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Box–Behnken design for the development of fluconazole-loaded classical ethosomes

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Abstract

Introduction: Ethosomes are soft and flexible vesicles mainly composed of phospholipids, ethanol and water. The presence of a high amount of ethanol ensure deeper drug penetration; however, an optimal formulation is necessary. This study aims to develop and characterize Fluconazole loaded classical ethosomes using Box-Behken design, in order to achieve to an optimal formulation having a minimal vesicle size, low polydispersity index, high zeta potential and good entrapment efficiency (% EE).

Methods: Fluconazole ethosomes were prepared using cold method and tested for vesicle size, polydispersity index, zeta potential and EE%. Box-Behken design was created using Design Expert® Software, where the impact of sonication time and amount of ethanol and soybean lecithin on resulting formulation were investigated.

Results: It was determined that increasing the concentration of ethanol up to an optimized limit reduces vesicle size and improves % EE. It was also observed that soybean lecithin concentrations affected positively vesicle size but negatively % EE. Whereas sonication time had an inverse effect both on, vesicle size and EE%**.** All prepared formulations showed a low polydispersity index and a good zeta potential indicating homogeneity and high stability. Therefore, the optimal formulation had % EE of 80.05±0.306 % and vesicular size of 226.501±5.34 nm with polydispersity index of 0.487±0.0078.

Conclusion: In summary, using Box-Behnken design can enhance the understanding of the correlations between the variables involved in ethosome formation and their effects on vesicle size, polydispersity index and % EE. The optimal formulation obtained can be incorporated into drug delivery systems to enhance skin permeation and antifungal activity.

Keywords: Ethosomes; Fluconazole; Cold method; Box-Behken design.

1. Introduction

Ethosomes are new non-invasive drug delivery systems and are currently the subject of extensive research, their size ranges from a few tens of nanometers to microns [1]. They are soft and flexible vesicles primarily composed of phospholipids, ethanol and water. The presence of a high amount of ethanol in their structure distinguishes them from other vesicular systems and allows them to alter the very dense alignment of lipid bilayers in the stratum corneum (SC), thereby ensuring deeper drug penetration [2]. Ethanol also imparts a net negative charge to the surface of the vesicles, providing them with improved stability due to electrostatic repulsion [3]. Additionally, ethosomes are less toxic and

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cause less skin irritation, making them suitable for transdermal delivery [4], they are easy to prepare, stable and safe to use, making various ethosomal preparations currently available on the market [5, 6]. However, it has been observed that when the ethanol concentration exceeds a specific level during the synthesis of ethosomes, it causes a subsequent increase in particle size, making the vesicular membrane more permeable, which can lead to reduced entrapment efficiency (% EE) and decreased stability of the ethosomes [4]. To achieve this, an optimal formulation of ethosomes is necessary to synthesize ethosomes with improved physicochemical properties, this is why statistical designed studies should be conducted under well-defined conditions. Among the currently available experimental design methods, Box-Behnken Design (BBD), it is a robust model of response surface methodology that evaluates the influence of multiple individual variables involved in an experiment with a minimal number of experimental trials, which not only saves time but also reduces costs related to experimentation [7]. Subsequently, two-dimensional graph (2D) contour plots and three-dimensional graph (3D) response surface plots are generated to analyze the correlation between the selected variables (both independent and dependent) and thus determine the appropriate experimental conditions for the best possible formulation.

Considering the challenges associated with Fluconazole, including its formulation in conventional pharmaceutical forms, the potential side effects and the risk of resistance development, recent approaches have focused on new transdermal delivery methods for antifungal drugs [1, 8]. These methods involve vesicular delivery systems like ethosomes, which can offer prolonged drug release, thereby minimizing side effects, reducing dosing frequency and enhancing treatment adherence[9].

This study aims to develop and characterize conventional ethosomes loaded with Fluconazole using a Box-Behnken design with three factors and three levels, in order to study the correlations between the independent variables (ethanol concentration, soybean lecithin concentration and sonication time) and the dependent variables (vesicle size, polydispersity index (PDI), zeta potential and % EE). The objective of this study is to achieve an optimal formulation characterized by a minimal vesicle size, low PDI, high zeta potential and good % EE of Fluconazole.

2. Material and methods

2.1. Method of development of Fluconazole-loaded ethosomal formulations

Ethosomes were prepared using the cold method [10] with absolute ethanol (VWR Chemicals, France) [25–45% (v/v)], soybean lecithin (AROMA-ZONE, France) [2–5% (w/v)], cholesterol (Alfa Aesar, USA) at 1% (w/v), Fluconazole (provided by MERINAL Laboratories, Algeria) 0.5 % (w/v) and distilled water. Soybean lecithin, cholesterol and Fluconazole were dissolved in ethanol while stirring vigorously with a magnetic stirrer (VELP Scientifica, Italy). The mixture was heated to 30 °C in a water bath for 10 minutes. Subsequently, distilled water heated at the same temperature was added slowly while the mixture was stirred at 700 revolutions per minute (rpm) for 15 minutes in a closed vessel (to prevent ethanol evaporation). After this, stirring continued for an additional 5 minutes at room temperature. The formed ethosomal suspension was then subjected to sonication using an ultrasonic sonicator (Vibra-Cell™, Sonics, USA) to reduce vesicle size. Sonication time applied were 5, 10, or 15 minutes (5 minutes per cycle) to avoid damaging the structure of the ethosomes [11]. Finally, the formulation was stored in the refrigerator at 4°C [12]. The optimized Fluconazole-loaded ethosomal formulation was selected and characterized for further studies.

2.2. Optimization of Fluconazole-loaded ethosomal formulations using Box-Behken Design

Several studies have revealed that the concentrations of ethanol, soybean lecithin and sonication time significantly affect particle size, PDI and % EE. In this work, both the individual and interactive effects of these variables on the characteristics of the ethosomal formulation were investigated.

Design Expert® Software was used for generating and evaluating the statistical experimental design, one of the sub-categories of experimental design methodology, the BBD. It helps in developing higher-order models that require fewer trials compared to other factorial designs. The BBD with three factors and three levels is one of the most suitable optimization techniques in response surface methodology. This method is ideal for exploring second-order polynomial models of quadratic response surfaces [7]. The selected independent and dependent variables for this design are listed in Table 1. Indeed, seventeen Fluconazole ethosomal formulations were developed and analyzed, according to Table 2 (A), vesicle size (Y1), PDI (Y2) and % EE (Y3) were selected as dependent variables (responses), while ethanol concentration (A) (%), soybean lecithin concentration (B) (%) and sonication time (C) (min) were selected as independent factors, keeping the Fluconazole and cholesterol concentrations constants.

An ANOVA test was conducted to evaluate the significance level of the tested factors on the selected responses as well as the interactions between these factors. The application of regression analysis is crucial to verify whether independent variables have a significant effect on responses. The 3D surface response graphs and 2D contour plots were also presented to show the effect of the independent variables on responses while keeping one of the independent factors constant.

Finally, to select the optimal formulation for further studies, the desirability function, which predicts the optimal levels of response variables, was calculated. The principle for choosing the optimal formulation was to achieve the smallest particle size, the lowest PDI, the highest absolute zeta potential and the highest % EE.

Table 1 List of independent and dependent factors used in the Box-Behnken design for the development of Fluconazole-loaded ethosomes

Factors	**Levels**		
Independent variables	**Minimum**	**Average**	**Maximum**
A= Ethanol (%)	25	35	45
B= Soy lecithin (%)	2	3.5	5
C= Sonication time (min)	5	10	15
Dependent variables (responses)	Target		
Y1= Particle size (nm)	Minimum		
Y2= PDI	Minimum		
Y3= EE (%)	Maximum		

2.3. Characterization

2.3.1. Particle Size, PDI and Zeta potential

The vesicle size, PDI and zeta potential of the Fluconazole-loaded classical ethosomes were measured using a Zetasizer (Malvern Instruments, United Kingdom) at 25 ± 1 °C. The samples were diluted with distilled water and then stirred before measurement. The mean and standard deviation of the measurements for three sample batches were reported in Table 1.

2.3.2. Entrapment efficiency (% EE)

First, maximum wavelength (λ_{max}) for pure Fluconazole was determined by scanning solution of Fluconazole in pure state in UV range from 200 to 400 nm against blank. An entrapment efficiency assessment of the developed formulations was conducted using an ultracentrifuge (Sigma 2-16 K, Germany). A total of 2 mL of each developed formulation was taken and centrifuged for 45 minutes at 15 000 rpm while maintaining the temperature at 4°C (refrigerated centrifuge). After centrifugation, the supernatant was collected and diluted. Subsequently, the free Fluconazole was analyzed using UV-visible spectroscopy (Optizen, South Korea) at a λ_{max} of 250 nm. The equation **(1)** was used to determine the % EE as follows:

$$EE\% = \frac{\text{Total Fluconazole - Non encapsulated Fluconazole}}{\text{Total Fluconazole}} \times 100 \qquad \textbf{(1)}$$

3. Results and discussion

To obtain the optimal formulation, the BBD generated a total of seventeen experimental runs. The results of these experiments, summarized in Table 2 (A), showed variations in vesicle size (Y1) ranging from 187.4 ± 1.2 nm to 334.9 ± 6.8 nm, PDI (Y2) ranging from 0.199 ± 0.02 to 0.576 ± 0.02, % EE (Y3) ranging from 72.44 ± 3.5 % to 80.23 ± 1.1 % and zeta potential from -15.5 ± 0.4 mV to -40 ± 0.3 mV.

It was noted that the quadratic model best fits the data from the seventeen formulations. This suggests that the relationship between the studied factors and the responses is likely non-linear. Table 2 (B) shows the R^2 values, standard deviation and coefficient of variation (CV) for the three responses, which help assess the quality of the model

fit and the variability of the responses across the different formulations. The model was found to be significant (p-value < 0.0001) for three responses; however, it was found to be non-significant (p-value > 0.0001) for the zeta potential, indicating that regardless of variations in the independent factors, the zeta potential remained within standard ranges. Therefore, it was excluded from the BBD model.

The 2D contour plots shows the relationship between independent variables and dependent variables (Figure 1).

Table 2 Observed responses for Fluconazole-loaded ethosomal formulations during development and optimization (A). Results of statistical and regression analyses produced by Box-Behken Design for all responses (B)

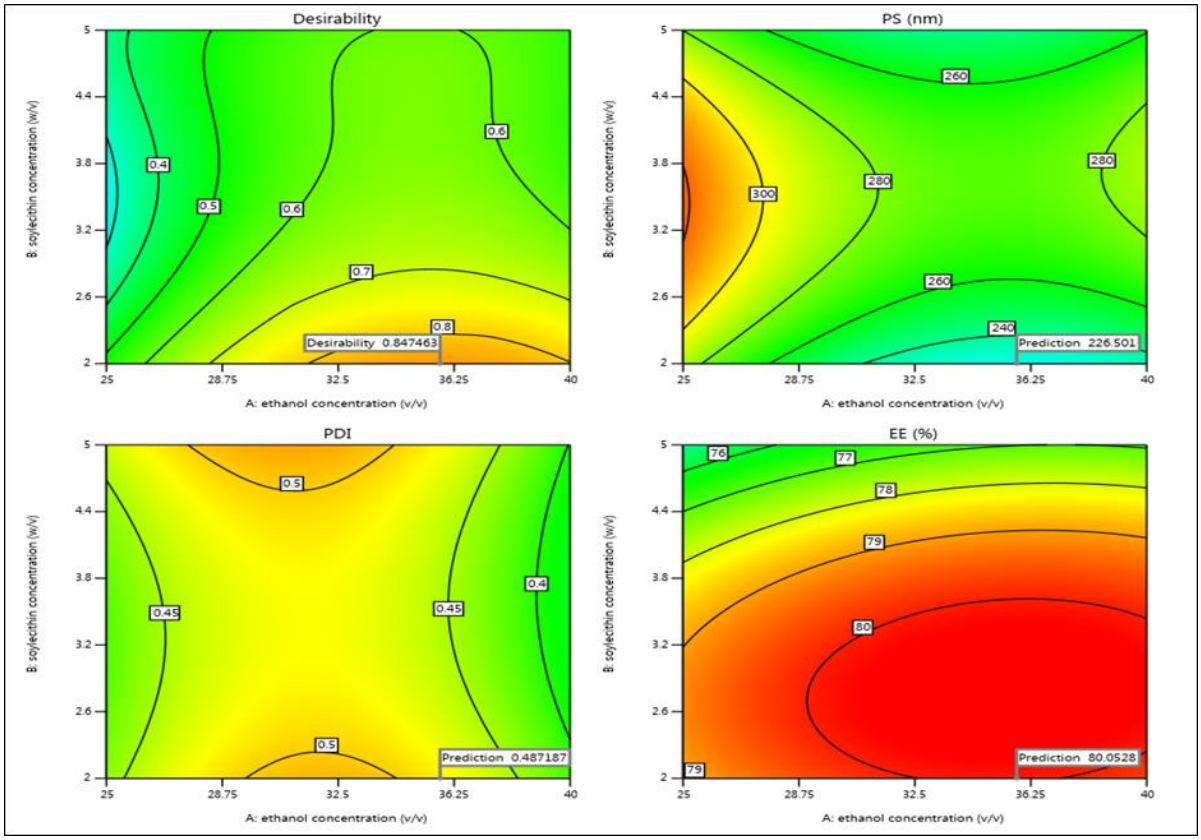
(A)

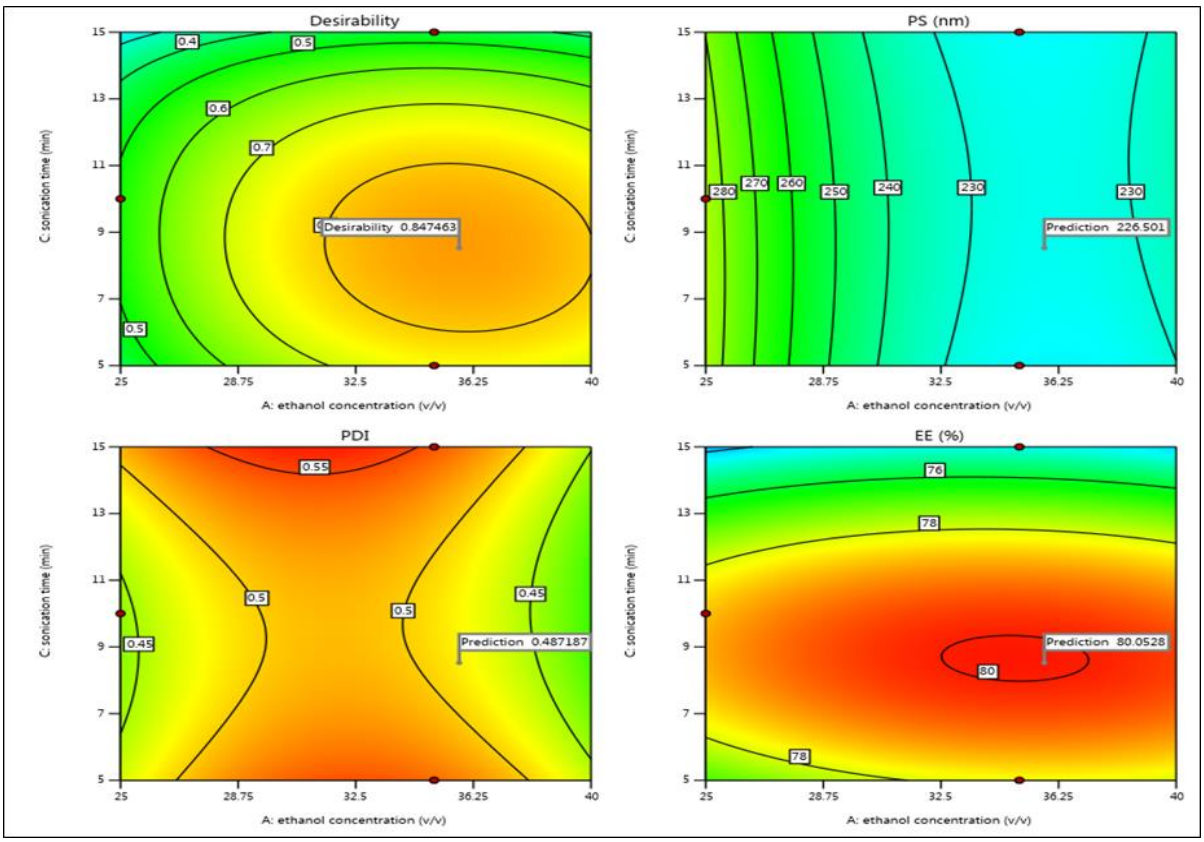
	Independent variables			**Dependent variables**			**PZ**
Formulation code	**A**	**B**	**C**	**Y1**	**Y2**	**Y3**	
F1	35	3.5	10	269.9±5.1	0.445±0.02	80.23±1.1	-16.6±0.3
F2	45	2	10	264.75±4.7	0.26±0.03	78.8±1.3	-29.6±0.2
F3	45	5	10	296.8±0.8	0.213±0.07	75.9±2.1	-15.9±0.6
F4	35	3.5	10	262.3±1.5	0.458±0.06	79.97±0.9	-20.3±0.4
F5	45	3.5	5	325.45±7.1	0.282±0.03	77.39±1.8	-18±0.1
F6	45	3.5	15	299.05±3.3	0.199±0.02	73.51±3.1	-15.9±0.4
F7	35	3.5	10	271.8±4.5	0.45±0.08	73.51±3.3	-21.45±0.1
F8	25	2	10	280±4.1	0.435±0.01	78.97±0.8	-29.6±0.3
F9	35	5	15	187.4±1.2	0.464±0.02	72.44±3.5	-36±0.05
F10	35	3.5	10	270.9±3.9	0.451±0.006	79.81±1.5	-40±0.3
F11	25	5	10	269.75±3.3	0.444±0.003	74.97±2.8	-20.5±0.5
F12	25	3.5	5	334.9±6.8	0.473±0.08	76.52±2.8	-18.5±0.5
F13	35	3.5	10	269.05±7.1	0.451±0.05	79.84±1.0	-15.5±0.4
F14	35	2	15	226.7±2.4	0.543±0.05	74.32±3.2	-17.9±0.05
F15	25	3.5	15	295.5±3.1	0.464±0.09	73.84±2.9	-16.6±0.2
F16	35	5	5	262.2±2.5	0.576±0.02	74.88±3.3	-40±0.05
F17	35	2	5	229.1±1.9	0.538±0.01	77.9±2.6	-34.7±0.05

(B)

Quadratic modèle	**R^2**	**R^2 adjusted**	**R^2 predicted**	**Standard deviation**	**Coefficient of variation (%)**
Response (Y1)	0.9901	0.9773	0.8742	5.34	1.97
Response (Y2)	0.9979	0.9952	0.9723	0.0078	1.86
Response (Y3)	0.9941	0.9866	0.9209	0.3060	0.3974

A: Ethanol (%). B: Soy lecithin (%). C: Sonication time (min). Y1: Particle size (nm). Y2: PDI. Y3: % EE. PZ: Zeta potential (mV)


Desirability
PS (nm)
PDI
EE (%)
B: soy lecithin concentration (w/v)
A: ethanol concentration (v/v)
Desirability 0.847463
Prediction 226.501
Prediction 0.487187
Prediction 80.0528


Desirability
PS (nm)
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Desirability 0.847463
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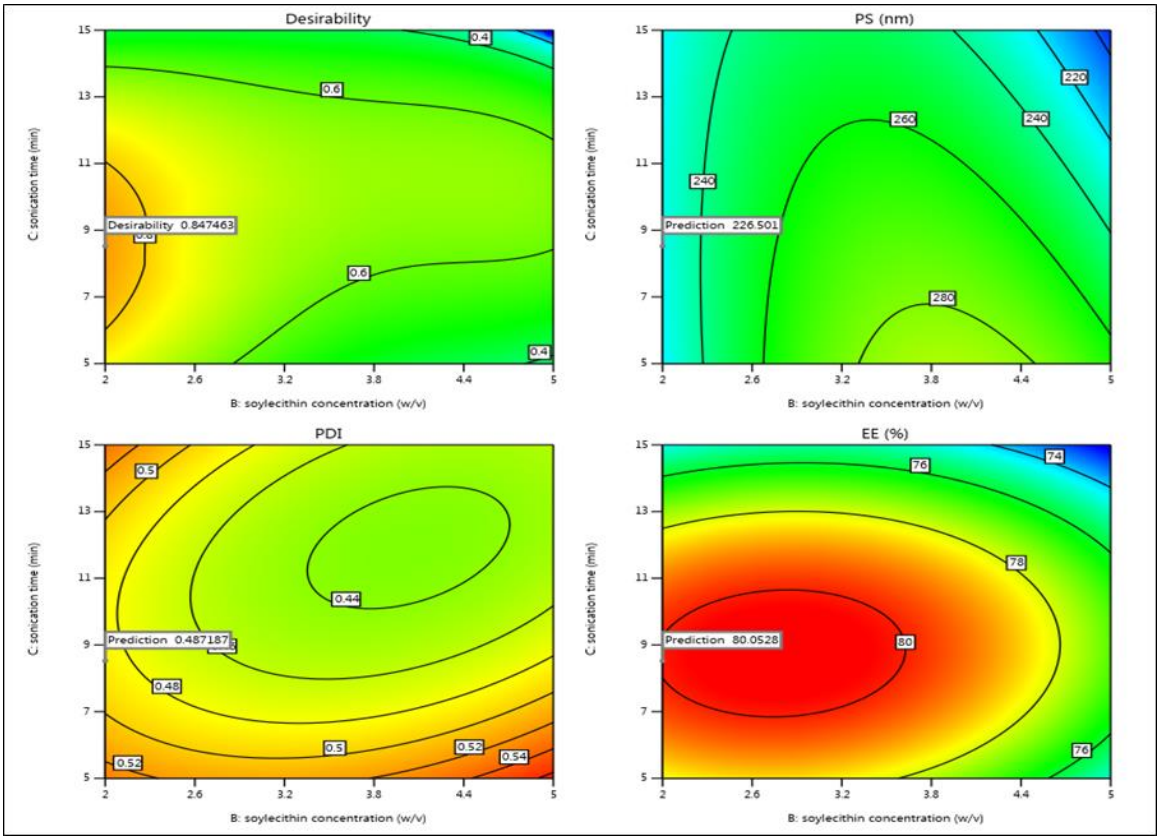

Figure 1 2D contour plots of the relationship between independent variables AB, AC, BC and dependent variables

3.1. Effect of formulation variables on the observed responses

The equations **(2)**, **(3)** and **(4)** presented below were derived using the best fit method to describe the main effect of the process variables (A, B, and C) and their interactions (AB, AC, BC) on the responses Y1, Y2 and Y3. In the regression equations, the sign and value associated with each variable represent the trend and magnitude of the terms influencing the responses. A positive sign reflects a synergistic effect, while a negative sign indicates an antagonistic effect [13].

From the regression equations, it was observed that a quadratic relationship between the dependent and independent variables is more appropriate, with better correlation between the variables. According to Design Expert® Software, R^2 value should be at least 0.80 and the difference between the adjusted and predicted R^2 values should be less than 0.2 to indicate a good and effective correlation. To further reinforce the results, an analysis of variance ANOVA was performed to assess the effect of each factor, it indicates that the quadratic regression model was significant and valid for each of the three responses studied (p-value < 0.0001). the high F-values obtained for each variable 77.5 (Y1), 366.9 (Y2) and 132 (Y3) implies the model is significant and well-suited for optimizing experimental conditions [4].

3.1.1. Impact of independent variables on particle size: Response 1 (Y1)

It is well known that the size of ethosomal vesicles is an important factor for effective transdermal delivery of the active ingredient [14]. The average vesicle sizes for the seventeen experimental trials ranged between the minimum and maximum values of 187.4 ± 1.2 nm to 334.9 ± 6.8 nm, with an average value of 271.33 ± 5.34 nm. The model that best fits the particle size data is a quadratic model and the polynomial equation **(2)** that best describes the model for the different concentrations of ethanol, phospholipids and sonication times is:

$$\boldsymbol{Particle\ size}\ (\boldsymbol{Y1}) = +268.21 + 0.7375\,A + 1.95\,B - 17.87\,C + 10.57\,AB + 3.25\,AC - 18.10\,BC + 48.49\,A^2 - 38.88\,B^2 - 2.98\,C^2 \qquad (\mathbf{2})$$

According to the equation **(2)**, ethanol and soybean lecithin concentrations showed a positive influence on vesicle size; however, sonication duration had a negative effect on vesicle size. The 2D contour plots (Figure 1) and the 3D graph (Figure 2) of particle size highlight the impact of the relationship between the different independent variables on the

variable Y1 (particle size). This visualization helps to understand, not only the interactions between variables, but also to identify the optimal conditions for the desired outcomes.

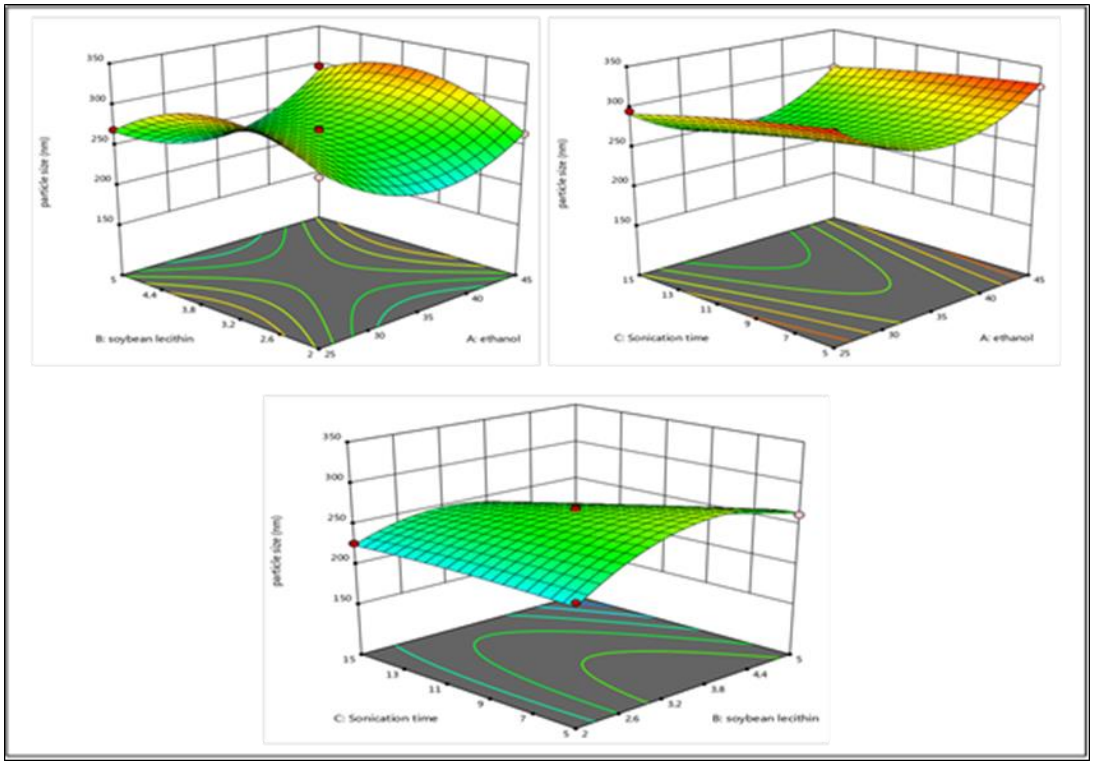

Figure 2 3D surface plot of the relationship between independent variables and particle size

Moreover, the combined effect of factors A and B as well as A and C revealed an increase in vesicle size. In contrast, the combined effect of factors B and C results in a decrease in vesicle size. Consequently, this suggests that the concentration of factors A and B and the sonication time (factor C) should neither be too high nor too low to achieve optimal vesicle size.

The ANOVA analysis revealed that the quadratic model was significant with a p-value< 0.0001. Furthermore, the difference between the adjusted R^2 value (0.9773) and the predicted R^2 value (0.8742) was less than 0.2, which likely confirms the linearity of the data. Additionally, the recorded R^2 value of (0.9901) and the adequate precision requirement of 34.61 > 4 indicate a sufficient signal and demonstrate the model's potential to guide the design space.

The results obtained suggest that the influence of each independent factor on particle size include the following:

At high ethanol concentrations and below specific levels, partial solubilization of the lipid bilayers (sublysis stage) can make the vesicular bilayers of ethosomes permeable, leading to a decrease in particle size [15]. However, an excessive amount of ethanol, specifically 45 %, in the ethosome structure can lead to an unstable membrane because the phospholipids dissolve rapidly in ethanol, resulting in a significant enlargement of the vesicles [16, 17]. Thus, an optimal ethanol concentration is important for preparing ethosomes of optimal size. Another explanation was provided by Vasanth and al. who suggested that a high ethanol concentration leads to intercalation of the hydrocarbon chains of lecithin, resulting in further reduction of the vesicle membrane thickness and causing a decrease in the average particle size [18]. This is consistent with Salem and al, who observed a general reduction in average vesicle size by increasing ethanol content from 10 % to 30 %. Ahmed and al also found the same antagonistic effect of ethanol concentration; they proposed an additional explanation that increased ethanol content leads to a reduction in the main transition temperature of the phospholipids, resulting in partial fluidization of the ethosome vesicles and the formation of small nanovesicles [19]. The same observation was made during the preparation of ethosomes by Limsuwan and al, who found that a higher ethanol concentration led to smaller sizes of the prepared ethosomes [20]. Finally, it was reported that ethanol levels above 40 % were not investigated because they would lead to failure in vesicle formation or degradation of already formed vesicles [17, 21].

Furthermore, a thicker matrix structure was formed and stiffened the vesicles when the concentration of soybean lecithin had been increased, which likely explains why the vesicles expanded. This finding is consistent with previous researches indicating that increasing phospholipid concentration slightly or moderately increases vesicle size [22-24]. The recommended amount of phospholipids to be used in an ethosomal formulation ranges from 0.5 % to 5 % [20], which was applied in the present experimental study. Comparable results have also been reported by Nasr and al. [17]. These results were also consistent with those reported by Morsi and al, who found that increasing the lecithin concentration can result in an increase in particle size [25]. Additionally, these results were corroborated by Prasanthi & Lakshmi, who reported that different phospholipid concentrations produce ethosomes of varying sizes without affecting % EE [26, 27].

It has been shown that sonication time had an inverse effect on particle size, its effect is clearly visible in these examples. This can be attributed to the fact that sonication reduces large droplets into nanodroplets, thus producing ethosomes with a small particles size [28]. Furthermore, it was found that the sonication process had a significant impact on the size of the vesicles.

3.1.2. Impact of independent variables on the PDI: Response 2 (Y2)

All the PDIs of the formulations ranged from 0.199 ± 0.02 (F6) to 0.5767 ± 0.02 (F16), with an average value of 0.4204 ± 0.0078 (Table 2 (A)). Only 3 formulations had values slightly above 0.5 without reaching 0.6. The impact of the independent variables on the PDI is illustrated by the equation **(3)**:

$$\boldsymbol{PDI(Y2)} = +0.4510 - 0.1077\,A - 0.0099\,B - 0.0249\,C - 0.014\,AB - 0.0185\,AC - 0.0292\,BC - 0.1444\,A^2 + 0.0314\,B^2 + 0.0479\,C^2 \qquad (\mathbf{3})$$

It was found that all the dependent variables had a negative impact on the PDI. The 2D contour plots (Figure 1) and The 3D graph (Figure 3) of PDI highlight the impact of the relationship between the different independent variables on the variable Y2 (PDI). This visualization helps to understand the interactions between the variables and to identify the optimal conditions for the desired outcomes.

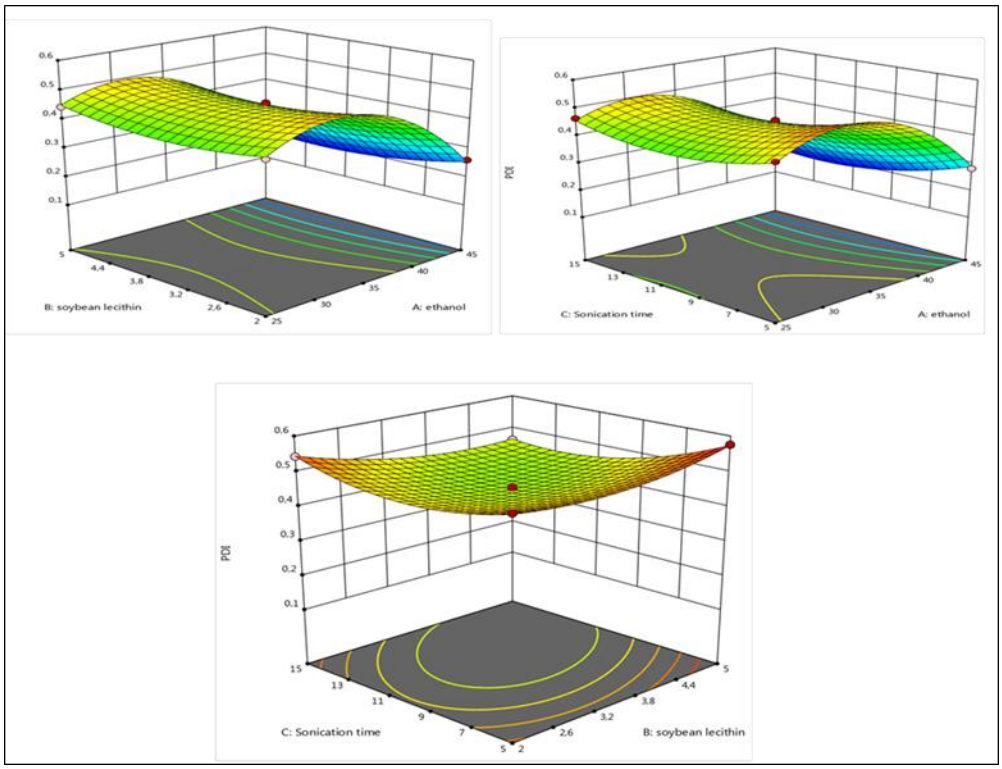

Figure 3 3D surface plot of the relationship between independent variables and PDI

Additionally, the combined effect of factors A and B, A and C, as well as B and C, revealed a decrease in PDI. Consequently, It would seem that the concentrations of A and B should not be too low and the sonication time (C) should be optimal to achieve a low PDI.

The ANOVA analysis revealed that the quadratic model was significant with a p-value< 0.0001. Furthermore, the difference between the adjusted R^2 value (0.9952) and the predicted R^2 value (0.9723) was less than 0.2, which likely confirms the linearity of the data. Additionally, the recorded R^2 value of (0.9979) and the adequate precision requirement of 61.90 > 4 indicate a sufficient signal and demonstrate the model's potential to guide the design space.

Particles tend to become more homogeneous as their PDI value approaches zero. As a number, the PDI can range from 0 (representing a completely uniform sample in terms of particle size) to 1 (representing a highly irregular, highly polydisperse sample with multiple particle size populations). The obtained values are acceptable and indicate a relatively homogeneous vesicle size distribution. According to Soleimanian and al, a PDI greater than 0.5 is not considered acceptable and reflects broad and heterogeneous size distribution values [29]. The decrease in PDI value with increasing lipid and ethanol concentrations can be attributed to the reduction in particle size at higher ethanol concentrations and the smoother vesicles with higher lipid content, resulting in less particle aggregation and increasing homogeneity. The decrease in PDI value with increased sonication time can also be explained by the fact that sonication reduces large particles, leading to smaller and more homogeneous particles.

3.1.3. Impact of independent variables on % EE: Response 3 (Y3)

Entrapment efficiency of a drug is a key parameter for estimating the amount of drug loaded into any delivery system; it helps assess the suitability of a delivery system for encapsulating the drug in question. % EE values of all formulations ranged from a minimum of 72.44 ± 3.5 % to a maximum of 80.23 ± 1.1 %, with an average value of 77.01 ± 0.3 % (Table 2 (A)). The model that best fits the % EE data is a quadratic model, and the equation **(4)** that best describes the model for the different concentrations of ethanol, phospholipids, and sonication times is:

$$\boldsymbol{EE}\%\ (\boldsymbol{Y3}) = +79.95 + 0.1625\ A - 1.47\ B - 1.57\ C + 0.275\ AB - 0.3\ AC + 0.285\ BC - 1.18\ A^2 - 1.61\ B^2 - 3.46\ C^2 \qquad (\mathbf{4})$$

The equation **(4)** reveal that ethanol concentration had a positive effect % EE, while soybean lecithin concentration and sonication duration had a negative impact. The model was found to be significant (p-value < 0.0001). The 2D contour plots (Figure 1) and The 3D graph (Figure 4) of % EE highlight the impact of the relationship between the different independent variables on the variable Y3 (% EE). This visualization helps to understand the interactions between the variables and to identify the optimal conditions for the desired outcomes.

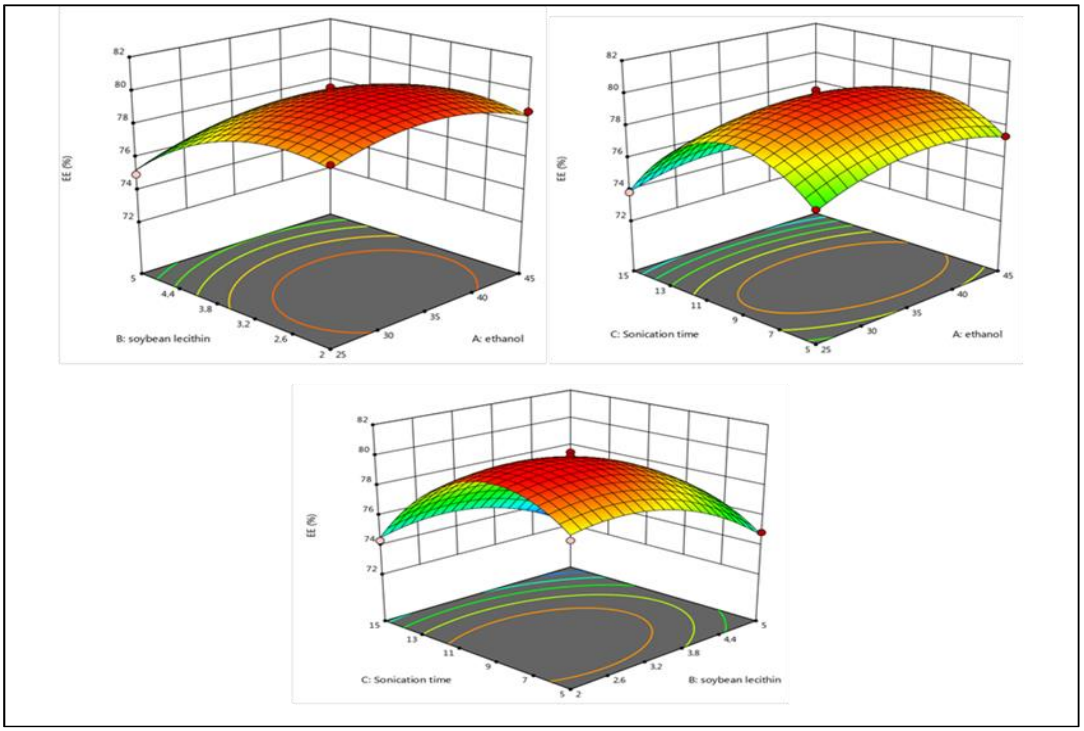

Figure 4 3D surface plot of the relationship between independent variables and % EE

The model for optimizing Fluconazole-loaded ethosomes was highly significant (p-value <0.0001); indeed, all variables showed a significant effect on the % EE of the prepared ethosomes.

Moreover, the combined effect of factors A and B, as well as B and C, showed an increase in % EE, while the combined effect of factors A and C had a negative effect. This suggests that the concentrations of factors A and B, as well as the sonication time (C), should not be too high or too low to achieve optimal encapsulation efficiency.

ANOVA analysis revealed that the quadratic model was significant with a p-value < 0.0001. Additionally, the difference between the adjusted R^2 value (0.9866) and the predicted R^2 value (0.9209) was less than 0.2, which likely confirms the linearity of the data. Furthermore, the recorded R^2 value of (0.9941) and the adequate precision required of 33.35 >4 indicate a suitable signal and demonstrate the model's potential to guide the design space.

Ethanol concentration, up to specific levels, had a positive impact on % EE of Fluconazole in classical ethosomes. This could be explained, according to Salem and al, by the solubilization effect of ethanol, which increases the fluidity of the ethosome vesicle membranes, leading to greater Fluconazole capture [30]. A high % EE is undoubtedly preferred as it allows for sufficient delivery of the drug to the site of action [31]. Beyond a specific level of ethanol, entrapment efficiency tends to decrease because ethanol can solubilize the phospholipids in the ethosomal membrane and cause destabilization of this membrane by forming pores in the phospholipid bilayers, making it permeable and thus reducing % EE [32].

Additionally, phospholipids can increase the number of vesicular bilayers formed, thereby enhancing the drug retention capacity of the ethosomes and increasing the % EE [33]. At the end of the results of this study, the increase in vesicle size due to the incorporation of a larger amount of phospholipids could explain the initial increase in Fluconazole entrapment at low ethanol concentrations.

Finally, it is important to highlight that the sonication process can significantly reduce the size of ethosomes, which could affect the entrapment efficiency of the ethosomes [34]. Some of Fluconazole may escape during centrifugation, a process necessary for determining the % EE [35], resulting in a decrease in % EE.

3.1.4. Evaluation of Zeta potential

Vesicular charge is a crucial characteristic that can affect vesicle properties such as stability and vesicle-skin contact. Therefore, the zeta potential of all Fluconazole-loaded classical ethosome formulations was measured. Due to their composition, ethosomes have negative charges because of the presence of the phosphate group in soybean lecithin [36]. Ogiso and al also mentioned that the negative charge of the zeta potential in the ethosomal system is mainly attributed to the high ethanol content in these nanovesicles. Ethanol imparts negative charges to the polar head groups of phospholipids, creating electrostatic repulsion [37]. When the zeta potential of a nanoparticle is between -10 and +10 mV, it is considered nearly neutral. However, when it is above +30 mV or below -30 mV, it is considered highly cationic or anionic and is regarded as highly stable because there is repulsion between the particles that prevents aggregation.

High ethanol concentrations, typically between 20% and 40%, have been associated with high zeta potentials, which delays the formation of aggregates through electrostatic repulsion and increases vesicle stability. Interestingly, vesicles can precipitate and become unstable at ethanol concentrations lower or higher than the mentioned range. Indeed, a high ethanol concentration in ethosomes can lead to steric stabilization of the vesicles by imparting a net negative charge to the surface of ethosomes [38]. Thus, ethosomes are prevented from aggregating due to electrostatic repulsion, which provides them good stability [38]. Ethanol molecules can distribute both in the external lipid bilayers and in the internal aqueous region of the vesicles [39]. The hydrophilic terminal hydroxyl groups of ethanol molecules, which distribute within the vesicular lipid bilayers, can interact with the external hydrophilic phase, leading to the formation of hydrogen bonds [39]. Another explanation is that the hydroxyl groups of ethanol molecules can also form hydrogen bonds with each other, resulting in a net negative charge on the ethosomes [40].

As shown in Table 2 (A), nearly all the prepared formulations had a zeta potential value ranging from -15.5 ± 0.4 mV to -40 ± 0.3 mV, this negative charge could stabilize the ethosomal system [41]. This indicates high stability and a low tendency to aggregation using all the tested ingredients at all levels of the variables used.

3.2. Selection of the optimal formulation

Based on the analysis of the studied variables, optimization was carried out using the desirability function approach. When multiple responses were evaluated in an experimental design, it was not always possible to achieve optimal responses for each factor in a single run. To address multiple response problems, various statistical methods can be

used; one of the most commonly employed methods is the desirability function [42]. To find the desired optimal formulation that meets all the constraints of the studied variables, a weight factor of 1 was chosen for all individual desirability in this work. The best solution, with the highest desirability (0.847), a % EE of 80.05 ± 0.306 % and vesicular size of 226.501 ± 5.34 nm with PDI of 0.487 ± 0.0078 was selected.

3.3. Results of characterization of the optimal formulation

According to the values provided by BBD, two optimal Fluconazole ethosomal formulation were carefully prepared and characterized based on parameters aimed at achieving minimal particle size, minimal PDI with a high absolute value of zeta potential and maximum % EE. PDI value was 0.465 ± 0.007, (< 0.5), indicating that the formulation was highly consistent overall. The average particle size was 265.1 ± 2.8 nm. The stability of the formulation was confirmed by measuring the zeta potential, which was -27.3 ± 0.85 mV showing a good stability. Finally, % EE was 80.13 ± 0.16% demonstrating a good entrapment efficiency of Fluconazole.

The observed results for particle size (Figure 5 **(A)**), PDI and the % EE obtained, are listed in Table 3 alongside the values predicted by the software. The zeta potential results are presented in the (Figure 5 **(B)**).

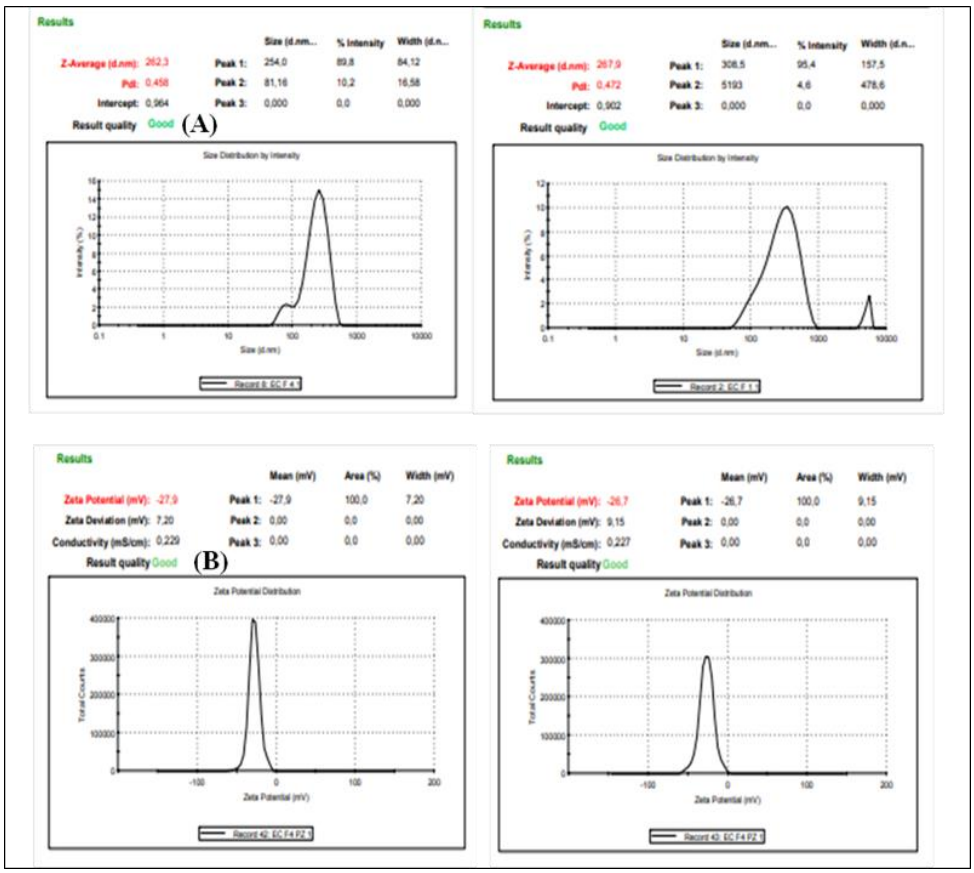

Figure 5 Observed results of particle size (A) and Zeta potential (B) of the optimal formulations

Table 3 Results of the independent responses for the tested optimal formulations

Independent responses	Solution 1	Solution 2	Obtained average	Predicted average	Confidence interval 95%	Standard deviation
Particle size (nm)	267.9	262.3	265.1	258.56	[247.98-269.137]	2.8
PDI	0.472	0.458	0.465	0.480	[0.465-0.496]	0.007
EE (%)	80.3	79.97	80.13	80.44	[79.83-81.05]	0.16

According to the values provided by BBD, the optimal Fluconazole classical ethosomal formulation showed a good zeta potential and was carefully developed based on parameters aimed at achieving the minimal particle size, the minimal PDI with the highest % EE. As shown in Table 3, the observed means were close to the predicted values and were within the confidence intervals, indicating the validity of the model for the preparation of Fluconazole-loaded classical ethosomes. The selected formulation was proposed for further studies

4. Conclusion

In summary, using the Box-Behnken design for optimizing Fluconazole-loaded classical ethosome formulations can enhance the understanding of the correlations between the variables involved in ethosome formation and their effects on vesicle size, PDI and drug % EE. It was observed that the optimal ethosome formulations exhibited a small vesicle size, a good PDI, a favorable zeta potential and a high % EE. Therefore, this study successfully developed and synthesized classical Fluconazole-loaded classical ethosomes and thoroughly identified and optimized the key factors involved in the experimental process, minimizing the number of experiments and resulting in time and cost savings. The optimal formulation obtained can make possible the synthesis of drug delivery systems (gel, ointment, cream, etc.) based on Fluconazole loaded classical ethosomes to enhance skin permeation and antifungal activity.

Future works will include studies on antifungal activity, stability and toxicity to develop a safe and effective antifungal ethosomal drug. Additionally, it will be important to study and optimize the scale-up to industrial production.

Compliance with ethical standards

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Disclosure of conflict of interest

We declare that there are no conflicts of interest in relation to this document.

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