

DEVELOPMENT OF LIPID-BASED VESICLES OF TERBINAFINE GEL FOR SKIN DELIVERY BY 3²FULL FACTORIAL DESIGN

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ABSTRACT

Objective: Terbinafine is a poorly water-soluble and highly permeable allylamine antifungal (BCS-II) drug. In this study, we looked at the possibility of using ethosomes as vesicular lipid nanocarriers to enhance the transdermal delivery of terbinafine.

Methods: Using a 3² full factorial design, the ethosomal formulation with different soya lecithin and cholesterol concentrations was improved and optimized. The influence of independent variables, namely soya lecithin and cholesterol concentration in ethosomes was determined by estimating dependent variable including the particle size, polydispersity index, zeta potential, entrapment efficiency, and *in vitro* drug release. To improve the residence time of ethosomes on the topical application, the ethosomes were incorporated into the carbopol gel. 1% w/v of Carbopol 934 P gel-embedded Terbinafine ethosomes were used to study medication release and skin interactions.

Results: Optimized ratios of soya lecithin and cholesterol was used to prepare vesicles. Formulation had a particle size of 1207.39±2.71 nm, entrapment efficiency of 94.46±0.47%, and *in vitro* diffusion of 51.27±0.16%. It was found that the growth of fungus *Aspergillus niger* and *Candida albicans* were inhibited by Ethosomal Gel. However, ethosomal gel had more inhibitory activity on *Aspergillus niger* compared to positive control.

Conclusion: The current study suggests that ethosomal vesicles may improve transdermal dispersion without causing skin irritation. Terbinafine-loaded ethosomes have the potential to be one of the most important transdermal application techniques for the treatment of fungi-related disorders.

Keywords: Ethosomes, Terbinafine hydrochloride, Optimization, Anti-fungal, Transdermal enhancement

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INTRODUCTION

Onychomycosis, specifically DLSO (Distal Subungual Onychomycosis), is the most common nail disease worldwide, causing nail discoloration, thickening, and onycholysis, and often affecting the adjacent skin. Risk factors include persistent nail trauma, psoriasis, diabetes, poor circulation, HIV, immune suppression, and smoking [1]. Environmental factors like humid environments, occlusive footwear, and genetic predisposition can be a triggering factor for the disease, and recurrence after treatment is common. Onychomycosis is clinically classified into different types. Treatment involves the use of antifungal medications, either topical or oral, depending on the severity and type of infection. However, antifungal medications can have side effects, and it's important to follow prescribed dosage and duration of treatment. In some cases, a combination of topical and oral antifungal medications may be needed for effective treatment [2, 3].

Oral and topical medications are both effective in treating fungus nail infections. Microscopy and culture have shown that the main objective of treatment is to completely get rid of the organism. Almost all the well-conducted studies define this as the primary endpoint [4]. Clinical cure and improvement are secondary objectives that are measured using a rigid scoring system based on clinical abnormalities in the nail apparatus. Side effects are unavoidable with all dosage forms. To avoid side effects, a transdermal drug delivery system can be used [5]. This can also have direct action on the affected area and also helps in overcoming the side effects caused by conventional methods.

Transdermal medication delivery has been widely studied for its potential benefits, including reduced adverse effects and avoidance of first-pass hepatic clearance and intestinal breakdown. However, the epidermis acts as a barrier, limiting the transport of most molecules through the skin. Various methods to enhance skin permeation have been proposed, such as penetration enhancers, vesicles, iontophoresis, and microneedles [6]. Ethosomes, which are soft lipid vesicles made primarily of phospholipids, alcohol, and water, have shown promise in transdermal drug delivery due to

their high flexibility and capacity to pass through the skin. Phospholipids with different compositions are used in varying amounts in ethosomal formulations. Ethosomes offer a potential solution to improve skin permeability and enhance transdermal drug delivery [7, 8].

Ethosomes are a novel and non-invasive drug transport system that allows drugs to penetrate the epidermal layers and reach systemic circulation. They are suitable for drugs with low membrane penetration and can incorporate hydrophilic, lipophilic, or amphiphilic drugs as shown in fig. 1. Ethosomes are soft vesicles made of phospholipids, water, and 20-45% ethanol and can be unilamellar or multilamellar [9, 10]. They act as a reservoir for drug delivery and provide sustained release. Ethosomes containing more ethanol have increased topical drug delivery, as ethanol is an effective penetration enhancer. Ethosomes are efficient transdermal and dermal drug delivery systems that allow drugs to enter deep skin layers or the systemic circulation. Ethosomes can improve drug delivery in both non-occlusive and occlusive conditions. Ethosomes have shown promise for the delivery of various proteins and peptides, making them a preferable option for delivery of these molecules [11, 12].

This is almost universally regarded as the main endpoint in adequately conducted studies [13]. Clinical cure and improvement are secondary objectives that are measured using a rigid scoring system based on clinical abnormalities in the nail apparatus. This therapy was time-consuming and costly, but it had a 90% success rate. As a result, in addition to oral administration, an alternative delivery method for Terbinafine that is effective against a variety of fungal illnesses is required. Transdermal application is one of the most popular methods for overcoming the disadvantages of oral delivery and delivering both systemic and topical benefits [14]. Except for lipophilic medications with a molecular weight of less than 500 Da, the stratum corneum serves as the main barrier in the transdermal route for drug distribution through the skin.

The skin's lipid bilayer can be disrupted by phospholipids and Ethosomes' high alcohol concentrations, which can make drugs

more permeable to the stratum corneum barrier. Ethosomes' unique ability to entrap weakly water-soluble medicines is employed to increase skin penetration. The goal of the current work was to entrap Terbinafine in vesicles to localise its distribution, enhance its solubility and availability at the application site for a

systemic effect, and lower the dose and GI side effects. The idea is that encapsulating Terbinafine in vesicles could be important for transdermal delivery of medication against fungi while simultaneously getting around oral delivery restrictions and reducing skin rashes [15, 16].

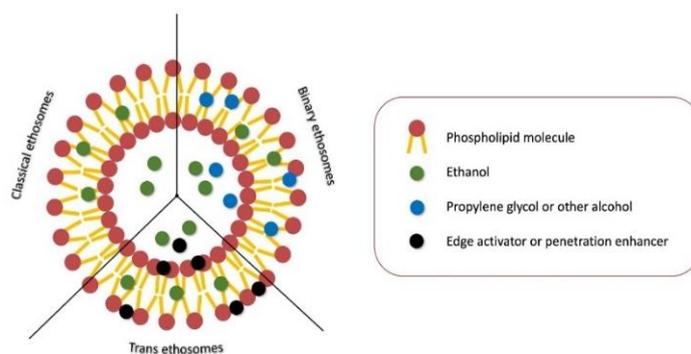


Fig. 1: Structure of ethosomes

In the current work, we have used a cold method procedure and a 3^2 -factorial design to optimise the various excipient concentrations in Terbinafine ethosomes. Soy lecithin and cholesterol concentrations were optimised using a 3^2 -factorial design to establish transdermal efficacy, and optimised Terbinafine ethosomes were integrated into the gel base and their characteristics were examined. Additionally, the stability, permeability, and *in vivo* permeability of terbinafine ethosomal gel were examined.

MATERIALS AND METHODS

Materials

Terbinafine was purchased from Max Med laboratories Pvt ltd. in Tamilnadu, India. SRL Chemicals provides soya lecithin, cholesterol, propylene glycol, carbopol 934P, and triethanolamine. Methanol was provided by Advent Chem Bio Pvt. ltd., and ethanol was provided by

Changshu Hongsheng Fine Chemical. All of the other chemicals used in this study are of analytical standard.

Method of preparation

Several formulations with varying compositions were created and used to optimise Terbinafine-loaded Ethosomes. The ethosomes were prepared using the cold method, followed by the ultrasonication method, as shown in fig. 2. Calculated amount of Soya lecithin, Cholesterol and Terbinafine was added to 10 ml ethanol in a covered vessel and was stirred using magnetic stirrer at 100-150 rpm at room temperature until all components dissolve and merge together to form a solution. At about 30 °C, 5 ml of Propylene glycol was added and after some time water was added dropwise for about 30-40 min to obtain a milky white suspension. Size reduction was done by using ultrasonication for 20 min [17, 18].

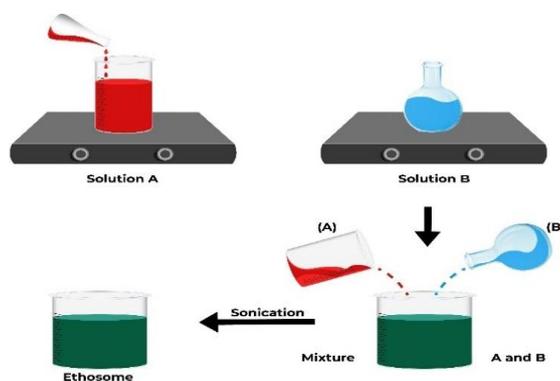


Fig. 2: Cold method preparation of ethosome

Optimization and design of experiments

Terbinafine-loaded Ethosomes were created using design-expert 12 software and a 3^2 -full factorial design. In this design, the best experimental performance with the fewest recurrences among nine experimental runs was determined by combining three dependent variables-EE% (Entrapment Efficiency) (Y1), Particle size (Y2) and *in vitro* diffusion (Y3)-with two independent variables-Soy lecithin (X1) and Cholesterol (X2).

Characterization of a drug-loaded ethosomal formulation

Entrapment efficiency

Using the ultracentrifugation method, the entrapment efficiency of Terbinafine ethosomes suspension kept overnight at 4 °C was

evaluated. To Eppendorf tubes containing PBS (Phosphate Buffer Solution), pH 7.4, the ethosomes formulation containing 1 ml of terbinafine was added. Samples were centrifuged for two hours at 15,000 rpm with a fixed-angle rotor. After being separated, the top clear layer was diluted with PBS pH 7.4 and examined with a UV-Visible spectrophotometer to determine absorbance at 223 nm.

$$\text{Entrapment efficiency \%} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100$$

Particle size

The laser diffraction method and a particle size analyser (DTS Version 5.03, Malvern) were used to determine the average diameter of sonicated vesicles.

SEM (Scanning Electron Microscopy)

The Ethosome vesicles were viewed through Quanta FEG. Images were captured on a scanning electron microscope after the materials were fully dried. (Thermoscientific Apreo S)

TEM (Transmission electron microscopy)

Terbinafine 1% aqueous solution was used to stain the samples negatively. Ethosomal solutions were dried on a tiny carbon-coated grid before staining. The excess solution was blotted away. After drying, the specimen was seen under a microscope at 10-100-fold enlargement (JEOL Japan's JEM-2100 Plus).

Zeta potential

The repulsion or attraction of charges between particles is quantified by the zeta potential. A Zeta potential analyzer (DTS Version 5.03, Malvern) was used to find the zeta potential at 25 °C. The mean zeta potential and electrophoretic mobility values were acquired directly from the measurement.

DSC (Differential Scanning Calorimetry)

Using a differential scanning calorimeter, terbinafine differential scanning calorimetric studies were conducted (Perkin Elmer). In the sample and control compartments of the furnace, 10 mg of the sample was added to an aluminium pan. Samples were heated to 400 °C while being continuously flushed with nitrogen gas at a rate of 5 °C/min and flow rate of 50 ml/min.

XRD (X-ray diffraction study)

An X-ray diffraction was used to conduct XRD study of terbinafine formulation. The samples were kept in a sample container, and the powder X-ray diffraction pattern was captured using an X-ray tube and a source of two 10-90° 2.2 KW copper anodes. The sample was checked with a lynx-eyed detector before being filtered with a Ni filter (Bruker D8Advance).

In vitro release study

In vitro drug release study was carried out using Dialysis bag diffusion technique (110 bag with a molecular weight cut-off of 3500 Da). The dialysis bag included a tightly sealed suspension of 2 ml (10 mg TH) customised ethosomes in 250 ml of phosphate buffer pH 7.4. On a hot plate digital magnetic stirrer, the entire diffusion cell setup was kept at 37 °C±0.5 °C with constant stirring at 100 rpm. Precise 5 ml aliquots of the samples were taken out of the media at intervals of 1h, 2h, 3h, and 8h and replaced with an equivalent volume of fresh medium. The absorbance of all the samples were measured by using UV Spectrophotometer at 223 nm and the drug release was calculated with the help of a calibration curve [19, 20].

Preparation of ethosomal gel

Three distinct batches of carbapol 940; 0.25g, 0.50g, and 0.75g, were placed in a beaker with 50 ml of distilled water. Solution was stirred using a magnetic stirrer for 2 h. 1 drop of triethanolamine was added to the above solution and made up to 100 ml with purified water; further, the solution was continuously stirred using magnetic stirrer in order to ensure that it is free of bubbles. The ethosomal formulation with high entrapment effectiveness and decreased vesicular size was centrifuged for 30 min at 4 °C at 12000 rpm to separate the ethosomal vesicles. The ethosomal sediment containing only the entrapped drug was collected and dispersed in the gel. Carbapol gel base was gently mixed to produce a total medication corresponding to 1%w/w Terbinafine [21, 22].

Characterization of formulation of ethosomal gel

Table 13 shows the results for gelling time, gelling capacity, and viscosity. For gelling ability, the grade++ was superior. Finally, it was decided that the ideal research vehicle would be 0.2 percent Carbapol 934 P/0.6 % HPMC.

In vitro permeation studies

Franz diffusion cell was used to conduct *in vitro* skin permeation studies. In this case, the dialysis membrane (in order to mimic the

skin) was kept in between the donor compartment and the receptor compartment. Further 1g of the gel (equivalent to 2 mg of medication) was added to the dialysis membrane. Phosphate buffer 7.4 was used as a medium and magnetic stirrer was used in order to ensure the temperature maintenance and uniform distribution of the drug. Around 1 ml of the samples were collected from the receptor compartment at 0.5h, 1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h, which was then replaced with equal volumes of medium [23, 24].

Total amount of the drug permeated through the dialysis membrane was calculated in mg/cm². The permeability coefficient was calculated by dividing the steady-state drug flux (mg/h/cm²) by the slope of the linear part of the curve.

Antifungal activity

Preparation of potato dextrose agar medium

The potato dextrose agar medium was prepared by dissolving 20 gm of potato infusion, 2 gm of dextrose and 1.5 gm of agar in 100 ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121 °C for 15 min. The autoclaved medium was mixed well and poured onto 100 mm petri plates (25-30 ml/plate) while still molten [25].

Petri plates containing 20 ml potato dextrose agar medium was seeded with 72 hr culture of fungal strain (*Candida albicans* and *Aspergillus niger*). Agar medium was drilled with holes and different concentration of gel (50, 100, 250 and 500 µg/ml) was added. The plates were then incubated at 28 °C for 72 h. The anti-fungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA) [26, 27].

RESULTS AND DISCUSSION

Material selection for the ethosomal formulation

The amounts of cholesterol and soy lecithin, which are essential building blocks for ethosomal vesicles, can affect particle size and entrapment effectiveness. Phospholipids are the primary components of the ethosomal membrane and contribute to the vesicle's stability and fluidity. Soybean phosphatidylcholine is a common ingredient. Soy lecithin can operate as a surfactant, lowering vesicle surface tension and enhancing their capacity to fuse with cell membranes, improving medication absorption. Soy lecithin has been extensively investigated in ethosomes and has shown promising results in improving drug delivery, particularly for topical and transdermal applications [28, 29].

Cholesterol is a type of lipid that can be used as a component of ethosomes, which are lipid-based vesicles used for drug delivery. Cholesterol is a key component of cell membranes and can improve the stability and integrity of ethosomes. Cholesterol can help to modulate the fluidity of the vesicle membrane, which can influence drug release and absorption. It can also enhance the stability of the vesicles. Surfactants can be used to stabilize the vesicles and improve their size distribution. Examples of surfactants commonly used in ethosomal formulations include Tween 80, Span 80, and sodium cholate [30, 31].

Overall, the selection of materials for ethosomal formulation depends on the specific application and properties of the active substance being delivered. A detailed understanding of the materials' physicochemical properties is required to assure the ethosomal carrier system's stability and efficiency.

Analysis of factorial design

To prepare an ethosomes suspension, extensive literature research and formulation trials are required. The quantity, concentration, and amount of soy lecithin used can all change the characteristics of the ethosomes formed, therefore, these are all regarded as essential elements in creating a stable and effective ethosomal system. Using a 3²-factorial experimental approach, the physicochemical characteristics of ethosomes loaded with terbinafine were ascertained.

Design of experiments

The following statistical model, which included interactive and polynomial terms, was used to evaluate the responses:

$$Y = \beta_0 + \beta_1 A + \beta_2 A^2 + \beta_3 AB + \beta_4 A^2 + \beta_5 B^2 + \beta_6 AB + \beta_7 AB^2$$

In this scenario, Y is the dependent variable, β_0 represents the arithmetic mean response across the nine runs, and β_1 represents the computed coefficient for factor A. The primary effects of the amounts of A and B demonstrate the average result when the components were altered one at a time from low to high values. The answer changes when two factors are adjusted at once, as shown by the interaction terms (AB). The results demonstrate a considerable influence of the independent factors on particle size and entrapment efficiency [32].

The 3² full factorial design offered 9 runs for two independent variables, soy lecithin and cholesterol, each at three different levels (high, medium, and low), in the experimental design. The results of the ANOVA are shown in tables 10, 11, and 12, and they show that each model was significant (p < 0.05) overall. Non-significant terms (p > 0.05) were removed from equations to simplify models, resulting in:

The relationship between the response model equation and particle size:

$$Y_1 = \beta_0 + \beta_1 (A) + \beta_2 (B) + \beta_3 (AB)$$

$$\text{Particle size (nm)} = -182.85000 * (A) + 197.78333 * (B) - 254.87500 * (AB)$$

As a result, the model equation for %EE changed to:

$$Y_2 = \beta_0 + \beta_1 (A) + \beta_2 (B)$$

$$\text{Entrapment Efficiency} = 77.14111 - 7.86500 * (A) + 18.97000 * (B)$$

The following was the model equation that related the percent maximum drug release as a reaction at the eighth hour:

$$Y_3 = \beta_0 + \beta_1 (A) + \beta_2 (B) + \beta_3 (AB)$$

$$\% \text{ Drug Release Maximum at 8th hour} = +49.16667 - 6.53000 * (A) + 4.56667 * (B)$$

According to the model equation above, a positive sign for a factor denotes a positive correlation or synergistic interaction with the responses, whereas a negative sign for a factor denotes a negative correlation or antagonistic interaction with the answers.

Table 1: Factors that influence formulation design

Factors	Coded values	Actual values ($\times 10^{-2}$ M)
Cholesterol (A)	-1	1
	0	2.5
	1	4
Soya lecithin (B)	-1	0.5
	0	1.25
	1	2

Characterization of terbinafine ethosomal formulation

Particle size

The particle size and zeta potential of ethosomes were examined using a zeta sizer, and the results are displayed in table 2. All experimental trials had particle sizes that ranged from 706 nm to 1433 nm.

Composition of the 3²-factorial terbinafine-loaded Ethosome

Response parameters based on the outcomes of the forecast profiler, the values for cholesterol and soy lecithin have been set at low 706.1 and high 1433.6, respectively. Cholesterol and soy lecithin are combined in a 2:1 ratio for the run that results in the lowest vesicle size. The interaction graph shows that the independent variables (A and B) interact strongly. In ANOVA table 3, the model's f-value of 5.619 suggests that it is significant. P-values for significant model variables are 0.0468 or below or less than 0.0500. The significance of the model term AB in this circumstance is therefore established, as well as the relationship between independent variables and the response (particle size) (fig. 3).

Entrapment efficiency

The formulations' entrapment effectiveness was assessed using the centrifuge technique. With an estimated 94.46% entrapment

efficiency, batch NET 3 achieved the best performance. The low and high values for soy lecithin and cholesterol are 62 and 94.46, respectively.

Model f-value of 56.768 in ANOVA table 4 indicates that the model is significant. The fact that the p-value was 0.0037-less than 0.0500-shows the importance of the model's parameters. In this case, the model term B is essential. It was developed by predicting how individuals will react to low (-1) and high (+) concentrations of a component.

According to the interaction graph, the significant interactions between Span 60 (A) and Cholesterol (B) account for the major response disparities. When viewed as response contours (two elements at once), the contour lines represent lines of equal response (fig. 4).

In vitro drug release studies

Terbinafine release from the formulations was studied using a Franz diffusion cell at 37 °C, and the amount of the drug released was quantified using a UV spectrophotometer. The drug release studies were completed for every trial run. Soya lecithin and cholesterol were established at 35.41 and 68.94, respectively, based on the predicted profiler's results.

Table 2: Composition of the 3²-factorial terbinafine-loaded Ethosome

Formulations	Particle size (nm) (Y1)	Entrapment efficiency (Y2)	In vitro studies (at 8 th h) (Y3)
NET1	933.4±0.23	76.5±0.23	68.94±0.26
NET2	1158.8±0.14	90.37±0.14	54.31±0.14
NET3	1678±0.31	94.46±0.43	51.27±0.16
NET4	759.7±0.66	62±0.15	58.24±0.66
NET5	1433.6±0.54	89.09±0.42	43.21±0.28
NET6	1236.7±0.48	93±0.28	52.78±0.21
NET7	981±0.36	42.86±0.43	35.41±0.34
NET8	977±0.87	79.85±0.16	46.21±0.36
NET9	706.1±0.94	91.43±0.18	54.72±0.22

Data are expressed as mean±SD (n=3)

Table 3: ANOVA results for particle size

Source	Sum of	df	mean	F-value	p-value
Particle size (nm) (2FI)					
Model	6.131E+05	3	231719.8	5.61918	0.0468*
A-Soyalecithin	2.309E+05	1	200604.7	5.59492	0.0643
B-Cholesterol	1.494E+05	1	234709.5	4.10104	0.0998
AB	2.598E+05	1	259845.1	2.59801	0.0444
*Significance					
R ² Value - 0.7					

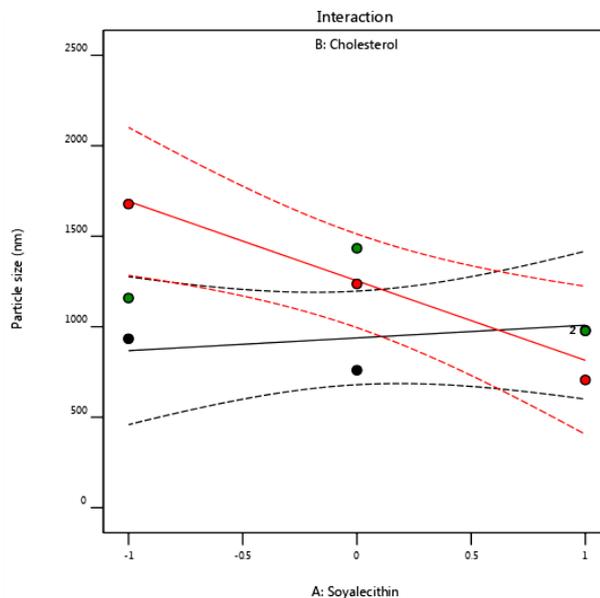


Fig. 3: Effect of factor A and B on particle size

Table 4: ANOVA results for entrapment efficiency

Source	Sum of	df	Mean	F-value	p-value
EE (Linear)					
Model	2389.315	5	477.80	56.76816	0.0037*
A-Soyalecithin	371.1494	1	371.1494	43.61897	0.0071
B-Cholesterol	1585.165	1	1585.165	186.26734	0.0009
*Significance					
R ² Value - 0.98					

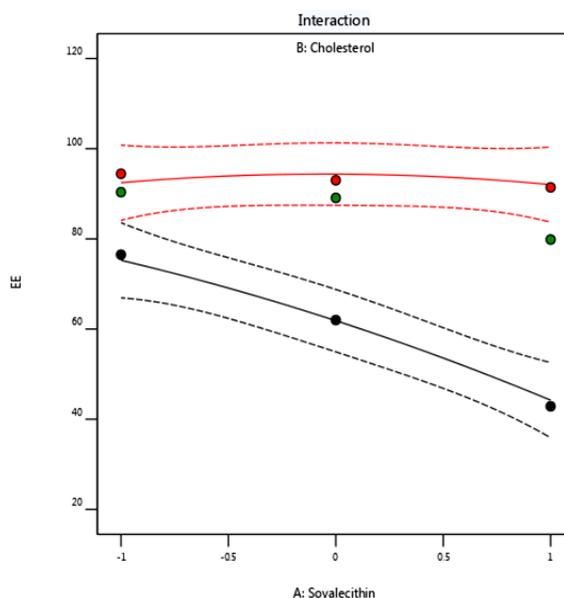


Fig. 4: Effect of factor A and B on entrapment efficiency

The ANOVA table 5 demonstrated that the model was significant and that the model terms were significant with a model f-value of 7.11 and a p-value of 0.0297. The interaction graph showed that the AB

interaction was a major model term in particular. The three factors were visualised simultaneously by the response contours, which were represented by the contour lines (fig. 5).

Table 5: ANOVA results of percentage drug release at 8th h

Source	Sum of	df	Mean	F-value	p-value
% DR Max at 8 th h (Linear)					
Model	587.26	2	195.75	7.11033	0.0297*
A-Soyalecithin	242.954	1	242.95	8.83226	0.0311
B-Cholesterol	2.43	1	2.43	1.688406	0.7782
*Significance					
R ² Value - 0.81					

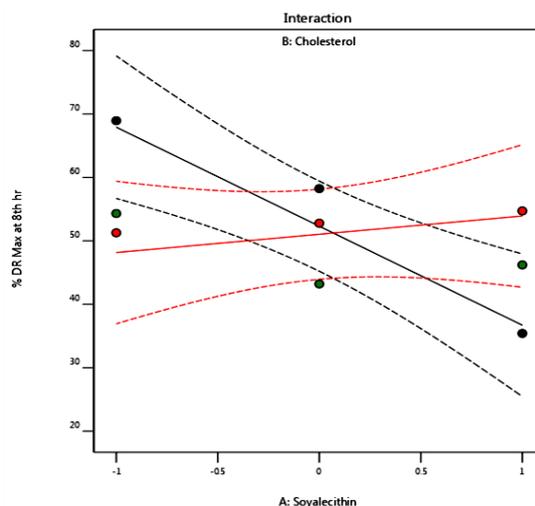


Fig. 5: Effect of factor A and B on *in vitro* drug release

Outcome of soya lecithin and cholesterol

Effect of soya lecithin concentration on entrapment efficiency and particle size

The particle size and entrapment effectiveness of a product can be greatly affected by soy lecithin. Soy lecithin functions as an emulsifying agent, lowering the surface tension between immiscible phases to enhance the stability and homogeneity of emulsions and dispersions. Soy lecithin can influence the particle size by affecting the rate of nucleation and growth of particles during production. Higher concentrations of soy lecithin can lead to smaller particle sizes due to increased surface activity and better stabilization of the emulsion or dispersion. This is seen when soy lecithin content is changed from level 1 to level 0. Soy lecithin can also alter entrapment effectiveness or the proportion of active chemicals that are contained within the particle. The presence of soy lecithin can create a lipid bilayer around the active ingredient, which can improve the solubility and stability of the active ingredient and prevent its degradation or release during storage or administration [33].

However, the effect of soy lecithin on particle size and entrapment efficiency can be influenced by other factors such as the type and concentration of active ingredient, the manufacturing process, and the formulation conditions. Therefore, it is important to carefully consider the specific application and requirements of the product when using soy lecithin as an emulsifying agent [34].

Influence of cholesterol levels on particle size and entrapment effectiveness

When creating ethosomes, which are lipid-based nanoparticles used for drug delivery, cholesterol is frequently used as a structural component. The efficiency of ethosome entrapment and the particle

size are both influenced by the amount of cholesterol in the formulation [35].

The size and stability of the particles may be impacted by cholesterol's effects on the ethosomes lipid bilayer's packing and fluidity. Due to the lipid bilayer's greater stiffness, higher cholesterol concentrations can cause a reduction in particle size. The efficacy of drug increased as cholesterol concentration was raised from -1 to 0 levels. Highcholesterol levels make it difficult for a gel to go into a liquid phase, making the resulting bilayers more rigid. The phase transition temperature can be eliminated, stable vesicles can be formed, and medication leakage can be stopped by using a low concentration of cholesterol at level 1 [36].

Based on the observations, it was determined that the chosen ethosomal formulation, which was optimised (Soya lecithin: Cholesterol 5:1), had high drug EE%, tiny particle size, and a higher percentage of drug release at eight hours, as well as having spherical shaped vesicles.

Optimized formulation Soya lecithin (A): Cholesterol (B) 5:1-EE%: 62%; Particle Size: 759.7%; Maximum drug release at 8h: 58.24%.

The hypothesised regression model was real and statistically significant according to ANOVA performed at significance level of 5%, as shown in table 3 and 10. The EE% of Terbinafine Ethosome, particle size, and drug release at the eighth hour were all significantly impacted by the type of Soy lecithin cholesterol ratio and its interaction, it was also discovered. Fig. 6, 7, and 8's surface response curve depicts the proportional impact of various process parameters on particle size, entrapment effectiveness, and drug release at the 8th h.

Optimization of formulation of terbinafine loaded ethosome

In the current study, a desirability function was used to maximize both responses at the same time using numerical optimization approach in

the Design-Expert software. As a result, a comprehensive grid search over the domain was used to identify the optimum desirability value

using Design-Expert software. The final optimized formula was obtained after evaluating all the parameters as seen in table 6.

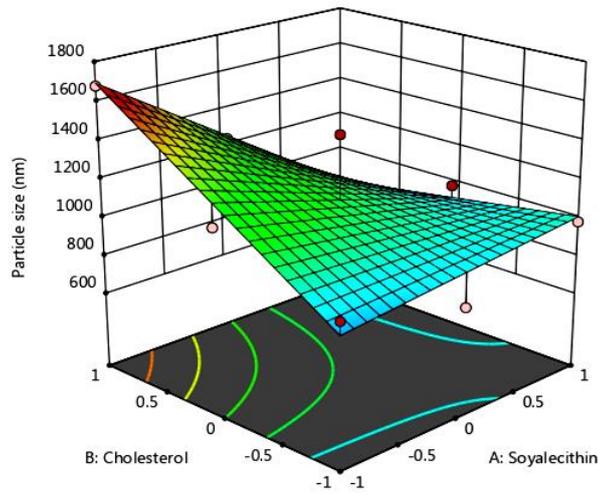


Fig. 6: Three-dimensional surface response plot demonstrating the relative effect of distinct process parameters on particle size

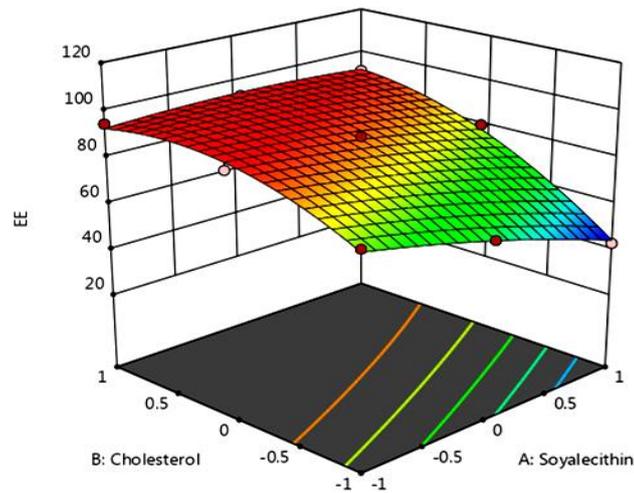


Fig. 7: A plot in three dimensions shows the relative impact of different process parameters on %EE

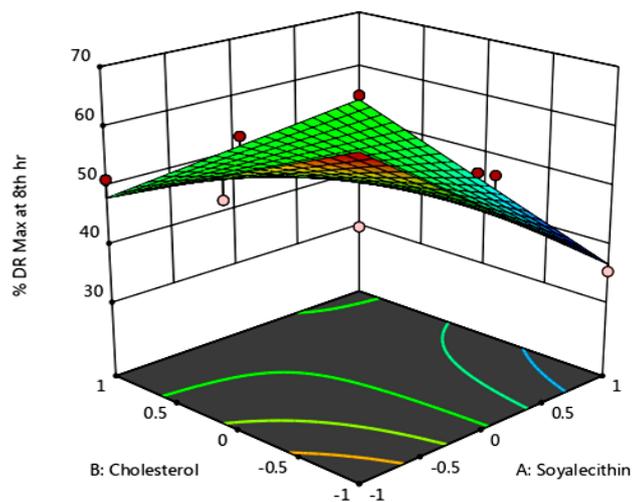


Fig. 8: Three-dimensional surface response plot illustrating the relative impact of several process parameters on drug release at 8 h

Table 6: Optimized ethosome factors

Ethosome factors	Level	Actual value ($\times 10^{-2}$ M)
Soya lecithin	0.3092	2.5
Cholesterol	0.8615	0.5

Table 7: Validation of the predicted optimized ethosomes

Response	Predicted Mean	Observed Mean
Entrapment Efficiency	94.0365	94.46 \pm 0.43
% Drug Release at 8 th hour	51.6233	51.27 \pm 0.16
Particle Size (nm)	1107.7	1207 \pm 0.14

Data are expressed as mean \pm SD (n=3)

The optimised ethosomal formulation's particle size and zeta potential are evaluated using a zeta sizer. The Zeta potential was

discovered to be 1.18mV, while the particle size was measured to be 1207 nm (fig. 9 and 10).

	Mean (mV)	Area (%)	St Dev (mV)
Zeta potential (mV): 1.18	Peak 1: 1.18	100.0	3.44
Zeta Deviation (mV): 3.44	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm): 0.321	Peak 3: 0.00	0.0	0.00

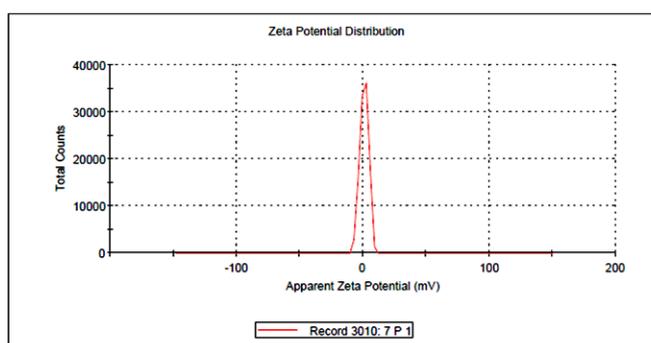


Fig. 9: Zeta potential of the optimized formulation

	Size (d.n...)	% Intensity:	St Dev (d.n...
Z- average (d.nm): 1207	Peak 1: 1281	100.0	164.5
Pdl: 0.395	Peak 2: 0.000	0.0	0.000
Intercept: 0.860	Peak 3: 0.000	0.0	0.000

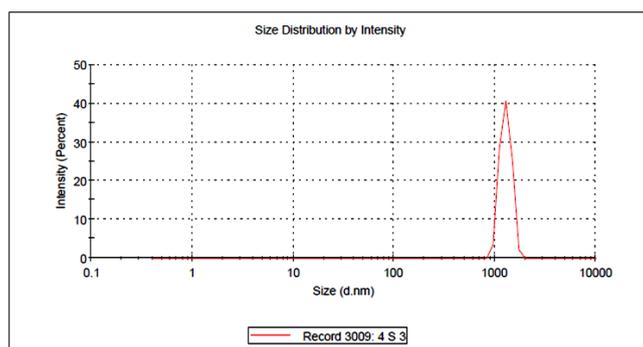


Fig. 10: Particle size of the optimized formulation

SEM and TEM analysis

The optimized formulation micrographs by scanning electronic microscopy revealed spherical droplets at the nanometre scale. The findings show that the particles had a spherical shape and that no drug crystals were found inside of them (fig. 11).

Transmission electron microscopy has been used to identify the size, morphology, and form of ethosomes (TEM).

According to the image it indicates that Terbinafine is distributed fairly and primarily spherical (fig. 12).

Differential scanning calorimetry and X-ray diffraction study

A method known as DSC (differential scanning calorimetry) is used to analyse a drug's physical condition, thermal behaviour, and potential interactions with other substances. Terbinafine was found to have a melting point of 199.32 °C and an endothermic peak of 208.68 °C. Terbinafine, cholesterol, and soya lecithin's physical mixture had an endothermic peak at 201.48 °C. Based on the findings, it was determined that all other excipients, including soy lecithin and cholesterol, were optimally suitable due to their compatibility with the medication, as seen in (fig. 13).

When compared to the improved formulation, Terbinafine's XRD pattern was strong, distinct, and consistent with crystalline powder, as opposed to the improved formulation's peak loss and more diffuse peaks (fig. 14). This illustrates the transition from Terbinafine's crystalline form to its amorphous form.

In vitro drug release kinetics

The ethosomal formulations NET1–NET9's cumulative % release was displayed in the table 2. The range of ethosomal formulations'

cumulative percentage drug release was 35.41% to 68.94%. The modified formulation has zero-order kinetics for release.

The drug release is proportional to the square root of time, as shown by Higuchi's correlation coefficient, showing that the release of terbinafine is diffusion-controlled. The n value for the ethosomal formulation of terbinafine in the Korsmeyer Peppas model was 0.9, which is consistent with anomalous or non-fickian diffusion. (table 8).

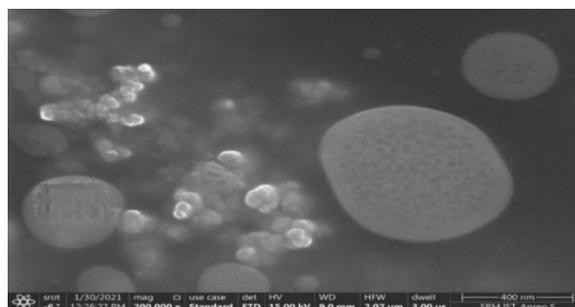


Fig. 11: SEM analysis of optimized terbinafine formulation

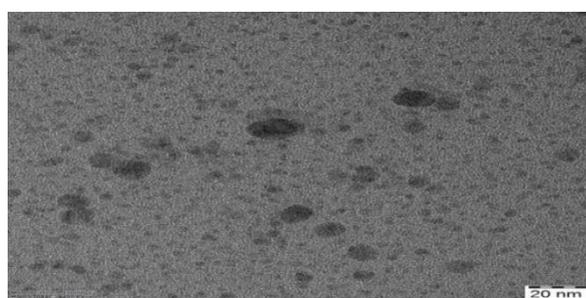
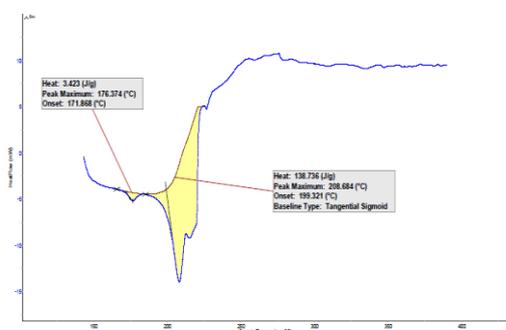
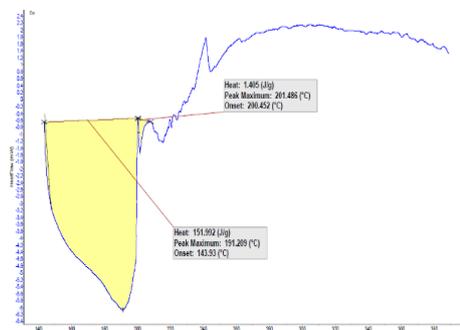


Fig. 12: TEM analysis of optimized formulation of terbinafine

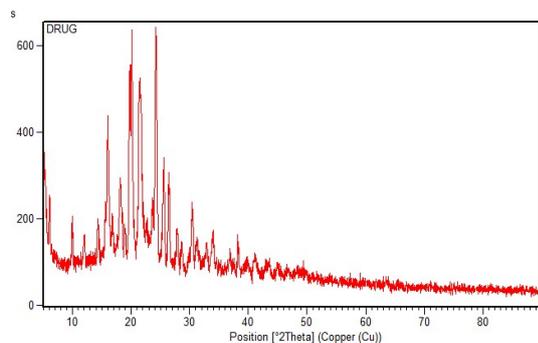


(a)

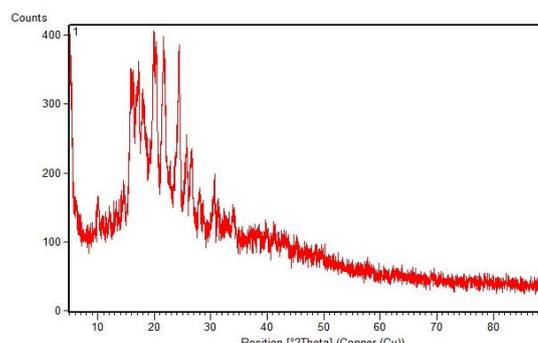


(b)

Fig. 13: DSC thermograms of a) Terbinafine b) Terbinafine, soya lecithin and cholesterol



(a)



(b)

Fig. 14: XRD of a) Terbinafine b) Optimized formulation

Table 8: Optimised terbinafine loaded ethosomal formula release kinetics

Formulation	Higuchi	Korsemeyer peppas	Zero order	First order	Hixson-crowell
	r ²	n	r ²	r ²	r ²
Optimized formulation	0.9791	0.9	0.9982	0.9978	0.973

Formulation and characterization of terbinafine loaded carbopol gel

The pH, Spreadability, and viscosity of F8 ethosomal formulation were examined in EG1, EG2, and EG3 gel formulations with various Carbopol 934P concentrations. The developed gel showed pH 6.8

non-irritancy at room temperature and Spreadability of 14.5 g cm/sec, which is an indication of a simple gel spreading with the application of light shearing force. At 25 rpm, the prepared gel had the appropriate viscosity of 3.37±0.21 PaS. The ethosome-loaded gel had the desired properties, according to a comprehensive investigation [37, 38].

Table 9: Evaluation parameters of ethosomal gel

Terbinafine gel formulation	pH	Gelling capacity	Viscosity PaS
EG1	6.7 ±0.6	+	3.02 ±0.03
EG2	7.0 ±0.3	+++	3.37±0.21
EG3	6.9 ±1.1	++	3.11 ±0.14

Data are expressed as mean±SD (n=3)

In vitro permeation study

The developed optimised ethosomal terbinafine loaded gel EG2, which contains 0.5g of Carbopol 934 P, was selected as the optimised formulation since it has an acceptable viscosity gelling ability.

The Terbinafine gel, which was based on carbopol 934 P, constituted a straightforward aqueous solution with terbinafine scattered throughout the matrix. Through permeation studies using a straightforward drug-loaded gel, the permeability capabilities of terbinafine are ascertained.

The total amount of medication absorbed over the duration of 1 to 8 hours is displayed in the table 10. The flux as well as the permeability coefficient of the samples is given in table 11. It was discovered that the flux Jss for the gel was 0.0018 (mg/cm²/min). The enhanced gel's permeability coefficient was discovered to be 0.0009 cm²/min. It was discovered that the formulation had higher

flux and permeability coefficients, showing that ethosome gel with Carbopol had increased drug release and permeability coefficient [39, 40].

Table 10: Cumulative % drug permeation

Time (h)	Cumulative % drug permeation
1	9.32 ±0.78
2	17.42 ±0.18
3	22.5 ±0.34
4	31.43 ±0.48
5	40.12 ±0.86
6	48.32 ±0.15
7	52.8 ±0.87
8	60.74 ±1.36

Data are expressed as mean±SD (n=3)

Table 11: Optimised terbinafine-oaded ethosomal gel formulation's total drug permeation

Formulation	Flux (Jss) mg/cm ² /h	Permeability coefficient (Kp) cm ² /h
Optimized Formulation	0.0018	0.0009

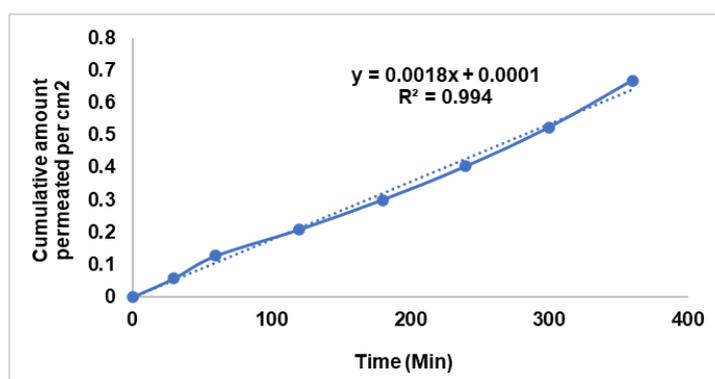


Fig. 15: Amount of drug permeation of terbinafine optimised gel formulation

Anti-fungal activity

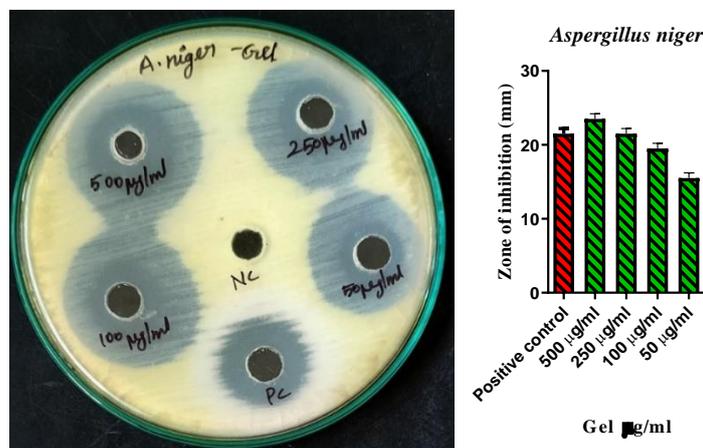
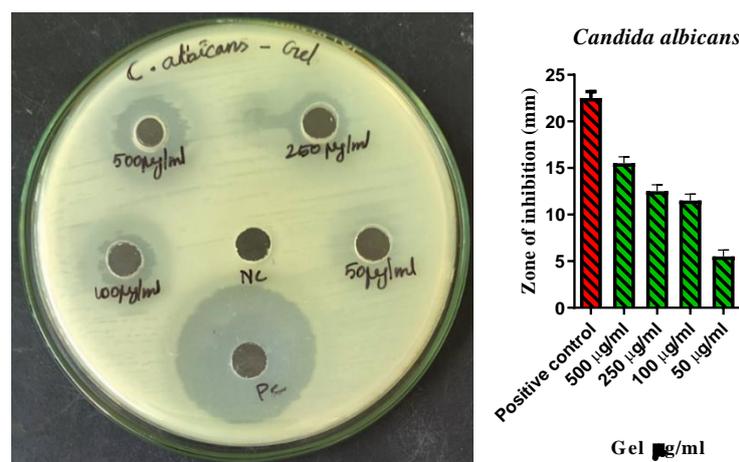
By using the well diffusion technique, the *in vitro* antifungal efficacy of the Terbinafine-loaded ethosomal formulation is examined. As a positive control, amphotericin B is utilised. The table shows the zone of inhibition of Gel sample in two different fungal strains. The fungal strains used are *Aspergillus niger* and

Candida albicans. The formation of the zone of inhibition is observed clearly around the wells containing the Terbinafine-loaded ethosomal gel. Terbinafine loaded ethosomal gel shows better activity when compared to that of the Positive control. The values of the Terbinafine-loaded ethosomal gel's zone of inhibition against fungi are listed in the table 12 and shown in the fig. 15 and 16.

Table 12: Mean zone of inhibition obtained by sample gel against *Candida albicans* and *Aspergillus niger*

S. No	Name of the test organism	Name of the test sample	Zone of inhibition (mm)				
			500 $\mu\text{g/ml}$	250 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$	Positive control (10-20 $\mu\text{g/ml}$)
1.	<i>Aspergillus niger</i>	Gel	23.5 \pm 0.7	21.5 \pm 0.7	19.5 \pm 0.7	15.5 \pm 0.7	21.5 \pm 0.7
2.	<i>Candida albicans</i>		15.5 \pm 0.7	12.5 \pm 0.7	11.5 \pm 0.7	5.5 \pm 0.7	22.5 \pm 0.7

Data are expressed as mean \pm SD (n=3)

Fig. 16: Effect of sample gel against *Aspergillus niger*Fig. 17: Effect of sample gel against *Candida albicans*

Stability studies

When the ethosomal vesicle formulations were tested three months later, it was discovered that they had greater drug retention than formulations kept at room temperature. A detailed examination revealed that as temperature rises, the lipid bilayer becomes more fluid, leading to vesicle leakage. When kept at room temperature,

Ethosomal gel was significantly more stable than Ethosomal solution, as evidenced by the drug entrapment estimation results in table 13.

According to the aforementioned findings, ethosomal suspension's size and entrapment effectiveness showed negligible changes at refrigeration temperature, whereas vesicle size did not change significantly at 25 °C [41].

Table 13: Stability studies data for optimized Terbinafine loaded Ethosome

Storage conditions	Duration (Month)	Particle size (nm)	Entrapment efficiency (%)
4 °C \pm 1 °C	1	1207 \pm 1.24	93.23 \pm 0.03
	2	1219 \pm 2.18	92.03 \pm 0.09
	3	1227 \pm 3.08	91.87 \pm 0.11
25 °C \pm 2 °C / 60 \pm 5% RH	1	1191 \pm 3.60	88.21 \pm 0.26
	2	1188 \pm 4.02	87.96 \pm 0.31
	3	1187 \pm 5.88	86.21 \pm 0.34

Data are expressed as mean \pm SD (n=3)

Table 14: Stability studies for terbinafine loaded ethosomal gel

Storage conditions	Duration (Month)	pH	Viscosity (Pa. S)
4 °C± 1 °C	1	6.6 ±0.29	3.23 ±0.31
	2	6.4 ±0.12	3.00 ±1.20
	3	6.3 ±0.16	2.98 ±0.79
25 °C±2 °C/60+5% RH	1	6.0 ±3.60	3.37 ±0.21
	2	5.7 ±0.39	3.00 ±0.49
	3	5.0 ±0.49	2.98 ±0.34

Data are expressed as mean±SD (n=3)

CONCLUSION

The Terbinafine loaded ethosome was prepared using the cold method, focusing on soy lecithin and cholesterol variables to determine particle size and entrapment effectiveness. The ethosome was then confirmed through SEM and TEM analysis, revealing stable formulations with better percentage entrapment efficiency. The optimal soya lecithin and cholesterol ratio resulted in high entrapment efficiency. The particle size ranged from 706 to 1433 nm. Statistical analysis using ANOVA and regression model was used to create a mathematical model. The optimized formulation underwent *in vitro* antifungal experiments, showing improved activity against fungal strains. The well diffusion technique was used, with the gel showing the highest activity against *Aspergillus niger*. The optimization approach 3² factorial design proved helpful in determining developmental goals and evaluating variables' effects by pre-determining the ratios of soya lecithin and cholesterol. The research findings suggest Terbinafine Ethosomal gel could be used as an anti-fungal delivery system in the future. However, further studies are required to prove the same.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally

CONFLICTS OF INTERESTS

The authors declare no conflict of interest

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