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Original Article

Development and Evaluation of Piperine-Loaded Phytosomal Gel for Improved Antifungal Activity Against *Candida albicans*

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ABSTRACT

Background: Phytosomes have recently emerged as a promising nano-vesicular drug delivery system for improving the therapeutic utilisation of herbal extracts and isolated phytoconstituents. Compared with conventional dosage forms, phytosomal systems offer several advantages including enhanced skin permeation, improved bioavailability, reduced dose requirement, lower toxicity and minimised skin irritation. **Objectives:** The present study proposed the development of a piperine-loaded phytosomal gel as a nano-drug delivery system against *Candida albicans*, one of the most commonly encountered pathogenic yeasts associated with various skin-related fungal infections. **Materials and Methods:** Piperine-loaded phytosomes were prepared using the solvent evaporation technique employing soya lecithin and cholesterol as the main vesicle-forming components. The prepared phytosomal formulations were evaluated for particle size, PDI, zeta potential, entrapment efficiency and drug content to identify the optimised batch. The optimised formulation was characterised using FT-IR, DSC, PXRD, FEG-SEM and Cryo-HRTEM analysis. Further, the optimised phytosomal formulation was incorporated into a Carbopol 934 based gel and evaluated for antifungal activity against *C. albicans* using the Kirby-Bauer disc diffusion method. **Results:** The study successfully demonstrated the formation of nano-sized phytosomal vesicles with an average size of approximately 200 nm along with desirable physicochemical characteristics and possible conversion of crystalline piperine into an amorphous state. Furthermore, antifungal studies indicated significantly improved inhibitory activity of the piperine-loaded phytosomal gel against *C. albicans*. **Conclusion:** The piperine-loaded phytosomal gel may be regarded as a promising therapeutic strategy for the treatment of fungal infections.

Keywords: Effective inhibition, Fungal infections, Hydrophobic, Phytosomes, Stability.

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INTRODUCTION

In recent years, plant-derived medicinal products, available as extracts or isolated compounds, have been recognised as beneficial for the treatment and management of various diseases. Their efficacy is primarily due to their diverse chemical structures and pharmacological mechanisms, which contribute to minimising the development of resistance against disease, while maintaining low toxicity.[1–3] Despite this, the therapeutic use is limited by several factors, including poor solubility and limited permeability.[4,5] These limitations can be overcome through the development of different Novel Drug Delivery Systems (NDDS) including nanoparticles, liposomes, phytosomes and nanoemulsions, which primarily enhance drug availability at the site of action through solubility enhancement and controlled release actions. Moreover, requirement of lower doses compared to conventional dosage forms helps to decrease tissue toxicity. Therefore, several researches are ongoing worldwide to develop their novel formulations for therapeutic use.[6–11] Phytosomes are one of the types of vesicular drug delivery system, belongs to NDDS, developed to enhance the absorption and bioavailability of herbal extracts or isolated phytoconstituents through oral as well as topical routes. The word 'phytosome' consists of two parts: 'phyto', represent plant while 'some', stands for structure similar to a cell.[12] This novel delivery approach has demonstrated significant advantages in various scientific studies by improving the therapeutic performance of extracts and phytoconstituents such as *Ginkgo biloba* extract[13], *Rubia cordifolia* extract[14], curcumin[15,16] and silybin.[17,18] In phytosomes, the plant extract or active constituent forms a molecular complex with phospholipids mainly phosphatidylcholine, present in soya lecithin, which

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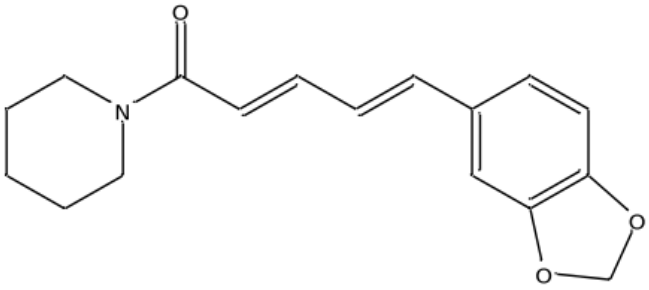


Figure 1: Chemical structure of piperine.

enhances lipid compatibility. Since phosphatidylcholine is also a natural component of biological and skin membranes, hence it facilitates improved permeation and targeted delivery of the drug, particularly in topical applications.[19,20] Besides enhancing skin permeation and retention, phytosomes offer several additional advantages, including protection of phytoconstituents from oxidative degradation, reduction in the required drug dose and decreased skin irritation.[21,22]

Among various natural products, piperine ($C_{17}H_{19}NO_3$) (Figure 1), an alkaloidal compound obtained from black pepper and long pepper, holds a crucial role due to its wide range of therapeutic applications. It has been reported to exhibit antitumour, antioxidant, antifungal and antidepressant activities.[23–26] Piperine is also well known for its ability to enhance the oral bioavailability of several drugs and phytoconstituents when taken together.[27,28] However, in the case of topical application, particularly for the management of skin diseases like fungal infections, use of piperine is limited due to its hydrophobic nature, which results in poor aqueous solubility and consequently inadequate penetration through the stratum corneum to achieve the desired therapeutic levels at the site of action.[29,30] These limitations can be overcome through the application of NDDS, specifically by converting them into nano-systems like phytosomes to improve its solubility, skin permeation and therapeutic efficacy.[31–33] Accordingly, various nano-formulations of piperine, either alone or in combination with other drugs, have been developed for topical applications.[29,34–35]

Candida albicans is one of the most frequently encountered yeasts responsible for a variety of skin-related diseases, particularly among immunocompetent individuals. It primarily affects areas such as the spaces between fingers and toes, armpits, nails and other moist regions. The common symptoms of these diseases include redness, itching and irritation; however, if they are not treated properly, they can result in serious complications and may at times be life-threatening for individuals who are at risk.[36–38] Various antifungal agents, such as clotrimazole, fluconazole and nystatin, are utilised in different formulations for treatment.[39–41] Because of the ongoing increase in antifungal resistance, natural products are being emphasised for their effectiveness against multidrug-resistant fungal infections.[42] Several studies have indicated that black pepper (*Piper nigrum*) extract and its principal bioactive constituent, piperine, exhibit significant antifungal activity against various dermatological fungal pathogens, including *C. albicans*, while maintaining relatively low toxicity.[43–46] Piperine has been reported to exert antifungal effects against *C. albicans* with Minimum Inhibitory Concentration (MIC) of 78 µg/mL[47], by inhibiting biofilm formation, disrupting the integrity of fungal cell membranes and suppressing hyphal growth.[48,49]

In light of the significant antifungal activity of piperine, which is limited by its hydrophobic nature, the current study aimed to develop a piperine-loaded phytosomal nano-drug delivery system and evaluate its physicochemical characteristics and antifungal efficacy against *C. albicans* as future option for the management of fungal infections. Although numerous piperine-based nanoformulations and topical delivery systems have been documented, this study focuses specifically on a piperine-loaded phytosomal system integrated into a Carbopol 934 topical gel to determine the efficacy of antifungal effectiveness of piperine in comparison to its traditional gel formulation.

MATERIALS AND METHODS

Materials

Piperine was procured from Yucca Enterprises, India. For antifungal evaluation, the freeze-dried culture of *Candida albicans* (MTCC 227 / ATCC 10231) was sourced from the Microbial Type Culture Collection and Gene Bank (MTCC), India. Sterile filter paper discs were procured from TM Media, India. Sabouraud Dextrose Agar (SDA), soya lecithin, cholesterol, Dichloromethane (DCM), Carbopol 934, glycerine, propylene glycol, triethanolamine and methyl paraben were purchased from CDH Fine Chemicals, India. Laboratory-prepared double-distilled water was utilised for all experimental procedures and all chemicals and reagents used were of analytical grade.

Methods

λ_{max} Determination of Piperine

UV-visible spectroscopy was employed for the determination of λ_{max} of piperine using methanol, as also investigated in the previous study, over a scanning range of 190-400 nm.[50] The wavelength showing maximum absorbance was noted and used for further studies.

Compatibility Studies

Compatibility studies of piperine with selected excipients and the corresponding physical mixture were conducted by FT-IR, DSC and PXRD analysis. As a confirmatory step for further processing, a physical mixture containing components in the same proportion and quantity as the optimised formulation was formulated.

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Table 1: Preparation of phytosome batches.

Phytosome batches	Piperine: Soya lecithin ratio	Piperine (mg)	Soya lecithin (mg)	Cholesterol (mg)	Solvent (DCM/ mL)
F-1	1:1	19.5	19.5	1.95	20
F-2	1:2	19.5	39.0	3.90	20
F-3	1:3	19.5	58.5	5.85	20
F-4	1:4	19.5	78.0	7.80	20

Preparation of Phytosomes Loaded with Piperine

The solvent evaporation technique was used for the preparation of piperine-loaded phytosomes.[12,51] To optimise the formulation, four different batches were prepared as shown in (Table 1). For phytosome preparation, piperine, soya lecithin and cholesterol were weighed accurately in the required quantities. DCM was used to separately dissolve piperine, soya lecithin and cholesterol with continuous stirring at 35-40°C until clear and homogeneous solutions were obtained. Under constant stirring, the cholesterol solution was added gradually to the solution of lecithin. The piperine solution was then added slowly to the prepared lipid phase and the mixture was stirred continuously for 30 min at 35-40°C to facilitate the formation of a piperine-phospholipid complex. The resulting complex solution containing piperine and phospholipids was introduced into a round-bottom flask and the organic solvent was evaporated under vacuum at 40°C using a rotary evaporator to form a thin lipid layer on the inner wall of the flask. A dried white-coloured piperine-loaded phytosomal complex was formed as a thin lipid film, collected carefully and preserved in an airtight amber coloured container until further analysis. The dried piperine-loaded phytosomal complex (piperine-loaded phytosomes) was accurately weighed and reconstituted in purified water in accordance with gel preparation requirements followed by sonication with a probe sonicator to obtain a consistent phytosomal dispersion. The dried piperine-loaded phytosomal complex was used for solid-state characterisation studies including Fourier Transform Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC), powder X-ray DIFFRACTIOn (PXRD) and field emission gun-scanning electron microscopy (FEG-SEM). The reconstituted phytosomal dispersion was subjected to particle size, Polydispersity Index (PDI) and zeta potential evaluation. Additionally, Cryo-HR Transmission Electron Microscopy (Cryo-HRTEM) was used to evaluate vesicular morphology and size in the hydrated state. The process was repeated three times to check the reproducibility of results.

Characterisation Studies

Selection of Optimised Batch

For the selection of the optimised piperine-loaded phytosomes among the four different batches, parameters such as particle size, PDI and zeta potential along with entrapment efficiency as well as drug content were considered. Measurements were carried out three times and the obtained results were presented as mean±Standard Deviation (SD) (n=3). The selected optimised batch was further characterised using techniques such as FT-IR, DSC, PXRD, FEG-SEM and Cryo-HRTEM.

Determination of Particle Size, Polydispersity Index (PDI) and Zeta Potential

Dynamic Light Scattering (DLS) analysis was performed using a Litesizer DLS 500 (Anton Paar GmbH, Austria) to determine the mean particle size, PDI and zeta potential of the piperine-loaded phytosomal formulations, commonly utilised by the researchers.[19] The deionised water was used for the sample preparations and analysed at room temperature.

Entrapment Efficiency (%)

To assess the entrapment efficiency of piperine within the phytosomal complex, samples were evaluated using the centrifugation method as described by F. Xu *et al.*[52] In details, the piperine-loaded phytosomal complex was dispersed in the methanol, the samples were then centrifuged at 5000 rpm for 60 min at 4°C. The supernatant obtained after centrifugation was carefully separated and the concentration of unentrapped (free) piperine was measured using a UV-vis spectrophotometer at 342 nm using methanol as the solvent. The entrapment efficiency (%) was calculated as-

$$\text{Entrapment efficiency}\ (\%) = \frac{\text{Total amount of piperine} - \text{Amount of free piperine}}{\text{Total amount of piperine}} \times 100$$

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Drug Content (%)

The prepared piperine-loaded phytosomal complex (50 mg) was dispersed in 20 mL methanol and was sonicated for 15-20 min to dissolve the piperine completely. The solution was filtered, suitably diluted with methanol and the volume was adjusted prior to determine the piperine concentration by UV spectrophotometry at 342 nm with previously established calibration curve and linear regression equation similar to the previously conducted study.[53] Following equation was used to calculate drug content (%).

$$\text{Drug content}\ (\%) = \frac{\text{Amount of piperine found in the sample}}{\text{Amount of piperine used in the formulation}} \times 100$$

Fourier Transform Infrared Spectroscopy (FT-IR) Study

The FT-IR study of piperine, soya lecithin, cholesterol, physical mixture, blank phytosomes (without piperine) and piperine-loaded phytosomes was carried out using IRAffinity-1 FT-IR spectrophotometer (Shimadzu Corporation, Japan) to evaluate possible molecular interactions and functional group characterisation as used in the previous study.[54] FT-IR spectra of the samples were obtained using a KBr disk system fitted with a germanium-coated KBr beam splitter and a DLATGS detector within the scanning range of 400-4000 cm^{-1}.

Differential Scanning Calorimetry (DSC) Study

The DSC study of piperine, soya lecithin, cholesterol, physical mixture, blank phytosomes (without piperine) and piperine-loaded phytosomes was carried out using DSC1 instrument (Mettler Toledo, Switzerland) to determine changes in thermal behavior and crystallinity as used in the previous study.[55] The samples, accurately weighed and sealed in aluminium pans were subjected to heating from 40 to 300°C in a nitrogen purge of 20 mL/min at scan rate of 5°C /min. The thermograms produced were evaluated using STAR software (version 11.0).

Powder X-ray Diffraction (PXRD) Study

To determine the crystalline behavior of piperine, soya lecithin, cholesterol, physical mixture, blank phytosomes (without piperine) and piperine-loaded phytosomes, powder X-ray diffraction studies were performed using a Rigaku Miniflex-600 X-ray Diffractometer (Japan) equipped with a Theta-Theta (θ-θ) goniometer.[56] The scanning range was maintained from 10° to 90° (2θ) with a step increment of 0.02° (2θ). The resulting diffraction data were interpreted by PDXL software in reference to the Crystallography Open Database (COD) database.

Field Emission Gun-Scanning Electron Microscopy (FEG-SEM) Study

Surface morphological analysis of piperine, blank phytosomes (without piperine) and piperine-loaded phytosomes was carried out using a Field Emission Gun-Scanning Electron Microscope with EDS, WDS and Schottky field emission gun (JEOL JSM-7600F, Japan) following a procedure similar to that reported by N.B. Banarase *et al.*[56] The samples were analysed under an accelerating voltage of 15 kV.

Cryo-HR Transmission Electron Microscopy (Cryo-HRTEM) Study

Cryo-HRTEM presents several benefits over conventional TEM because it operates effectively at cryogenic temperatures. This technique helps maintain the native condition of sample and reduces structural shrinkage or damage during analysis, particularly useful for phytosomal studies. Unlike conventional TEM, Cryo-HRTEM generally does not need staining or chemical fixation, making it beneficial for the precise identification of vesicle structures.[57,58]

The morphology, average particle size and appropriate formation of piperine-loaded phytosomes were analysed with Cryo High-Resolution Transmission Electron Microscope (JEOL NEOARM 200F/JEM-ARM200F). Sample vitrification was done using a cryo-plunger (Leica EM GP2). High-resolution images were taken at cryogenic temperatures with a cold field emission gun. Imaging was performed at accelerating voltages between 80 and 200 kV.

Stability Study

The accelerated stability study of piperine-loaded phytosomes was performed following ICH Q1A(R^2) guidelines by storing the samples in closed vials at 40±2°C /75±5% RH for 6 months. The stored samples were examined for any changes in physical appearance and crystallinity state through DSC and PXRD analysis.[59]

Preparation of Piperine-Loaded Phytosomal Gel

The prepared piperine-loaded phytosomes were incorporated into the Carbopol based gel.[60] For this purpose, five different gel formulations (G-1 to G-5; Table 2) were prepared using trial-and-error method. In brief, the Carbopol 934, as gelling agent, was dispersed in the suitable quantity of water with continuous stirring. The dispersion was allowed to stand overnight to ensure complete hydration and swelling of Carbopol 934. The propylene glycol and glycerine, as humectants, were then added with continuous stirring. The piperine-loaded phytosomes, corresponding to five times the MIC of piperine against *C. albicans*, dispersed in the small amount of water, were then incorporated and required pH was adjusted using triethanolamine

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Table 2: Preparation of piperine-loaded phytosomal gel batches.

Ingredients used	Gel batches				
	G-1	G-2	G-3	G-4	G-5
Piperine-loaded phytosomes	Equivalent to 390 µg/mL	Equivalent to 390 µg/mL	Equivalent to 390 µg/mL	Equivalent to 390 µg/mL	Equivalent to 390 µg/mL
Carbopol 934 (g)	1.0	1.0	1.0	1.0	1.0
Propylene glycol (mL)	2.0	4.0	6.0	8.0	10.0
Glycerine (mL)	1.0	2.0	3.0	4.0	5.0
Triethanolamine (mL)	q.s.	q.s.	q.s.	q.s.	q.s.
Distilled water (mL)	q.s. to 50 g	q.s. to 50 g	q.s. to 50 g	q.s. to 50 g	q.s. to 50 g

followed by volume make up to 50 g using distilled water to get homogenous clear gel. Evaluation of the prepared gel batches was carried out further for optimisation purposes. The optimised batch was then evaluated for the antifungal activity against *C. albicans* without incorporation of preservative. After the antifungal evaluation, a preservative (methyl paraben), 0.25 g, was added to maintain the long-term stability.

Evaluation of Gel Batches

To select the optimised formulation, the gel batches were evaluated for different parameters including viscosity, consistency, homogeneity and spreadability. Measurements were performed three times and the results were reported as mean±SD ($n=3$). Statistical significance was analysed using one-way ANOVA with Tukey's *post hoc* test, where $p<0.05$ was considered significant.

Viscosity

The prepared gel formulations were evaluated for viscosity using a Brookfield digital viscometer (DVEERVTJ0, AMETEK Brookfield, MA, USA) using spindle no. 6 under controlled conditions of 25±1°C and 10 rpm. The viscosity was noted in centipoise (cP).

Consistency and Homogeneity

The evaluation of the gel batches for consistency and homogeneity was done manually by examining their smoothness, uniformity, grittiness and phase separation. Consistency was based on how easily the gel spreads with slight force and homogeneity was determined by the absence of lumps and the overall uniformity of the formulation.[61]

Spreadability

The spreadability of the gel batches was tested using the parallel plate method. In this method, 0.5 g of gel was put between two glass plates, and a weight of 220 g was applied for 1 min. After that, the diameter of the gel spread was measured as spreadability.[62]

In vitro Antifungal Activity by Disc Diffusion Method

The prepared piperine-loaded phytosomal gel was subjected to antifungal activity testing using disc diffusion (Kirby-Bauer) method. In brief, the freeze-dried culture of *C. albicans* was suspended in 0.4 mL of sterile water and allowed to stand for 20 min, followed by transfer to SDA plates and incubation at 37 1°C for 48 h. The selected well grown colonies were harvested and suspended in sterile saline water with turbidity adjusted to 0.5 McFarland standard (1×10^6 CFU/mL). Subsequently, 100 µL of the prepared inoculum was uniformly spread onto SDA plates with the help of sterile glass spreader. Sterile filter paper discs (6 mm) impregnated with plain piperine gel and piperine-loaded phytosomal gel equivalent to 390 µg/mL piperine (For the present antifungal evaluation, a concentration equivalent to five times the MIC was selected; therefore, the target piperine concentration was calculated as 78 µg/mL × 5=390 µg/mL. This MIC value refers to free piperine and was not determined for the phytosomal formulation)[63] were placed on the inoculated plates separately. Blank phytosomal gel (without piperine), gel base, DMSO (0.5% v/v), and fluconazole (25 µg/disc) were used as controls. Following incubation at 37°C for 48 h, the zones of inhibition were determined in millimeters with the help of an antibiotic zone reader. The process was carried out in triplicate.[64]

RESULTS AND DISCUSSION

λ_{max} Determination of Piperine

The λ_{max} of piperine in methanol was found to be 342 nm. The calibration plot constructed for concentrations ranging from 4-20 µg/mL at this wavelength followed the linear regression equation y = 0.0489x - 0.006 and demonstrated a correlation coefficient (R^2) of 0.9915 (Figures 2 and 3). This λ_{max} of piperine (342 nm in methanol) was chosen for further evaluation studies, and the obtained linear regression equation was used for the quantitative estimation of piperine in the prepared formulations.

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Table 3: Particle size, zeta potential, PDI, entrapment efficiency and drug content of phytosome batches.

Phytosome batches	Particle size (nm)	Zeta potential (mV)	PDI	Entrapment efficiency (%)	Drug content (%)
F-1	284.1±0.76	-45.1±0.26	0.262±0.10	72.34±0.4	71.22±0.35
F-2	179.6±0.24	-38.4±0.21	0.145±0.06	89.32±0.14	92.41±0.08
F-3	332.9±0.62	-27.4±0.26	0.366±0.08	65.27±0.34	68.8±0.21
F-4	215.4±0.05	-36.2±0.38	0.227±0.08	48.02±0.23	56.54±0.3

Values are expressed as mean±SD (*n*=3).

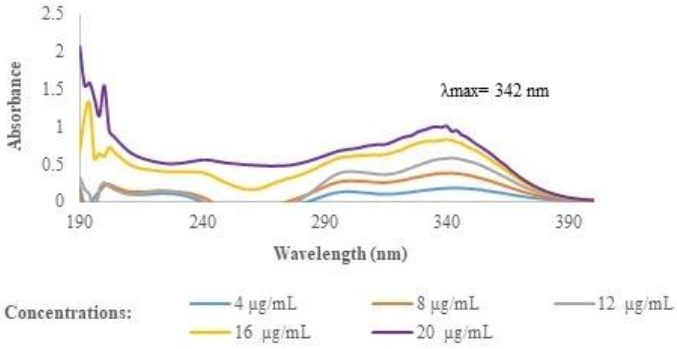


Figure 2: Overlay UV absorption spectra of piperine.

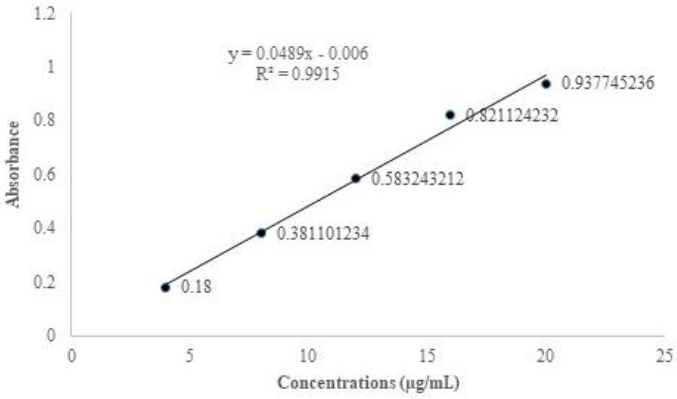


Figure 3: Plot of piperine concentration versus absorbance.

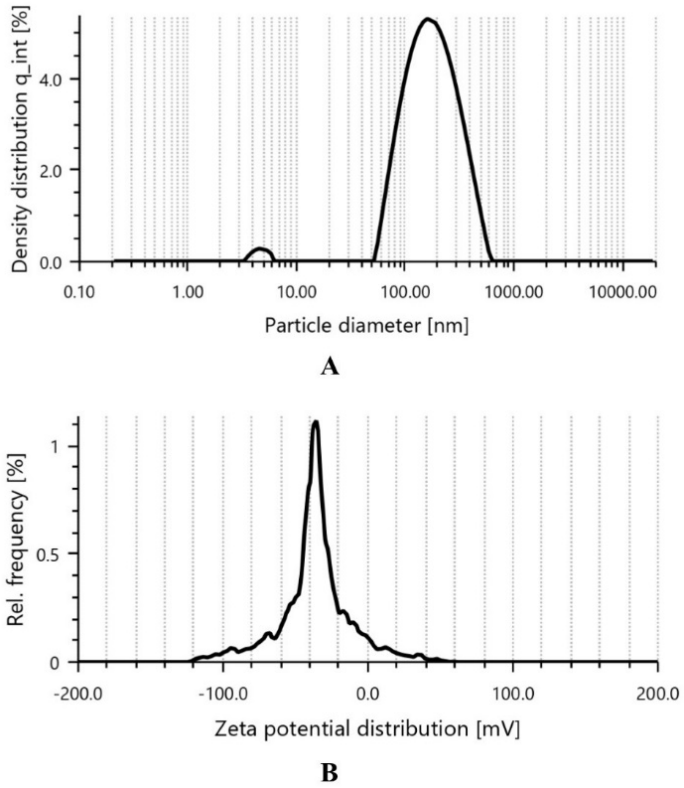


Figure 4: DLS analysis of piperine-loaded phytosomes (Batch F2) - (A) particle size distribution and (B) zeta potential.

Characterisation Studies

Selection of Optimised Phytosomal Batch

The results of four different batches of piperine-loaded phytosomal complexes (F1-F4), formulated with varying concentrations of constituents particularly soya lecithin and cholesterol, analysed for particle size, zeta potential, PDI, entrapment efficiency and drug content are tabulated in (Table 3).

The mean particle size of the phytosomes of formulated batches were ranged from 179.6±0.24 nm to 332.9±0.62 nm. Among the formulations, batch F-2 revealed the smallest particle size (179.6±0.24 nm) (Figure 4A). The results indicate that a relatively stable phytosomal complex was obtained at a piperine: soya lecithin ratio of 1:2. In contrast, on further increasing the lecithin concentration, an increase in particle size was observed, which may because of aggregation of phospholipid molecules (soya lecithin) during continuous stirring, as reported in earlier study.[65]

The PDI values, representing the degree of uniformity of particle size distribution, ranged from 0.145±0.06 to 0.366±0.08. Batch F-2 exhibited the minimum PDI among the formulations, indicating better uniformity in particle size distribution compared to the remaining batches.

On evaluation of zeta potential as an indicator of colloidal stability, all formulation batches resulted in negative values ranging from -27.4±0.26 mV to -45.1±0.26 mV. Batch F-1 showed the highest absolute zeta potential as -45.1±0.26 mV, reflecting excellent colloidal stability, followed by batch F-2 with a high zeta potential of -38.4±0.21 mV (Figure 4 B), which is also considered sufficient to maintain the colloidal stability of the phytosomal dispersion for the preparation of gel.

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Table 4: Evaluation of piperine-loaded phytosomal gel batches.

Evaluation parameters	Gel batches				
	G-1	G-2	G-3	G-4	G-5
Viscosity (cP)	17,420±110	15,740±105	13,720±95	12,180±90	11,750±120
Consistency	Slightly stiff and less spreadable	Moderately stiff and less spreadable	Smooth and good spreadability	Smooth and excellent spreadability	Very soft and runny
Homogeneity	Slight grittiness	Uniform and no lumps	Homogeneous	Excellent homogeneity, no lumps or phase separation	Homogeneous but less structured
Spreadability (cm)	4.2±0.10	4.8±0.12	5.4±0.12	6.1±0.02	6.6±0.14

Values are expressed as mean±SD ($n=3$). Statistical analysis was performed using one-way ANOVA. Differences were considered statistically significant at $p<0.05$.

The entrapment efficiency of the prepared phytosomal formulation batches varied significantly from 48.02±0.23% to 89.32±0.14%. Formulation F-2 showed the highest entrapment efficiency of 89.32±0.14%, possibly because of the formation of a stable phytosomal complex at a piperine: soya lecithin ratio of 1:2. While, F-1 formulation showed an entrapment efficiency of 72.34±0.40%, probably because of low soya lecithin content, while F-3 and F-4 also showed lower entrapment efficiency, which might because of higher concentration (saturation) of phospholipid content with respect to piperine.

The drug content (%) of the four phytosomal batches were ranged from 56.54±0.3% to 92.41±0.08%. In line with the entrapment efficiency, the highest drug content was also observed in the F-2 batch (92.41±0.08%). This higher drug content is possibly due to the optimised proportion of piperine and soya lecithin used for this batch. For this parameter as well, the decrease in drug content (%) in the F-3 and F-4 batches may be attributed to the higher proportion of lecithin compared to piperine.

The above evaluation studies indicated that, among the four formulations, F-2 exhibited the smallest particle size (179.6±0.24 nm), lowest PDI (0.145±0.06), highest entrapment efficiency (89.32±0.14%) and highest drug content (92.41±0.08%). Although, F-1 had a greater absolute zeta potential (-45.1±0.26 mV), F-2 also had a sufficiently high negative zeta potential (-38.4±0.21 mV), which shows it has good colloidal stability. Hence, F-2 was chosen as the optimised formulation due to its overall balance of small particle size, narrow particle size distribution, good colloidal stability, high entrapment efficiency and high drug content rather based on a single evaluation parameter.

FT-IR Study

FT-IR investigation confirmed the characteristic absorption peaks of piperine (1610.63 cm^{-1}/ aromatic C=C stretching, 1726.36 cm^{-1}/carbonyl stretching, 1260.54 cm^{-1}/ C-O-C vibration and 2928.07 cm^{-1}/aliphatic C-H stretching)[66], soya lecithin (1735.04 cm^{-1}/ester carbonyl, 1259.57 cm^{-1}/P=O stretching, 1118.76 cm^{-1}/phosphate group, and 2949.29 cm^{-1}/CH_2 stretching)[67] and cholesterol (3419.94 cm^{-1}/OH stretching) and 2949.29 cm^{-1}/C-H stretching) confirming their structures.[68] The physical mixture has shown the peaks at (1614.49 cm^{-1}, 1725.4 cm^{-1}, 2942.53 cm^{-1} and 3403.54 cm^{-1}), without significant shift or disappearance, representing absence of physicochemical incompatibility among its individual components. The FT-IR spectrum of piperine-loaded phytosomes showed the shifting of the aromatic C=C peak to a higher wavenumber from 1610.63 cm^{-1} to 1663.68 cm^{-1}, shifting of the soya lecithin peak from 1735.04 cm^{-1} to 1741.8 cm^{-1}, as well as broadening of the peak between 3349.53 cm^{-1} and 3395.83 cm^{-1} (Figure S1), suggesting possible molecular interactions between piperine and phospholipids.[54,69] It was well supported by the FT-IR spectrum of blank phytosomes (without piperine), which showed no occurrence of piperine-related peaks.

DSC Study

DSC analysis revealed a distinct endothermic peak of piperine at 131.86°C, consistent with the melting point previously reported in the literature.[70] This endothermic peak confirms the crystalline structure of piperine, as also supported by the PXRD and FEG-SEM studies. Similarly, cholesterol showed an endothermic peak at 146.03 °C,[71] while soya lecithin, instead of a sharp endothermic peak, revealed multiple transition ranges due to its amorphous nature.[72] The physical mixture showed the presence of melting point ranges for piperine, cholesterol and soya lecithin with slight variations. However, no new endothermic peaks were found, which suggests the absence of significant chemical interactions and incompatibilities among them. The blank phytosomes (without piperine) showed multiple melting transition states similar to the physical mixture, suggesting the formation of a lipid complex due to the disappearance of endothermic peak of piperine. In contrast, the said endothermic peak was observed at 127.18°C in the piperine-loaded phytosomes (Figure S2), with a shift of approximately 4.68°C compared with pure piperine representing the partial reduction of crystallinity as also observed in the PXRD studies which further helps in successful phytosomal complex formation.[54,73]

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PXRD Study

The PXRD diffraction pattern showed that piperine exhibited the sharp diffraction peaks at 2θ values of 13.7°, 14.38° and 19.3°, confirming its crystalline nature, supported well with the previous studies.[74] In contrast, soya lecithin did not show sharp diffraction peaks, indicating its amorphous nature.[75] The diffraction pattern of cholesterol displayed several distinct peaks, suggesting its crystalline structure.[76] The physical mixture exhibited most of the characteristic diffraction peaks corresponding to piperine and the remaining components, indicating simple mixing of piperine with the excipients without significant interaction and incompatibilities. However, the PXRD pattern of blank phytosomes (without piperine) exhibited a predominantly amorphous pattern, which can be resulted due to the formation of phospholipid vesicular structures and the characteristic peaks of piperine were absent. In contrast, the piperine-loaded phytosomes showed the characteristic diffraction peaks of piperine, but with marked reduction in intensity and slight peak broadening (Figure S3). This suggests that piperine was successfully incorporated into the phospholipid complex, resulting in reduced crystallinity and partial transformation of the crystalline form into a more amorphous state.[77]

FEG-SEM Study

Scanning electron microscopy revealed piperine crystals with plate-like, flat, lamellar morphology organised in the form of layered aggregations. The observed morphology supports the crystalline characteristics of piperine, as also reported by previous researchers.[78,79] FEG-SEM images of the piperine-loaded phytosomal complex showed rounded to irregular spherical vesicle-like structures in the form of aggregated masses. The observed morphology suggests the formation of nanosize phytosomes, also supported by DLS study, without the evidence of plate-like crystalline piperine morphology. These findings along with DSC and PXRD results, indicated the transformation of crystalline piperine into a partial amorphization within the phytosomal complex. The blank phytosomes (without piperine) showed flake-like aggregated structures with an amorphous appearance. Also, in the case of blank phytosomes, no specific crystalline or well-defined vesicular structures were observed compared to plate-like piperine crystals and the rounded to irregular spherical vesicles of the piperine-loaded phytosomal complex (Figure S4). The observed morphology of blank phytosomes may suggest phospholipid vesicle collapse, which can occur during vacuum drying prior to analysis.

Cryo-HRTEM Study

For the determination of the average particle size of the phytosomes by Cryo-HRTEM, multiple images were analysed and more than 450 measurable phytosomal assemblies were considered for size estimation. The Cryo-HRTEM images revealed the successful formation of spherical phytosomal assemblies in the nanometric range, exhibiting distinct multilamellar structures with clearly visible bilayer boundaries. The phytosomal assemblies exhibited an average size of approximately 200 nm (Figure S5), which was consistent with the particle-size results obtained from DLS and the morphological observations from FEG-SEM. Minor variations were noted between the particle size values obtained from DLS and Cryo-HRTEM. This variation can be explained by the differing principles of the two techniques. DLS measures the hydrodynamic particle size of phytosomes in dispersion, while Cryo-HRTEM provides the physical diameter of the phytosomal assemblies through direct visualisation and measurement from the images. Thus, the slight difference between the DLS and Cryo-HRTEM values is anticipated and does not indicate any inconsistency between the methodologies.

Stability Study

The stability study conducted over a duration of 6 months revealed no notable alterations in the color of the piperine-loaded phytosomes. Additionally, the DSC and PXRD analyses demonstrated that the reduced crystallinity of piperine within the phytosomal formulation remained consistent throughout the study period, similar to the original formulation. These findings clearly suggest that the piperine-loaded phytosomes developed are stable under accelerated conditions.

Selection of Optimised Gel Batch

The evaluation of piperine-loaded phytosomal gel batches (G1 to G5) for various parameters (Table 4) revealed viscosity values ranging from 11,750±120 to 17,420±110 cP. The decrease in viscosity from G1 to G5 may be due to the gradual increase in propylene glycol and glycerine concentration. The G-4 formulation batch showed a good viscosity of 12,180±90 cP, which is suitable for topical application and easy extrusion. Additionally, the G-4 formulation batch was superior in terms of smooth consistency and excellent spreadability compared to G1 to G3. However, G-5 had a very soft consistency, making it difficult to maintain its structure, thus unsuitable for prolonged retention on the topical site. In terms of homogeneity, G-1 showed slight grittiness, while G-2 and G-3 were relatively uniform and homogenous. Notably, G-4 demonstrated excellent homogeneity with no lumps or phase separation, indicating proper incorporation of all components. Although G-5 showed good homogeneity, it was less structured. The spreadability of the gel batches ranged from 4.2±0.10 to 6.6±0.14 cm. This increase in spreadability correlates well with the gradual decrease in viscosity.

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The G-4 gel batch exhibited optimal spreadability (6.1±0.02 cm), which is ideal for uniform topical application. While G-5 had the highest spreadability, its unsuitable viscosity and lack of structural uniformity could negatively affect its use. Therefore, based on all these evaluation results, G-4 was selected as the optimised gel formulation.

Antifungal Activity

The zone of inhibitions of piperine-loaded phytosomal gel, plain piperine gel, blank phytosomal gel (without piperine), gel base, DMSO (0.5% v/v) and fluconazole against *C. albicans* are given in (Table S1). On comparative study, it was found that the piperine-loaded phytosomal gel exhibited a higher zone of inhibition (23.2±0.4 mm) as compared to plain piperine gel (8.1±0.2 mm), approximately 85% of the mean inhibition-zone diameter produced by the reference drug (fluconazole), reflecting its significant antifungal activity. This improved inhibition may be associated with improved penetration of piperine into *C. albicans* by the phytosomal drug delivery system. While blank phytosomal gel (without piperine), gel base, and DMSO (0.5% v/v) did not show any observable zone of inhibition (Figure S6), confirming the absence of inhibition, which validated the noted results.

CONCLUSION

Piperine is recognised for its diverse beneficial pharmacological activities, exhibiting notable effectiveness against *C. albicans* as an antifungal agent. However, its clinical applications are limited due to its hydrophobic properties, which hinders adequate skin penetration. To effectively utilise it, this study has proposed and successfully developed a piperine-loaded phytosomal gel, serving as a nano-drug delivery system. The formulated phytosomal system was evaluated across various parameters and it was found that the optimised piperine-loaded phytosomes (F-2) demonstrated desirable characteristics, including minimal particle size, lowest PDI, high zeta potential, highest entrapment efficiency with high drug content (%). These findings were further supported by FT-IR, DSC, PXRD, FEG-SEM and Cryo-HRTEM studies, confirming the successful formation of nano-sized phospholipid vesicles and the transformation of crystalline piperine into an amorphous state. In the present study, the term 'piperine-loaded phytosomal system' is used to describe the phospholipid-associated formulation characterised by particle size, PDI, zeta potential and solid-state characterisation rather than a conventional liposomal system in which the active compound is merely entrapped within an aqueous vesicular compartment.

The *In vitro* antifungal evaluation of the piperine-loaded phytosomal gel revealed effective inhibition against *C. albicans*, likely due to enhanced penetration through the fungal cells. The six-month accelerated stability assessment revealed no significant changes in physical appearance and no considerable modifications in the solid-state characteristics as evaluated by DSC and PXRD. These results indicate that the physicochemical solid-state properties of the piperine-loaded phytosomal system were stable under the tested accelerated storage conditions. Nevertheless, for further investigation, assessing particle size, PDI, zeta potential, entrapment efficiency, drug content and antifungal activity both prior to and following stability testing will be regarded helpful for a thorough evaluation. Stability studies clearly indicated that the developed piperine-loaded phytosomes are suitable for practical applications. In conclusion, the piperine-loaded phytosomal gel as nano-drug delivery system may represent a promising future option for the management of fungal infections.

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ABBREVIATIONS

NDDS: Novel Drug Delivery Systems; ***C. albicans*:** *Candida albicans*; **MIC:** Minimum Inhibitory Concentration; **DCM:** Dichloromethane; **SDA:** Sabouraud Dextrose Agar; **FTIR:** Fourier Transform Infrared Spectroscopy; **DSC:** Differential Scanning Calorimetry; **PXRD:** Powder X-ray Diffraction; **FEG-SEM:** Field Emission Gun-Scanning Electron Microscopy; **PDI:** Polydispersity Index; **Cryo-HRTEM:** Cryo-HR Transmission Electron Microscopy; **DLS:** Dynamic Light Scattering; **DMSO:** Dimethyl Sulfoxide. **CFU:** Colony-Forming Unit.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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The authors received no financial support or sponsorship for the conduct of this study.

ETHICS/DATA AVAILABILITY STATEMENT

This study has included an *In vitro* antifungal study and did not involve any human participants or animal subjects; therefore, ethical approval was not required.

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AUTHOR CONTRIBUTIONS

Radha V. Kale: Writing- original draft, Conceptualization, Methodology, Validation, Investigation. **Koshish B. Gabhane:** Formal analysis, Supervision. **Nilesh B. Banarase:** Writing- original draft, Conceptualization, Methodology, Writing-review and editing, Validation, Investigation, Formal analysis, Supervision. **Vikrant L. Salode:** Writing-review and editing, Formal analysis, Supervision.

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