$2752.63 \pm 218.42^{\#}$	1043.13 ± 67.81
$AUC_{0-\infty}$ (h·ng/mL)	1028.36 ± 467.38	$4319.4 \pm 807.65^{\#}$	$4791.56 \pm 794.51^{\#}$	1378.86 ± 478.78
MRT (h)	14.13 ± 3.17	18.75 ± 4.87	14.5 ± 4.89	8.63 ± 6.788

All values are expressed as mean $\pm$ SD; $n=6$. AUC, area under the curve; C_{max} , maximum concentration of drug in plasma; K_{el} , elimination rate constant, MRT, mean residential time; $t_{1/2}$, elimination half-life.

* $p < 0.05$, compared to the SIL; $^{\#}p < 0.0001$, compared to the SIL and Silybon[®]

aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are significant biomarkers that play a direct role in hepatic injury and indicate the extent of damage induced by D-Gal [66]. In the biochemical analysis, it was observed that the D-Gal induced hepatic toxicity led to increase in the AST, ALT and the ALP levels compared to the control group. However, GS-16-OPT and GS-12-OPT showed significant reduction in the AST, ALT and ALP levels as compared to the plain SIL and Silybon® as shown in Fig. 5A, indicating effective protection against hepatocellular damage.

3.12.2 Estimation of Endogenous Antioxidant Enzymes

Catalase (CAT) and superoxide dismutase (SOD) are essential antioxidant enzymes that protect cellular macromolecules by eliminating reactive oxygen species (ROS). CAT decomposes hydrogen peroxide (H_2O_2) into water and oxygen [67], while SOD converts superoxide radicals to H_2O_2 , reducing oxidative damage [68]. Reduced activity of these enzymes in D-galactosamine (D-Gal)-treated rats indicates oxidative hepatic injury. In this study, treatment with the optimized GeminiSomes formulations restored CAT and SOD activity toward normal levels, outperforming both plain silybin and the marketed formulation (Silybon®). Glutathione (GSH), a major intracellular antioxidant, also plays a key role in neutralizing ROS and maintaining redox balance. Its depletion increases susceptibility to oxidative stress [69]. The optimized formulations significantly restored hepatic GSH levels, indicating improved antioxidant protection. D-Gal exposure elevated malondialdehyde (MDA) levels, a marker of lipid peroxidation and oxidative membrane damage [40]. Pre-treatment with silybin reduced MDA levels significantly ($p < 0.001$), attributed to its radical-scavenging ability. However, optimized GeminiSomes showed a greater reduction in MDA levels, confirming enhanced protection against oxidative damage (Fig. 5B).

3.12.3 Anti-Inflammatory Response

The presence of inflammatory cytokines, such as TNF- α and IL-6, significantly impacts liver inflammation, an essential aspect of acute and chronic liver disorders [70]. D-Gal significantly ($p < 0.0001$) enhanced hepatic toxicity, resulting in elevated levels of TNF- α and IL-6. The Gemini surfactant nanovesicle demonstrated a substantial reduction ($p < 0.0001$) in the concentrations of TNF- α and IL-6, restoring them close to control group (Fig. 5C).

3.12.4 Histopathological Evaluation

Histopathological analysis revealed that liver tissues from the control group exhibited typical hepatic architecture with only minor vascular congestion (Fig. 5D i). In contrast, the D-Gal-treated control showed extensive necrosis, cellular debris, neutrophilic infiltration, and apoptotic bodies, including single-cell necrosis with hyper-eosinophilic cytoplasm (Fig. 5D ii). Animals treated with plain silybin and the marketed formulation Silybon® (100 mg/kg) displayed reduced hepatic inflammation and necrosis (Figs. 5D iii and vi). Pre-treatment with the GS-16-OPT formulation resulted in moderate necrosis and inflammatory changes (Fig. 5D iv), whereas GS-12-OPT demonstrated the most prominent protective effect, showing only mild sinusoidal and vascular congestion with minimal inflammation compared to the positive control (Fig. 5D v) [40]. These findings correlate with biochemical and antioxidant data, confirming that optimized Gemini surfactant vesicles mitigate D-Gal-induced liver injury more effectively than plain SIL or Silybon®.

Overall, the results demonstrate that incorporation of Gemini surfactants into the lipid nanovesicles significantly enhanced formulation performance. GS-16-OPT and GS-12-OPT showed reduced particle size, narrow PDI, higher zeta potential, and improved entrapment efficiency, which can be attributed to the dual cationic head groups and hydrophobic alkyl chains promoting tighter bilayer packing and electrostatic stabilization. These structural features facilitated enhanced solubilization, cellular uptake, and transcellular transport, resulting in improved in vitro dissolution and ex vivo intestinal permeability. In vivo pharmacokinetics revealed higher systemic exposure and optimized absorption profiles. Finally, hepatoprotective studies confirmed superior restoration of biochemical, antioxidant, and inflammatory markers, along with improved histological architecture, demonstrating overall enhanced therapeutic efficacy of optimized GeminiSomes compared to pure SIL and Silybon®.

4 Conclusion

This study addresses the critical challenge of improving oral therapy for acute hepatitis by enhancing the bioavailability of silybin using Gemini surfactant-based lipid nanovesicles. A comprehensive approach involving preformulation studies, GS synthesis, analytical method development, and formulation optimization led to the creation

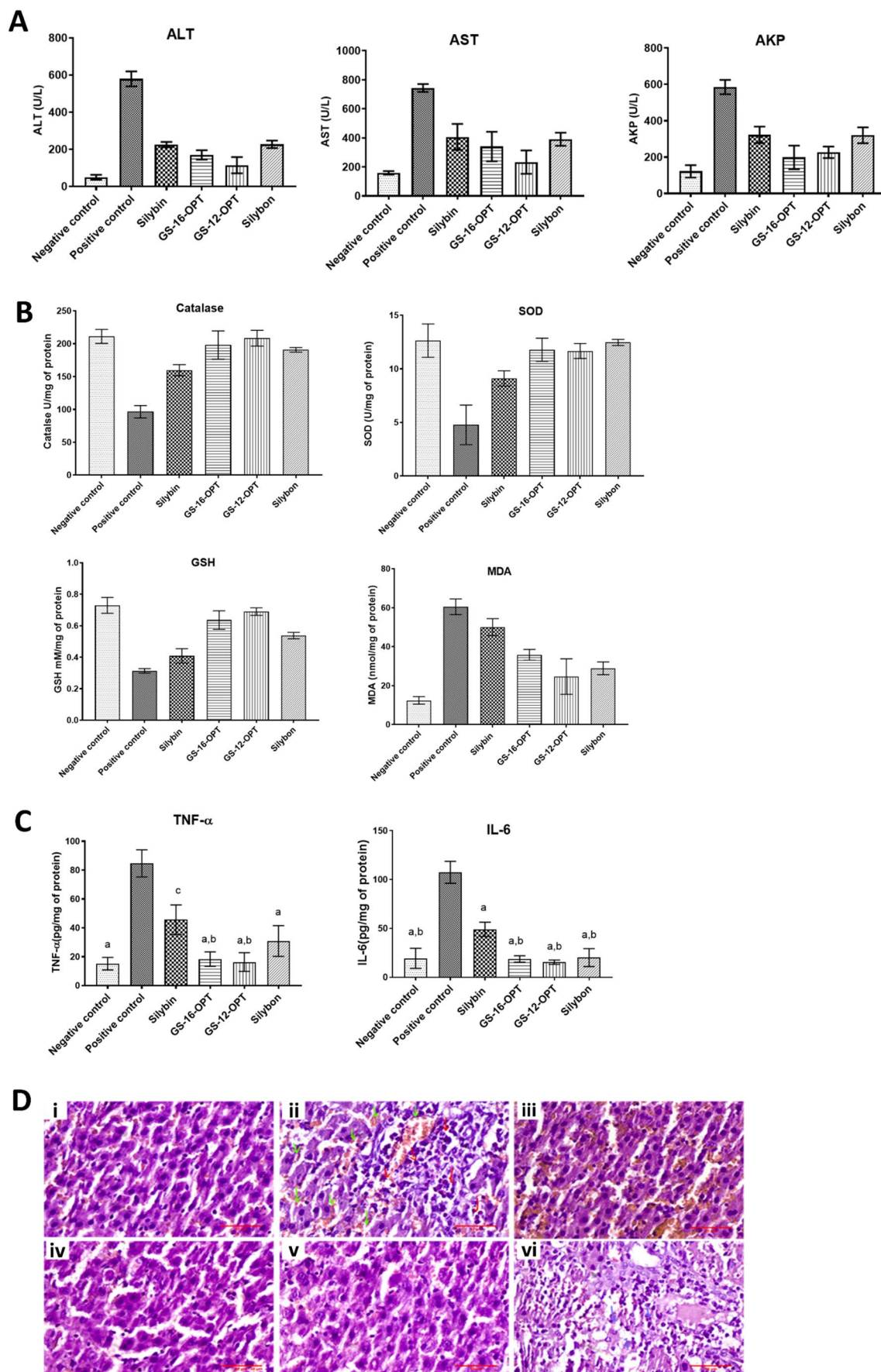


Fig. 5 Effect of various treatments on (A) biochemical markers (AST, ALT, ALP), (B) antioxidant enzymes (catalase, SOD, GSH, and MDA), and (C) inflammatory cytokines (TNF- α and IL-6) in rats subjected to D-galactosamine-induced hepatotoxicity. Data are presented as mean $\pm$ SD ($n=4$ per group). (a $p<0.0001$ vs. positive control; b $p<0.05$ vs. silybin group; c $p<0.05$ vs. positive control). (D) Histopathological examination of liver sections of different groups. (i) Normal Control, (ii) Positive control, (iii) Plain SIL, (iv) GS-16-OPT, (v) GS-12-OPT, and (vi) Silybin[®]. Red arrows indicate inflammatory cell infiltration associated with tissue injury and yellow arrows indicate necrotic changes

of a stable and biocompatible nanocarrier system. The optimized lipid-surfactant hybrid nanovesicles i.e. GeminiSomes exhibited favorable physicochemical properties and significantly enhanced permeability and uptake, as demonstrated through in vitro and ex vivo models. In vivo pharmacokinetic studies in Wistar rats showed a 4.6-fold improvement in oral bioavailability, while pharmacodynamic evaluations confirmed strong hepatoprotective efficacy in the D-galactosamine-induced hepatitis model. These findings underscore the translational potential of GS-based nanocarriers for enhancing the oral delivery of poorly bioavailable phytotherapeutics like silybin and present a promising strategy for advancing hepatoprotective drug therapy.

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Author Contributions **Gasper Fernandes: ** Writing - original draft, Writing - review & editing, Data curation, Methodology, Investigation, Formal analysis; **Naitik Jain: ** Writing - original draft, Writing - review & editing, Data curation, Formal analysis; **Ruchira Raychaudhuri: ** Methodology, Investigation; Data curation, Formal analysis; **Neha Kandpal: ** Investigation; **Rahul Pokale: ** Data curation, Formal analysis; **Raviraja N Seetharam: ** Writing - review & editing, Data curation, Methodology, Formal analysis; **Srinivas Mutalik: ** Conceptualisation, Writing - original draft, Writing - review & editing, Data curation, Formal analysis, Project administration, Validation, Resources and Supervision.

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Data Availability Data is presented in the main manuscript and the Supplementary Information.

Declarations

Ethical Statement The animal studies were conducted in accordance with protocol approved by the IAEC, Kasturba Medical College, Manipal (IAEC/KMC/95/2021).

Competing interests The authors declare no competing interests.

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References

- Ahmad, A. A. S., Dighade, S. J., & Khan, W. K. Y. (2025). A Review on Global Burden of Liver Diseases. *International Journal of Pharmaceutical Sciences*, 03(01). <https://doi.org/10.5281/zenodo.14722693>
- WHO sounds alarm on viral hepatitis infections claiming 3500 lives each day. (n.d.). Retrieved August 5 (2025). from <https://www.who.int/news/item/09-04-2024-who-sounds-alarm-on-viral-hepatitis-infections-claiming-3500-lives-each-day>
- Xiong, F., & Guan, Y. S. (2017). Cautiously using natural medicine to treat liver problems. *World Journal of Gastroenterology*, 23(19), 3388.
- Taru, V., Szabo, G., Mehal, W., & Reiberger, T. (2024). Inflammation in chronic liver disease: Hepatic injury, fibrosis progression and systemic inflammation. *Journal of Hepatology*, 81(5), 895–910. <https://doi.org/10.1016/j.jhep.2024.06.016>
- Pandey, B., Baral, R., Kaundinnayana, A., & Panta, S. (2023). Promising hepatoprotective agents from the natural sources: A study of scientific evidence. *Egyptian Liver Journal*, 13(1), Article 14. <https://doi.org/10.1186/s43066-023-00248-w>
- Singh, S., Sharma, A., Pandey, T., Gupta, S., Shukla, A., Singh, S. K., & Singh, A. (2025). Silibinin: A natural flavonoid with multifaceted anticancer potential and therapeutic challenges. *Medicinal Chemistry Research*, 34(10), 2115–2136. <https://doi.org/10.1007/s00044-025-03466-6>
- Loguercio, C., & Festi, D. (2011). Silybin and the liver: From basic research to clinical practice. *World Journal of Gastroenterology*, 17(18), 2288–2301. <https://doi.org/10.3748/wjg.v17.i18.2288>
- Thakur, N., & Judeh, Z. (2026). Layer-by-layer coating of silibinin-loaded cochleates with chitosan and alginate for enhanced oral delivery. *Chemical Engineering Research and Design*, 226, 1–13. <https://doi.org/10.1016/j.cherd.2025.12.029>
- Chang, C., Zhang, Y., Kuo, Y. C., Du, M., Roh, K., Gahler, R., Ibi, A., & Solnier, J. (2025). Novel micellar formulation of silymarin (Milk Thistle) with enhanced bioavailability in a double-blind, randomized, crossover human trial. *Pharmaceutics*, 17(7), Article 880. <https://doi.org/10.3390/pharmaceutics17070880>
- Javed, S., Kohli, K., & Ali, M. (2011). Reassessing bioavailability of silymarin. *Alternative Medicine Review*, 16(3), 239.

11. Zeng, Q., Liu, Z., Huang, A., Zhang, J., & Song, H. (2016). Preparation and characterization of silymarin synchronized-release microporous osmotic pump tablets. *Drug Design, Development and Therapy*, 10, 519–531. <https://doi.org/10.2147/DDDT.S91571>
12. Selc, M., Macova, R., & Babelova, A. (2024). Novel strategies enhancing bioavailability and therapeutical potential of silibinin for treatment of liver disorders. *Drug Design, Development and Therapy*, 18, 4629–4659. <https://doi.org/10.2147/DDDT.S483140>
13. Kiani, K., Ramezani, M., Jafari-Gharabaghlo, D., Amiri, I., Soleimani Asl, S., & Mirzaei, F. (2026). Silibinin-loaded PEGylated niosomal nanoparticles restore hippocampus histological changes, memory and learning, and downregulate the inflammasome pathway in Alzheimer's disease animal models. *Biochemical and Biophysical Research Communications*, 809, Article 153496. <https://doi.org/10.1016/j.bbrc.2026.153496>
14. Santos, R., Padilha Lorenzett, A. K., de Matos, G. C. G., de Souza Bonfim-Mendonça, P., Lima, V., & Mainardes, R. M. (2026). Factorial design-driven optimization of zein-chitosan nanoparticles for oral delivery of silibinin. *ACS Omega*, 11(10), 16673–16686. <https://doi.org/10.1021/acsomega.5c13167>
15. Mahgoub, S. M., Alawam, A. S., Rudayni, H. A., Allam, A. A., Shaban, A., Abdel Aleem Abdel Aziz, S., & Mahmoud, R. (2026). Silybum marianum-based nanoformulations for enhanced hepatoprotective and anticancer activity. *Journal of Pharmaceutical Innovation*, 21(2), Article 181. <https://doi.org/10.1007/s12247-025-10280-5>
16. Wu, J., Wen, L., Liu, X., Li, Q., Sun, Z., Liang, C., & Li, X. (2024). Silybin: A review of its targeted and novel agents for treating liver diseases based on pathogenesis. *Phytotherapy Research*, 38(12), 5713–5740. <https://doi.org/10.1002/ptr.8347>
17. Shetty, A., Keerikkadu, M., Bangera, P. D., Tippavajhala, V. K., & Rathnanand, M. (2025). An overview of advanced nanocarrier systems for ibuprofen delivery: Overcoming pharmacokinetic barriers and enabling targeted cancer therapy. *International Journal of Pharmaceutics: X*, 10, Article 100417. <https://doi.org/10.1016/j.ijpx.2025.100417>
18. Vambhurkar, G., Jain, N., Srinivasarao, D. A., Famta, P., Singh, S. B., & Srivastava, S. (2023). Drug Solubilization and Drug Release from Polymeric Micelles. In S. K. Singh, M. Gulati, S. Mutalik, M. Dhanasekaran, & K. Dua (Eds.), *Polymeric Micelles: Principles, Perspectives and Practices* (pp. 87–109). Springer Nature. https://doi.org/10.1007/978-981-99-0361-0_5
19. Wang, C., Cui, J.-J., Sheng, T., Xia, S., Li, J.-M., & Zhang, B.-F. (2025). Chitosan-decorated silibinin-hyaluronic acid conjugate for enhancing intestinal absorption and improving liver fibrosis. *International Journal of Biological Macromolecules*, 320, Article 146057. <https://doi.org/10.1016/j.ijbiomac.2025.146057>
20. Qelliny, M. R., Mustafa, W. W., Fatease, A. A., Alamri, A. H., Alany, R., & Abdelkader, H. (2025). Biofunctional excipients: Their emerging role in overcoming the inherent poor biopharmaceutical characteristics of drugs. *Pharmaceutics*, 17(5), Article 598. <https://doi.org/10.3390/pharmaceutics17050598>
21. Raychaudhuri, R., Nikam, A. N., Jain, N., Eedara, A., Kandpal, N., Ray, R., & Mutalik, S. (2026). Gemini surfactant-stabilized cubosomes for enhanced topical delivery of 5-fluorouracil in cutaneous squamous cell carcinoma. *International Journal of Pharmaceutics: X*, 100504. <https://doi.org/10.1016/j.ijpx.2026.100504>
22. Guerrero-Hernández, L., Meléndez-Ortiz, H. I., Cortez-Mazatan, G. Y., Vaillant-Sánchez, S., & Peralta-Rodríguez, R. D. (2022). Gemini and bicephalous surfactants: A review on their synthesis, micelle formation, and uses. *International Journal of Molecular Sciences*, 23(3), Article 1798. <https://doi.org/10.3390/ijms23031798>
23. Ahmady, A. R., Hosseinzadeh, P., Solouk, A., Akbari, S., Szulc, A. M., & Brycki, B. E. (2022). Cationic gemini surfactant properties, its potential as a promising bioapplication candidate, and strategies for improving its biocompatibility: A review. *Advances in Colloid and Interface Science*, 299, Article 102581. <https://doi.org/10.1016/j.cis.2021.102581>
24. Shukla, D., & Tyagi, V. (2006). Cationic Gemini surfactants: A review. *Journal of Oleo Science*, 55(8), 381–390. <https://doi.org/10.5650/jos.55.381>
25. Zana, R. (2002). Dimeric and oligomeric surfactants. Behavior at interfaces and in aqueous solution: A review. *Advances in Colloid and Interface Science*, 97(1), 205–253. [https://doi.org/10.1016/S0001-8686\(01\)00069-0](https://doi.org/10.1016/S0001-8686(01)00069-0)
26. Ahmad, M., & Sardar, N. (2023). Study on solution behavior of green gemini-conventional mixed systems and their solubilization capability towards phenanthrene. *Oriental Journal of Chemistry*, 39(5), 1214–1225.
27. Kouser Qadri, H., Shaheen, A., Rashid, S., Ahmad Bhat, I., Mohammad Rather, G., & Ahmad Dar, A. (2022). Micellization and gelation characteristics of Pluronic P123 and single ester-bonded cleavable cationic gemini surfactant: A potential system for solubilization and release of ibuprofen. *Journal of Molecular Liquids*, 366, Article 120311. <https://doi.org/10.1016/j.molliq.2022.120311>
28. Recent Progress in Gene Delivery Systems Based on Gemini-Surfactant - Qian—2025 - MedComm - Future Medicine - Wiley Online Library. (n.d.). Retrieved August 5 (2025). from <https://onlinelibrary.wiley.com/doi/full/https://doi.org/10.1002/mef2.70027>
29. Formation of hydrotropic drug/gemini surfactant based catanionic vesicles as efficient nano drug delivery vehicles (2020). *Colloid and Interface Science Communications*, 37, 100273. <http://doi.org/10.1016/j.colcom.2020.100273>
30. Radziunas-Salinas, Y., Domínguez-Arca, V., Pardo, A., Cambón, A., Taboada, P., & Prieto, G. (2025). Long-chain cationic gemini surfactants as drug retention adjuvant on liposomes. A methodological approach with atorvastatin. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1867(4), Article 184419. <https://doi.org/10.1016/j.bbamem.2025.184419>
31. Song, Y., Han, J., Feng, R., Wang, M., Tian, Q., Zhang, T., & Deng, Y. (2016). The 12-3-12 cationic gemini surfactant as a novel gastrointestinal bioadhesive material for improving the oral bioavailability of coenzyme Q10 naked nanocrystals. *Drug Development and Industrial Pharmacy*, 42(12), 2044–2054. <https://doi.org/10.1080/03639045.2016.1195399>
32. Cannito, S., Bincoletto, V., Turato, C., Pontisso, P., Scupoli, M. T., Ailuno, G., Andreana, I., Stella, B., Arpicco, S., & Bocca, C. (2022). Hyaluronated and PEGylated liposomes as a potential drug-delivery strategy to specifically target liver cancer and inflammatory cells. *Molecules*, 27(3), Article 1062. <https://doi.org/10.3390/molecules27031062>
33. Manikkath, J., Parekh, H. S., & Mutalik, S. (2020). Surface-engineered nanoliposomes with lipidated and non-lipidated peptide-dendrimeric scaffold for efficient transdermal delivery of a therapeutic agent: Development, characterization, toxicological and preclinical performance analyses. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik e V*, 156, 97–113. <https://doi.org/10.1016/j.ejpb.2020.09.001>
34. Fernandes, G., Pusuluri, S. L. A., Nikam, A. N., Birangal, S., Shenoy, G. G., & Mutalik, S. (2022). Solvent free twin screw processed Silybin nanophospholipid: In silico, in vitro and in vivo insights. *Pharmaceutics*, 14(12), Article 2729. <https://doi.org/10.3390/pharmaceutics14122729>
35. Naik, S., Jain, N., Theruveethi, N., & Mutalik, S. (2025). Peptide dendrimer and hyaluronic acid modified nanovesicles for ocular delivery of timolol maleate and siRNA. *Scientific Reports*, 15(1), Article 26074. <https://doi.org/10.1038/s41598-025-10960-9>

36. Jain, N., Shahrulkh, S., Famta, P., Shah, S., Vambhurkar, G., Srinivasarao, D. A., Sharma, A., Pandey, G., Wagh, S., Shinde, S., Khan, A., Kumar, P., & Srivastava, S. (2025). Combating breast cancer-associated metastasis using paclitaxel and tranilast-loaded human serum albumin nanoparticles. *Drug Development and Industrial Pharmacy*, 51(7), 786–798. <https://doi.org/10.1080/03639045.2025.2509861>
37. Kumar, N., Rai, A., Reddy, N. D., Raj, P. V., Jain, P., Deshpande, P., Mathew, G., Kuty, N. G., Udapa, N., & Rao, C. M. (2014). Silymarin liposomes improves oral bioavailability of silybin besides targeting hepatocytes, and immune cells. *Pharmacological Reports*, 66(5), 788–798. <https://doi.org/10.1016/j.pharep.2014.04.007>
38. Managuli, R. S., Wang, J. T., Faruqi, F. N., Kushwah, V., Raut, S. Y., Shreya, A. B., Al-Jamal, K. T., Jain, S., & Mutalik, S. (2019). Asenapine maleate-loaded nanostructured lipid carriers: Optimization and in vitro, ex vivo and in vivo evaluations. *Nanomedicine*, 14(7), 889–910. <https://doi.org/10.2217/nnm-2018-0289>
39. Tong, Y., Zhang, Q., Shi, W., & Wang, J. (2019). Mechanisms of oral absorption improvement for insoluble drugs by the combination of phospholipid complex and SNEDDS. *Drug Delivery*, 26(1), 1155–1166. <https://doi.org/10.1080/10717544.2019.1686086>
40. Mondal, M., Hossain, M. M., Hasan, M. R., Tarun, M. T. I., Islam, M. A. F., Choudhuri, M. S. K., Islam, M. T., & Mubarak, M. S. (2020). Hepatoprotective and antioxidant capacity of *Mallotus repandus* ethyl acetate stem extract against d-galactosamine-induced hepatotoxicity in rats. *ACS Omega*, 5(12), 6523–6531. <https://doi.org/10.1021/acsomega.9b04189>
41. CHEN, X., & Wang, T. (2019). Preparation and characterization of atrazine-loaded biodegradable PLGA nanospheres. *Journal of Integrative Agriculture*, 18, 1035–1041. [https://doi.org/10.1016/S2095-3119\(19\)62613-4](https://doi.org/10.1016/S2095-3119(19)62613-4)
42. Masoudipour, E., Kashanian, S., Azandaryani, A. H., Omidfar, K., & Bazayr, E. (2017). Surfactant effects on the particle size, zeta potential, and stability of starch nanoparticles and their use in a pH-responsive manner. *Cellulose*, 24(10), 4217–4234. <https://doi.org/10.1007/s10570-017-1426-3>
43. Singh, L. P., Bhattacharyya, S. K., Mishra, G., & Ahalawat, S. (2011). Functional role of cationic surfactant to control the nano size of silica powder. *Applied Nanoscience*, 1(3), 117–122. <https://doi.org/10.1007/s13204-011-0016-1>
44. Varshosaz, J., Pardakhty, A., Hajhashemi, V. I., & Najafabadi, A. R. (2003). Development and physical characterization of sorbitan monoester niosomes for insulin oral delivery. *Drug Delivery*, 10(4), 251–262. https://doi.org/10.1080/drd_10_4_251
45. Bnyan, R., Khan, I., Ehtezazi, T., Saleem, I., Gordon, S., O'Neill, F., & Roberts, M. (2018). Surfactant effects on lipid-based vesicles properties. *Journal of Pharmaceutical Sciences*, 107(5), 1237–1246. <https://doi.org/10.1016/j.xphs.2018.01.005>
46. Mohammed, A. R., Weston, N., Coombes, A. G. A., Fitzgerald, M., & Perrie, Y. (2004). Liposome formulation of poorly water soluble drugs: Optimisation of drug loading and ESEM analysis of stability. *International Journal of Pharmaceutics*, 285(1–2), 23–34. <https://doi.org/10.1016/j.ijpharm.2004.07.010>
47. Hao, Y., Zhao, F., Li, N., Yang, Y., & Li, K. (2002). Studies on a high encapsulation of colchicine by a niosome system. *International Journal of Pharmaceutics*, 244(1–2), 73–80. [https://doi.org/10.1016/s0378-5173\(02\)00301-0](https://doi.org/10.1016/s0378-5173(02)00301-0)
48. Gala, R. P., Khan, I., Elhissi, A. M. A., & Alhnan, M. A. (2015). A comprehensive production method of self-cryoprotected nano-liposome powders. *International Journal of Pharmaceutics*, 486(1), 153–158. <https://doi.org/10.1016/j.ijpharm.2015.03.038>
49. He, Y., Luo, L., Liang, S., Long, M., & Xu, H. (2019). Influence of probe-sonication process on drug entrapment efficiency of liposomes loaded with a hydrophobic drug. *International Journal of Polymeric Materials and Polymeric Biomaterials*, 68(4), 193–197. <https://doi.org/10.1080/00914037.2018.1434651>
50. Al Shuwaili, A. H., Rasool, B. K. A., & Abdulrasool, A. A. (2016). Optimization of elastic transfersomes formulations for transdermal delivery of pentoxifylline. *European Journal of Pharmaceutics and Biopharmaceutics*, 102, 101–114. <https://doi.org/10.1016/j.ejpb.2016.02.013>
51. Barbosa, R. M., Severino, P., Preté, P. S. C., & Santana, M. H. A. (2017). Influence of different surfactants on the physicochemical properties of elastic liposomes. *Pharmaceutical Development and Technology*, 22(3), 360–369. <https://doi.org/10.3109/10837450.2016.1163387>
52. Yang, W., Cao, Y., Ju, H., Wang, Y., Jiang, Y., & Geng, T. (2021). Amide Gemini surfactants linked by rigid spacer group 1,4-dibromo-2-butene: Surface properties, aggregate and application properties. *Journal of Molecular Liquids*, 326, Article 115339. <https://doi.org/10.1016/j.molliq.2021.115339>
53. Mirgorodskaya, A. B., Ya Zakharova, L., Khairutdinova, E. I., Lukashenko, S. S., & Sinyashin, O. G. (2016). Supramolecular systems based on gemini surfactants for enhancing solubility of spectral probes and drugs in aqueous solution. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 510, 33–42. <https://doi.org/10.1016/j.colsurfa.2016.07.065>
54. Abdullah, A. S., Sayed, I. E. T. E., El-Torgoman, A. M. A., Kalam, A., Wageh, S., & Kamel, M. A. (2022). Green synthesis of Silymarin-Chitosan nanoparticles as a new nano formulation with enhanced anti-fibrotic effects against liver fibrosis. *International Journal of Molecular Sciences*, 23(10), Article 5420. <https://doi.org/10.3390/ijms23105420>
55. N, S. (2019). In vitro hepatoprotective activity of *Indigofera barberi* gamble against d-galactosamine induced toxicity. *Journal of Pharmacognosy and Phytochemistry*, 8(1), 719–723.
56. Du, L., Yang, Y. H., Xu, J., Wang, Y. M., Xue, C. H., Kurihara, H., & Takahashi, K. (2016). Transport and uptake effects of marine complex lipid liposomes in small intestinal epithelial cell models. *Food & Function*, 7(4), 1904–1914. <https://doi.org/10.1039/C6FO00066E>
57. Akbari, A., Lavasanifar, A., & Wu, J. (2017). Interaction of cruciferin-based nanoparticles with Caco-2 cells and Caco-2/HT29-MTX co-cultures. *Acta Biomaterialia*, 64, 249–258. <https://doi.org/10.1016/j.actbio.2017.10.017>
58. Li, X., Chen, D., Le, C., Zhu, C., Gan, Y., Hovgaard, L., & Yang, M. (2011). Novel mucus-penetrating liposomes as a potential oral drug delivery system: Preparation, in vitro characterization, and enhanced cellular uptake. *International Journal of Nanomedicine*, 6, 3151–3162. <https://doi.org/10.2147/IJN.S25741>
59. Rizwanullah, M., Perwez, A., Alam, M., Ahmad, S., Mir, S. R., Rizvi, M. M. A., & Amin, S. (2023). Polymer-lipid hybrid nanoparticles of exemestane for improved oral bioavailability and anti-tumor efficacy: An extensive preclinical investigation. *International Journal of Pharmaceutics*, 642, Article 123136. <https://doi.org/10.1016/j.ijpharm.2023.123136>
60. Alama, T., Kusamori, K., Morishita, M., Katsumi, H., Sakane, T., & Yamamoto, A. (2019). Mechanistic studies on the absorption-enhancing effects of Gemini surfactant on the intestinal absorption of poorly absorbed hydrophilic drugs in rats. *Pharmaceutics*, 11(4), Article 170. <https://doi.org/10.3390/pharmaceutics11040170>
61. Lavanya, N., Muzib, Y. I., Aukunuru, J., & Balekari, U. (2016). Preparation and evaluation of a novel oral delivery system for low molecular weight heparin. *International Journal of Pharmaceutical Investigation*, 6(3), 148–157. <https://doi.org/10.4103/2230-973X.187351>
62. Chi, C., Zhang, C., Liu, Y., Nie, H., Zhou, J., & Ding, Y. (2020). Phytosome-nanosuspensions for silybin-phospholipid complex

- with increased bioavailability and hepatoprotection efficacy. *European Journal of Pharmaceutical Sciences*, 144, Article 105212. <https://doi.org/10.1016/j.ejps.2020.105212>
63. Wills, P. J., & Asha, V. V. (2006). Protective effect of *Lygodium flexuosum* (L.) Sw. (Lygodiaceae) against d-galactosamine induced liver injury in rats. *Journal of Ethnopharmacology*, 108(1), 116–123. <https://doi.org/10.1016/j.jep.2006.04.028>
 64. Keppler, D. O. R., Pausch, J., & Decker, K. (1974). Selective uridine triphosphate deficiency induced by d-galactosamine in liver and reversed by pyrimidine nucleotide precursors: Effect on ribonucleic acid synthesis. *Journal of Biological Chemistry*, 249(1), 211–216. [https://doi.org/10.1016/S0021-9258\(19\)43113-X](https://doi.org/10.1016/S0021-9258(19)43113-X)
 65. Kasravi, F. B., Wang, L., Wang, X., Molin, G., Bengmark, S., & Jeppsson, B. (1996). Bacterial translocation in acute liver injury induced by D- galactosamine. *Hepatology*, 23(1), 97–103. <https://doi.org/10.1002/hep.510230114>
 66. Wu, Y.-H., Hao, B.-J., Cao, H.-C., Xu, W., Li, Y.-J., & Li, L.-J. (2012). Anti-hepatitis B virus effect and possible mechanism of action of 3,4-*O*-dicaffeoylquinic acid *in vitro* and *in vivo*. *Evidence-Based Complementary and Alternative Medicine*, 2012, Article e356806. <https://doi.org/10.1155/2012/356806>
 67. Kale, M., Rathore, N., John, S., & Bhatnagar, D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: A possible involvement of reactive oxygen species. *Toxicology Letters*, 105(3), 197–205. [https://doi.org/10.1016/S0378-4274\(98\)00399-3](https://doi.org/10.1016/S0378-4274(98)00399-3)
 68. Kharpate, S., Vadnerkar, G., Jain, D., & Jain, S. (2007). Evaluation of hepatoprotective activity of ethanol extract of *Pterospermum acerifolium* stem leaves. *Indian Journal of Pharmaceutical Sciences*, 69(6), 850–850.
 69. Swarnalatha, L., & Reddy, P. N. (2012). Hepatoprotective activity of *Sphaeranthus amaranthoides* on D-galactosamine induced hepatitis in albino rats. *Asian Pacific Journal of Tropical Biomedicine*, 2(3, Supplement), S1900–S1905. [https://doi.org/10.1016/S2221-1691\(12\)60518-8](https://doi.org/10.1016/S2221-1691(12)60518-8)
 70. Niederreiter, L., & Tilg, H. (2018). Cytokines and fatty liver diseases. *Liver Research*, 2(1), 14–20. <https://doi.org/10.1016/j.livres.2018.03.003>

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