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Optimization of the experimental design parameters for synthesis of Fluconazole loaded transethosomes as nano-based antifungal vesicles

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Abstract

Introduction: Antifungal drugs formulated in conventional pharmaceutical forms are not fully effective due to various factors. New approaches have focused on transdermal delivery drugs, including transethosomes, where the mixture of ethanol and surfactant allows a deeper drug skin's penetration. Formulation parameters and process variables can make their optimization a considerable challenge. This study aimed to formulate Fluconazole-loaded transethosomes using a full-factorial design with the goal of optimizing formulation and process variables.

Method: Fluconazole transethosomes were developed using the cold Method. A 2^1,3^1 full-factorial design was created by Design Expert® Software, where the impact of surfactant's type and soy lecithin to surfactant ratio on resulting formulation were investigated. The formulations were tested for vesicle size, polydispersity index, zeta potential and entrapment efficiency.

Results: Formulations containing Tween®80 presented the smaller particle sizes and showed a considerable entrapment efficiency for Fluconazole, they were more homogenous and highly stable compared to those prepared with Span®80. The optimized soy lecithin to surfactant ratio of 90:10 with Tween®80 was deemed apt for the synthesis of transethosomes giving the optimal formulation with a small particle size (300.2±5.57 nm), a low PDI (0.203±0.004), a good zeta potential (−31.75±0.68 mV) and a high entrapment efficiency (88.11±0.74 %).

Conclusion: This study enabled the in-depth identification and optimization of the key factors involved in the experimental process, such as the type of surfactant used and the soybean lecithin-to-surfactant ratio. To ensure safety and effectiveness in use, this work provides the perspective of continuing the study by evaluating the antifungal activity, long-term stability and safety of Fluconazole-loaded transethosomes.

Keywords: Fluconazole; Transethosomes; Cold method; Surfactant; Full-factorial design

1. Introduction

Antifungal drugs formulated in conventional pharmaceutical forms, such as ointments, creams and gels, are not fully effective due to various factors, such as poor skin penetration, inability to reach target sites, short residence time, which necessitates high or frequent dosing, consequently reducing treatment adherence [1]. Fluconazole, for example, is a

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broad-spectrum azole antifungal drug used for the treatment of skin infections. It has a low penetration rate when applied topically and many side effects when administered orally [2].

Recently, new approaches have focused on transdermal delivery of antifungal drugs, including vesicular delivery systems such as liposomes and ethosomes. The initial studies published on these systems revealed that drug penetration through the skin was relatively enhanced with the advent of liposomes as a delivery system [3, 4]. A liposome is a vesicle composed of a bilayer of phospholipids and an aqueous compartment, with a size ranging from a few tens of nanometers to microns [2]. It was later found, however, that the rigid nature of liposomes was not favorable for satisfactory drug penetration through the skin [5]. To enhance drug delivery through the skin, next-generation vesicular carriers of nanoscale size are currently the focus of extensive investigations in scientific research. In recent years, researchers have studied nanoscale vesicles, such as transfersomes and ethosomes, for dermal/transdermal drug delivery [6-8]. Transfersomes are flexible vesicles and the presence of a phospholipid bilayer and a surfactant in their structure helps promote drug penetration through the skin [9-11]. On the other hand, researchers have also tested additional methods to enhance drug penetration through the skin using ethosomes. These systems are primarily composed of phospholipids, water and ethanol. For the preparation of ethosomes, a range of 10% to 40% ethanol is typically used [12]. Ethanol significantly enhances drug penetration and ensures that the active ingredients reach the epidermis. Indeed, ethanol contributes to skin fluidity by modulating the multiple lipid layers, allowing the structure to be flexible and penetrate deeper into the skin [13]. Moreover, it has been shown that ethosomes do not cause significant skin irritation [14].

Transethosomes represent the latest generation of ethosomes, in which surfactants are combined with ethanol to produce improved forms of ethosomes and transfersomes. The combination of surfactants with ethanol makes transethosomes more deformable and elastic, allowing drugs to penetrate deeper into the skin. Transethosomes exhibit the beneficial characteristics of both transfersomes and ethosomes [15, 16]. As described in the literature, transethosomal formulations have demonstrated their effectiveness in various skin conditions. They ensure better skin penetration, thus improving local retention as well as transdermal action [17, 18].

Despite these advantages, optimizing the composition of transethosomes remains a major and significant challenge. Several factors influence the formulation, such as the nature of the drug, formulation parameters and process variables. This study aimed to formulate Fluconazole-loaded transethosomes using a full-factorial design created with Design Expert® software, with the goal of optimizing formulation and process variables.

2. Material and methods

2.1. Multi-Level Factorial Design for Fluconazole-loaded transethosomal suspensions

A full-factorial design 2^1, 3^1 multi-level (Table 1) was created using Design Expert® software to determine the effect of different variables on Fluconazole-loaded transethosomal suspensions (provided by MERINAL Laboratories, Algeria) using a reduced number of experimental trials (eighteen trials).

In this chosen design, the effects of two independent variables were analyzed: the surfactant's type (1^{st} factor) at two levels, which are Tween® 80 (Merck, Germany) and Span® 80 (Merck, Germany), and the soybean lecithin (AROMA-ZONE, France) to surfactant ratio (LS:S) (2^{nd} factor) at three levels (80:20, 85:15 and 90:10) on four responses: particle size (Y1), polydispersity index (PDI) (Y2), zeta potential (Y3) and drug entrapment efficiency (% EE) (Y4).

The experimental design covered all possible formulations for incorporating Fluconazole into transethosomal suspensions. Experimental data were evaluated for significance using ANOVA with Design Expert® software. Desirability was calculated to select the optimal formulation after inputting the desired results. The established criteria for deciding the optimal formulation were the smallest particle size, the lowest PDI, the highest zeta potential and finally the highest % EE.

Table 1 List of formulation factors for the multi-level factorial design used to develop Fluconazole-loaded transethosomes

Independent variables	**Minimum**	**Average**	**Maximum**
X1= Surfactant's type	Tween® 80		Span® 80
X2= Soy lecithin : Surfactant ratio (w/w)	80 :20	85 :15	90 :10
Dependent variables (responses)	Target		
Y1= Particle size (nm)	Minimum		
Y2= PDI	Minimum		
Y3= Zeta potential (mV)	Maximum		
Y4= EE (%)	Maximum		

2.2. Method of preparing Fluconazole-loaded transethosomes

The Fluconazole-loaded transethosomal dispersions were prepared using a modified cold method initially developed by Touitou and al. [19]. The organic and aqueous phases were prepared separately. The organic phase contained soybean lecithin, cholesterol (Alfa Aesar, USA) and Fluconazole dissolved in absolute ethanol (VWR Chemicals, France) at room temperature under vigorous stirring at 1200 revolutions per minute (rpm) using a magnetic stirrer (VELP Scientifica, Italy). Distilled water was then added dropwise using a syringe and the mixture was stirred at 700 rpm for 20 minutes to produce the required transethosomal suspension. The surfactant (Tween® 80 or Span® 80) was added to the aqueous or organic phase, respectively. The resulting suspension underwent sonication using an ultrasonic sonicator (Vibra-Cell™, Sonics, USA) to reduce vesicle size at a frequency of 20 kHz. To avoid damaging the structure of the ethosomes, the sonication duration was 10 minutes (5 minutes per cycle). Finally, the formulations were stored in the refrigerator and then characterized. Table 2 presents the composition of the different Fluconazole-loaded transethosomal formulations. The constant compositions were chosen from an optimal formula obtained in a previous study using Box-Behnken Design for the formulation of classical ethosomes, where the independent variables studied were ethanol and soybean lecithin concentrations and sonication time. In this study, we focused exclusively on the impact of surfactant's type and its proportion used, given that the surfactant is the component added to classical ethosomes to obtain transethosomes.

Table 2 Components of Fluconazole-loaded transethosomes

Components	**Quantities**
Soybean lecithin/ Surfactant ratio (Tween® 80, Span® 80)	80: 20 85: 15 90: 10 (Total: 3.5 %)
Fluconazole (%)	0.5
Cholesterol (%)	1
Ethanol (%)	35
Distilled water (%)	Up to 100

2.3. Characterization of transethosomes

2.3.1. Analysis of particle size and polydispersity index (PDI)

The particle size and PDI were measured for all Fluconazole-loaded transethosomes using dynamic light scattering (DLS) at 25°C with the particle size measurement system (Zetasizer, Malvern Instruments, UK). The colloidal dispersion of Fluconazole-loaded transethosomes was diluted with distilled water before being subjected to measurements. The PDI is used to describe the particle size distribution for effective and stable formulations.

2.3.2. Measurement of Zeta Potential

The surface charge of the vesicles is useful for preventing vesicle aggregation and improving formulation stability. The samples for zeta potential analysis were prepared by diluting the samples with distilled water and assessed using the Malvern Zetasizer to determine their zeta potential [20].

2.3.3. Determination of Entrapment Efficiency (% EE)

First, the maximum wavelength (λ_{max}) for pure Fluconazole was determined by scanning a solution of pure Fluconazole in the UV range of 200 to 400 nm against a blank. A specific volume of Fluconazole-loaded transethosomes was then diluted and filtered using a nylon syringe filter (0.22 µm pore size) and analyzed with a UV spectrophotometer at λ_{max} 250 nm against a blank of empty transethosomal suspension. The ultracentrifugation method was used to determine % EE, where free (non-encapsulated) Fluconazole was separated from the transethosomal suspension using a refrigerated centrifuge at 4°C (Sigma 2-16 K, Germany) at 15,000 rpm for 45 minutes [21]. The supernatant, supposed to contain the non-entrapped drug, was diluted with a certain amount of ethanol and then measured using a UV-visible spectrophotometer (Optizen, South Korea) at 250 nm. % EE of the vesicles was then calculated using the following equation:

$$\% \text{ EE} = \frac{\text{Total Fluconazole} - \text{Non encapsulated Fluconazole}}{\text{Total fluconazole}} \times 100$$

2.4. Optimization of the Formulation

Several studies have revealed that the type of surfactant and the phospholipid-to-surfactant ratio have a notable effect on particle size, PDI, zeta potential and % EE. In this work, the individual and interactive effects of these variables on the characteristics of Fluconazole-loaded transethosomal formulations were studied.

Design-Expert® was used for the generation and evaluation of the statistical experimental design. Eighteen Fluconazole-loaded transethosomal formulations were developed and analyzed. According to Table 3 (A), vesicle size (Y1), PDI (Y2), zeta potential (Y3) and % EE (Y4) were selected as dependent responses, while surfactant type (X1) and the soybean lecithin-to-surfactant ratio (X2) were selected as independent factors (responses). Results were considered significant when p-value < 0.05. All results are expressed as mean ± standard deviation. ANOVA was conducted to assess the significance level of the tested factors on the selected responses and their interactions.

Graphs were also presented to show the effect of independent variables on particle size, PDI, zeta potential and % EE. To select the optimal formulation, the desirability function, which predicts the optimal levels of response variables, was calculated.

3. Results and discussion

Two different independent variables were used, including surfactant's type (X1) and phospholipid-to-surfactant ratio (X2) (Table 1). The independent variables were evaluated using a multi-level factorial design (2^1, 3^1), resulting in eighteen different Fluconazole-loaded transethosomal formulations, as shown in Table 3 (A). All formulations were prepared using the cold method and then evaluated for particle size, PDI, zeta potential and % EE to determine the optimal formula.

The amount of ethanol was kept constant at 35% (v/v), cholesterol at 1% (w/v), Fluconazole at 0.5% (w/v), total soybean lecithin + surfactant at 3.5% (w/v) and sonication time at 10 minutes. The lecithin-to-surfactant ratio was varied at three levels 80:20, 85:15 and 90:10. The study revealed different types of responses depending on the surfactant type and the various ratios used.

Table 3 Observed responses for Fluconazole-loaded transethosomal formulations during development and optimization (A), ANOVA analysis results for all responses (B), Statistical analysis results produced by the Design Expert® Software (C).

(A)	**Independent factors**		**Dependent factors**			
Run	**X1**	**X2**	**Y1 (nm)**	**Y2**	**Y3 (mV)**	**Y4 (%)**
FT-1	Tween® 80	90:10	300.2±2.9	0.211±0.01	-31.75±0.5	88.35±2.3
FT-2	Span® 80	90:10	514.8±7.3	0.315±0.05	-19.25±0.35	83.21±1.3
FT-3	Tween® 80	85:15	427.9±3.5	0.21±0.01	-28.05±0.23	81.59±1.9
FT-4	Span® 80	85:15	522.1±5.5	0.298±0.04	-13.8±0.73	96.15±0.5
FT-5	Tween® 80	85:15	435.1±3.8	0.2±0.01	-27.9±0.77	82.3±5.6

FT-6	Span® 80	90:10	513.9±6.1	0.31±0.04	-18.9±0.61	83.5±2.7
FT-7	Tween® 80	80:20	476.75±4.2	0.157±0.005	-21.75±0.66	81.39±2.3
FT-8	Tween® 80	90:10	310.5±2.1	0.198±0.009	-30.3±0.78	87.9±1.2
FT-9	Tween® 80	85:15	420.5±2.2	0.209±0.01	-27.3±1.04	81±2.0
FT-10	Span® 80	80:20	573.4±8.9	0.269±0.03	-13.35±0.79	96.15±0.3
FT-11	Tween® 80	90:10	289.9±1.4	0.2±0.01	-33.2±0.26	88.1±1.0
FT-12	Tween® 80	80:20	472.4±2.7	0.154±0.003	-22.2±0.31	79.9±2.1
FT-13	Span® 80	90:10	516.1±7.3	0.32±0.06	-19.6±0.28	83±0.9
FT-14	Span® 80	85:15	524.8±7.4	0.289±0.04	-14.2±0.1	95.69±0.2
FT-15	Span® 80	85:15	526.3±8.1	0.294±0.04	-14.4±0.3	95.8±1.1
FT-16	Span® 80	80:20	570.7±8.3	0.265±0.003	-13.7±0.45	96±0.9
FT-17	Tween® 80	80:20	481.1±3.8	0.159±0.006	-21.3±0.38	83.2±2.7
FT-18	Span® 80	80:20	572.9± 9.5	0.27±0.03	-13±0.2	96.3±1.0

(B)	**F value**	**p-value**
Response (Y1)	891.75	<0.0001
Response (Y2)	488.73	<0.0001
Response (Y3)	346.74	<0.0001
Response (Y4)	253.07	<0.0001

(C) Modele	**R^2**	**Adjuste R^2**	**Predicted R^2**	**Adequate precision**	**Standard deviation**	**Coefficient of variation (%)**
Response (Y1)	0.9973	0.9962	0.9940	84.6289	5.57	1.19
Response (Y2)	0.9951	0.9931	0.9890	57.1833	0.0048	1.99
Response (Y3)	0.9931	0.9903	0.9845	46.6456	0.6832	3.2
Response (Y4)	0.9906	0.9867	0.9789	34.2533	0.7410	0.8446

X1 (A): Surfactant Type, X2 (B): Soy Lecithin: Surfactant Ratio Y1: Particle Size, Y2: PDI, Y3: Zeta Potential, Y4: % EE

The data revealed responses Y1, Y2, Y3 and Y4 within the ranges of 289.9 ± 1.4 to 573.4 ± 8.9 nm for particle size, 0.154 ± 0.003 to 0.32 ± 0.06 for PDI, -33.2 ± 0.26 to -13 ± 0.2 mV for zeta potential and 79.9 ± 2.1 to 96.3 ± 1.0% for % EE.

The F-value is a statistical measure used in analysis of variance ANOVA to determine if the means of several groups are statistically different from each other. It is calculated as the ratio of the variance between groups to the variance within groups. In other words, it compares the variability due to the effect of the independent variable to the variability due to random error. A high F-value indicates that the variability between groups is much greater than the variability within groups, suggesting that the independent variable has a significant effect on the dependent variable. The F-values for the model responses Y1, Y2, Y3 and Y4 were 891.75, 488.73, 346.74 and 253.07, respectively, indicating that the model was significant.

Along with the F-value, the associated p-value is generally examined. A low p-value (often less than 0.05) indicates that the observed differences are statistically significant, meaning they are unlikely to be due to chance. The model significance was confirmed by ANOVA with a p-value < 0.05. The p-values for all responses were <0.0001.

The multiple regression terms (R^2) were also analyzed. The difference between the adjusted and predicted R^2 values was <0.2, this indicated that the model predicted the responses well [22]. It is important to note that adequate precision measures the signal-to-noise ratio, and a ratio >4 is desirable [19] . For responses Y1, Y2, Y3 and Y4, the ratios were 84.6289, 57.1833, 46.6456 and 34.2533, respectively. Thus, this model could be used for the desired design [23].

3.1. Effect of formulation variables on observed responses

The distribution, diffusion, drug release mechanisms and skin deposition are significantly impacted by particle size [24]. Particle size and the amount of drug loaded into the nanoparticles are influenced by several parameters, including the chemical structure, the quantity and nature of the components used, as well as the experimental methodologies [25]. Characterization tests performed on the eighteen Fluconazole-loaded transethosomal formulations provided significant insights into the variables discussed below. These formulations were tested for particle size, PDI, zeta potential and % EE, as shown in Table 3 (A).

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

Generally, smaller vesicle sizes are more effective for drug penetration through the skin. In transethosomes, vesicle size can be regulated and modified by the surfactant [26]. The particle size results for the prepared transethosomes are presented in Table 3 (A), they ranged between 289.9 ± 1.4 nm and 573.4 ± 8.9 nm. The results clearly indicated a statistically significant relationship between the types of surfactant used; the lecithin-to-surfactant ratio and the vesicle size of the transethosomal suspensions, with p-values < 0.0001.

The graphs illustrating the effects of different independent factors on the vesicle size of Fluconazole-loaded transethosomes showed, on one hand, that the vesicle size varied according to X1 (type of surfactant). Specifically, the vesicle size was smaller for Fluconazole-loaded transethosomes using Tween® 80 as the surfactant, regardless of the lecithin-to-surfactant ratio used. On the other hand, they showed that the vesicle size decreased with the increase in X2 (lecithin-to-surfactant ratio), as illustrated in Figure 1 **(A)** and **(B)**.

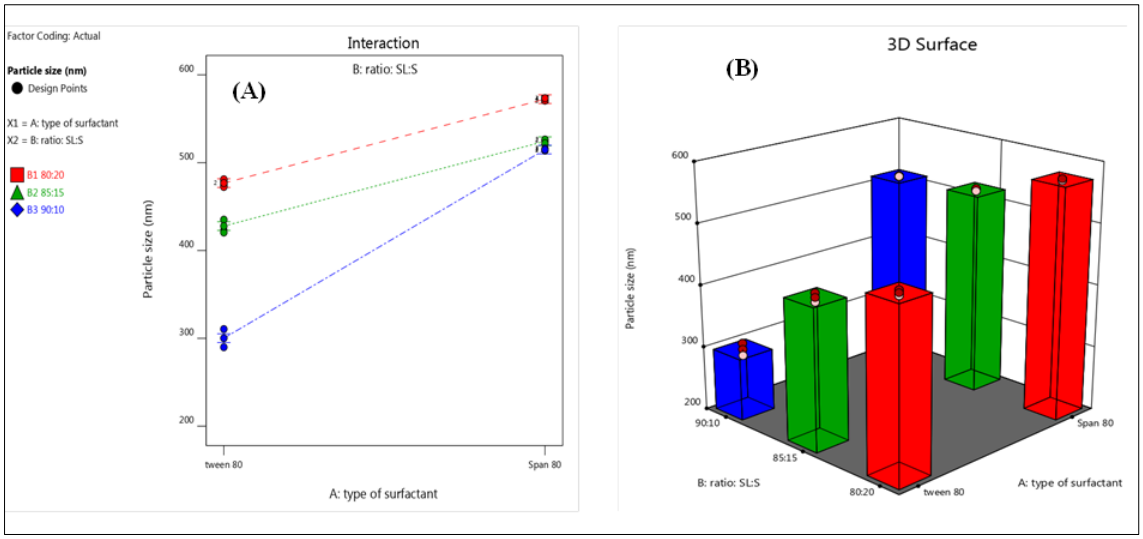

Figure 1 Effect of different independent variables on the vesicle size of Fluconazole-loaded transethosomes. (A): Interactions, (B): 3D Surface

It should be noted that the type of surfactant (Tween® 80, Span® 80) used in this experimental study had an impact on the particle size of the transethosomal suspensions. Specifically, formulations containing Tween® 80 resulted in smaller particle sizes compared to formulations containing Span® 80. These results can be attributed to the hydrophilic-lipophilic balance (HLB) value of the surfactant, which inversely affects the particle size [27]. Thus, Span® 80, with a low HLB value (4.3), resulted in an increase in particle size. This could also be attributed to the possible fusion of vesicles and the formation of aggregates caused by Span® 80 as the surfactant [28]. Non-ionic surfactants are amphiphilic molecules whose surface activity properties depend on the balance between their hydrophilic and hydrophobic regions. Surfactants with HLB value between 3 and 8 are suitable for forming bilayer membrane vesicles and are commonly referred to as water-in-oil (W/O) emulsifiers [29]. Since non-ionic surfactants lack charged groups in their hydrophilic head, they are less toxic and have broader compatibility compared to ionic surfactants, which is why Tween® 80 and Span® 80 were used is the study. In addition to the HLB value of a non-ionic surfactant, the surfactant's structure has a

significant impact on the geometric formation of its vesicle. This is attributed to critical formulation parameters (CPP), including the volume, the critical length and the surface area of the hydrophilic group. Larger vesicles form when the hydrophilic portion of the molecule decreases relative to the hydrophobic portion, as an increase in the length of the alkyl chain leads to an increase in CPP value [27]. This explains why Span® 80 resulted in the largest particle sizes.

On the other hand, the reduction in vesicle size when the lecithin: surfactant ratio increased from 80:20 to 90:10 could be due to the reduced amount of surfactant, which leads to incomplete vesicle maturation and consequently a decrease in their size [6]. The same result was observed by Qushawy and al., who found that a lecithin: surfactant ratio of 90:10 resulted in smaller transethosomes sizes compared to the 80:20 ratio [30]. This reveals that higher lecithin: surfactant ratio are desirable for controlling vesicle size within a lower range. However, it should be noted that other studies have found this effect to be insignificant on the vesicle size of transethosomes [31, 32].

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

The size distribution is another crucial parameter expressed by a dimensionless value called the PDI. This parameter is a significant indicator for characterizing the homogeneity of nanoparticles in a dispersed system and ranges from 0.0 to 1.0. The closer the PDI value is to zero, the more homogeneous the vesicles are [33].

As shown in Table 3 (A), the PDI values for the eighteen formulations prepared ranged from 0.154 ± 0.003 to 0.32 ± 0.06. The results indicated a statistically significant relationship between the type of surfactant used, the lecithin-to-surfactant ratio and the PDI with p-values < 0.0001.

The graphs representing the effects of the different independent factors on the size distribution of the Fluconazole-loaded transethosomes showed, on one hand, that the PDI varied with X1 (type of surfactant); specifically, it was lower for Fluconazole-loaded transethosomes using Tween® 80 as surfactant, regardless of the LS: S ratio used. On the other hand, the PDI decreased with the reduction in X2 (LS: S ratio), as illustrated in Figure 2 **(A)** and **(B)**:

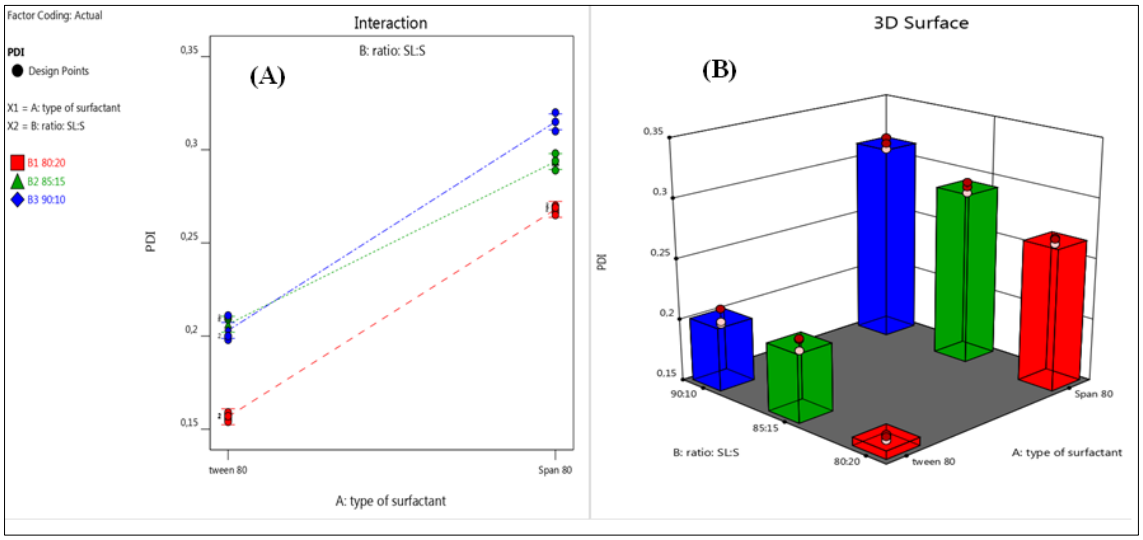

Figure 2 Effect of different independent variables on PDI of Fluconazole-loaded transethosomes. (A): Interactions, (B): 3D Surface

It was observed that only one formulation had a PDI value of 0.3 and none had a PDI value higher than 0.5. The obtained PDI values were thus within the acceptable range (< 0.5), indicating uniform vesicle size, showing a narrow and homogeneous distribution in the prepared suspensions with a low likelihood of aggregation, resulting in higher physical stability [33]. Indeed, a PDI greater than 0.5 is not considered acceptable and reflects heterogeneous and broad particle size distribution values [27, 34].

When the PDI is below 0.5, the system is monodispersed, meeting the requirements for drug delivery. It is clear that vesicles with a PDI under 0.5 are considered minimal or show no aggregation and their low PDI indicates a stable formulation system [35]. Lipids and surfactants are essential ingredients in transethosomal formulations that can impact the PDI [36]. A higher surfactant concentration can lead to more homogeneous vesicles and a lower PDI.

Increased surfactant concentration was associated with a lower PDI. Thus, a higher lecithin: surfactant ratio resulted in a larger PDI, while a lower lecithin: surfactant ratio led to a smaller PDI and more uniform vesicles [37].

3.1.3. Impact of independent variables on zeta potential: Response 3 (Y3)

A crucial factor in the stability of a colloidal dispersion system is the zeta potential [28, 37], It represents a measure of the extent of electrostatic repulsion or attraction between particles and is known to affect stability. Its measurement provides detailed insights into the causes of dispersion, aggregation, or flocculation and can be applied to improve the formulation of ethosomes. Nearly all particulate or macroscopic materials in contact with a liquid acquire an electrical charge on their surfaces [2].

The zeta potential values for each formulation indicate the potential stability of the vesicles and are listed in Table 3 (A). The nine Fluconazole-loaded transethosomal formulations based on Tween® 80 showed zeta potential values ranging from -21.3 ± 0.38 mV to -33.2 ± 0.26 mV, while those based on Span® 80 ranged from -13 ± 0.2 mV to -19.6 ± 0.28 mV. The results clearly showed a statistically significant relationship between the type of surfactant used, the lecithin-to-surfactant ratio and the zeta potential, with p-values <0.0001.

The graphs representing the effects of different independent factors on the zeta potential of Fluconazole-loaded transethosomes demonstrated that the zeta potential varied with X1 (type of surfactant); transethosomes using Tween® 80 as surfactant showed a better stability, regardless of the lecithin-to-surfactant ratio. On the other hand, the zeta potential decreased with the increase in X2 (lecithin-to-surfactant ratio) as shown in Figure 3 **(A)** and **(B)**:

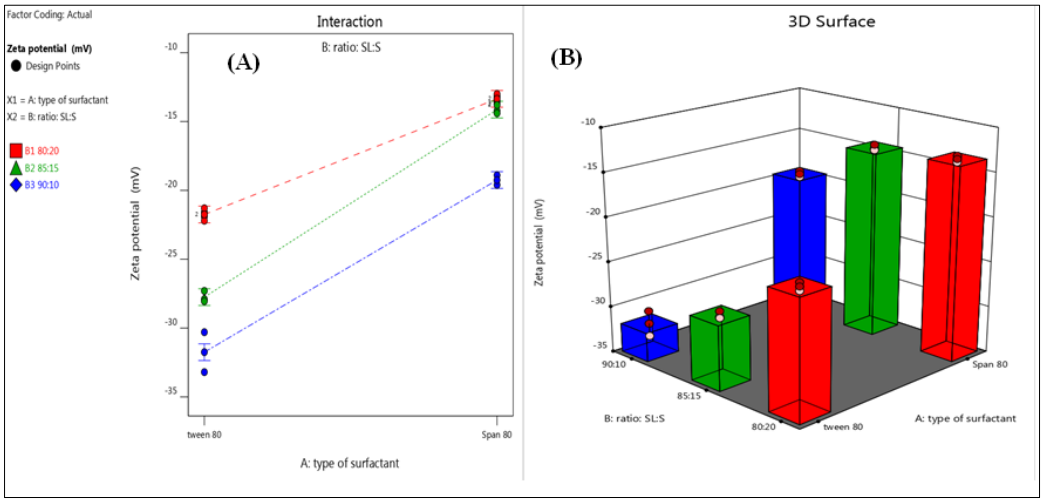

Figure 3 Effect of different independent variables on the zeta potential of Fluconazole-loaded transethosomes. (A): Interactions, (B): 3D surface

In general particles can be stably dispersed when the zeta potential is greater than 30 mV. Additionally, a zeta potential lower than 20 mV indicates good stability, while a potential below 5 mV suggests rapid aggregation [2]. The negative zeta potential is attributed to the high ethanol content, which imparts negative charges to the polar groups of phospholipids, creating electrostatic repulsion that reduces the aggregation of transethosomal vesicles and enhances their stability [17] .

The zeta potential values of Fluconazole-loaded transethosomes prepared with Tween® 80 showed increased electrostatic repulsion and greater vesicle stability compared to those prepared with Span® 80. This implies that Tween® 80 usage prevents vesicle fusion and contributes to their enhanced stability. The zeta potential indicates that transethosomes containing a higher lecithin: surfactant ratio exhibited a lower negative absolute zeta potential value. Therefore, increasing the lecithin concentration and using Tween® 80 contribute to improved vesicle stability.

3.1.4. Impact of independent variables on % EE: Response 4 (Y4)

As a parameter for evaluating the amount of Fluconazole entrapped in the transethosomes, the % EE for the prepared formulations was calculated. As indicated in Table 3 (A), high values of up to 96.3 ± 1.0 % were obtained for formula FT-18. The lowest % EE value was observed in FT-12 with a rate of 79.9 ± 2.1 % of entrapped Fluconazole. The results

clearly showed a statistically significant relationship between the type of surfactant used, the ratio of lecithin to surfactant and the zeta potential with p-values <0.0001.

The graphs representing the effects of different independent factors on the % EE of Fluconazole-loaded transethosomes (Figure 4 **(A)** and **(B))** showed that, on one hand, % EE varied according to X1 (type of surfactant); in fact, it was lower for Fluconazole-loaded transethosomes using Tween® 80 as a surfactant, regardless of the lecithin-to-surfactant ratio used. On the other hand, they showed that % EE increased with the increase in X2 (lecithin: Tween® 80 ratio) while it decreased with the increase in X2 (lecithin: Span® 80 ratio).

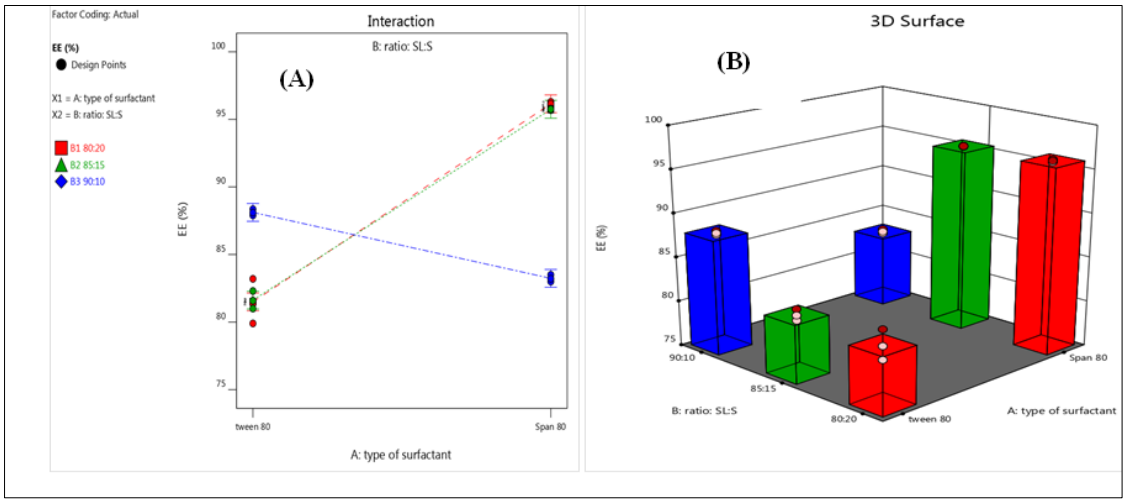

Figure 4 Effect of different independent variables on % EE of Fluconazole-loaded transethosomes. (A): interactions, (B): 3D surface

The high % EE values for Span® 80 could be related to the thermal conductivity (TC) of the surfactant, which is an important factor in explaining the surfactant's effects on the % EE of lipid-based vesicles. This could suggest that the higher the TC of the surfactant, the greater its ability to form a well-ordered structure and a less permeable bilayer, potentially improving the % EE. Tween® 80 also showed a considerable % EE for Fluconazole, likely due to its long carbon chain. These results are consistent with those of Abdallah and al., who reported that % EE of nystatin in prepared transfersomes was higher in the case of Span® 80 than in the case of Tween® 80 [38, 39].

It was also noted that % EE increased with a higher concentration of Span® 80, whereas it decreased with an increased concentration of Tween® 80. The reason for this is that the Tween® 80 used in the preparation of the formulations might be more efficiently incorporated into the membrane, resulting in a more permeable membrane, thus reducing % EE. A lower % EE can also be explained by the generation of mixed micelles when Tween® 80 is added at higher concentrations [17].

The results obtained were in good agreement with those of Balata and al., who found that an increase in surfactant concentration was accompanied by a decrease in % EE. They suggested that this could be due to the increased incorporation of the surfactant into the lipids, forming a more permeable vesicular membrane, thus decreasing % EE. The formation of mixed micelles when higher concentrations of surfactant are added is another explanation for a lower % EE, as they are more rigid and smaller in size [27].

3.2. Selection of the optimal formulation

Based on the analysis of the studied variables, the optimization was carried out using the desirability function approach. When multiple responses are evaluated in an experimental design, the optimal responses achieved individually for each factor do not always coincide in a single experiment. To address multiple response problems, various statistical methods can be used; one of the most commonly employed methods is the desirability function [40].

Tween® 80 and Span® 80 were used as surfactants and the characteristics of the resulting transethosomes were compared. It was observed that the size of the transethosomes formed using Span® 80 was less homogeneous and they were not stable; in contrast, those formed with Tween® 80 exhibited a smaller size and were highly stable. Thus, due

to its higher solubilization. Tween® 80 practically prevents the formation of aggregates and the fusion of vesicles [28]. Therefore, Tween® 80 was selected as the surfactant of choice and used with a lecithin: Tween® 80 ratio of 90:10.

After performing a full-factorial analysis for all formulation variables (surfactant's type and lecithin-to-surfactant ratio) using the Design-Expert® software and after studying their impact on various responses (vesicle size, PDI, zeta potential and % EE) based on the desired results (smallest particle size, lowest PDI, highest % EE and a good zeta potential) as indicated in Table 1, the software calculated the desirability for each formulation according to the desired outcomes. Among the eighteen developed formulations, the software provided the highest desirability score (0.698) to a formulation based on Tween® 80 with a lecithin-to-Tween® 80 ratio of 90:10.

This formulation is predicted to be optimal according to the software, as it would have a small particle size (300.2 nm), a low PDI (0.203), a good zeta potential (−31.75 mV) and high % EE (88.11%), as shown in Figure 5.

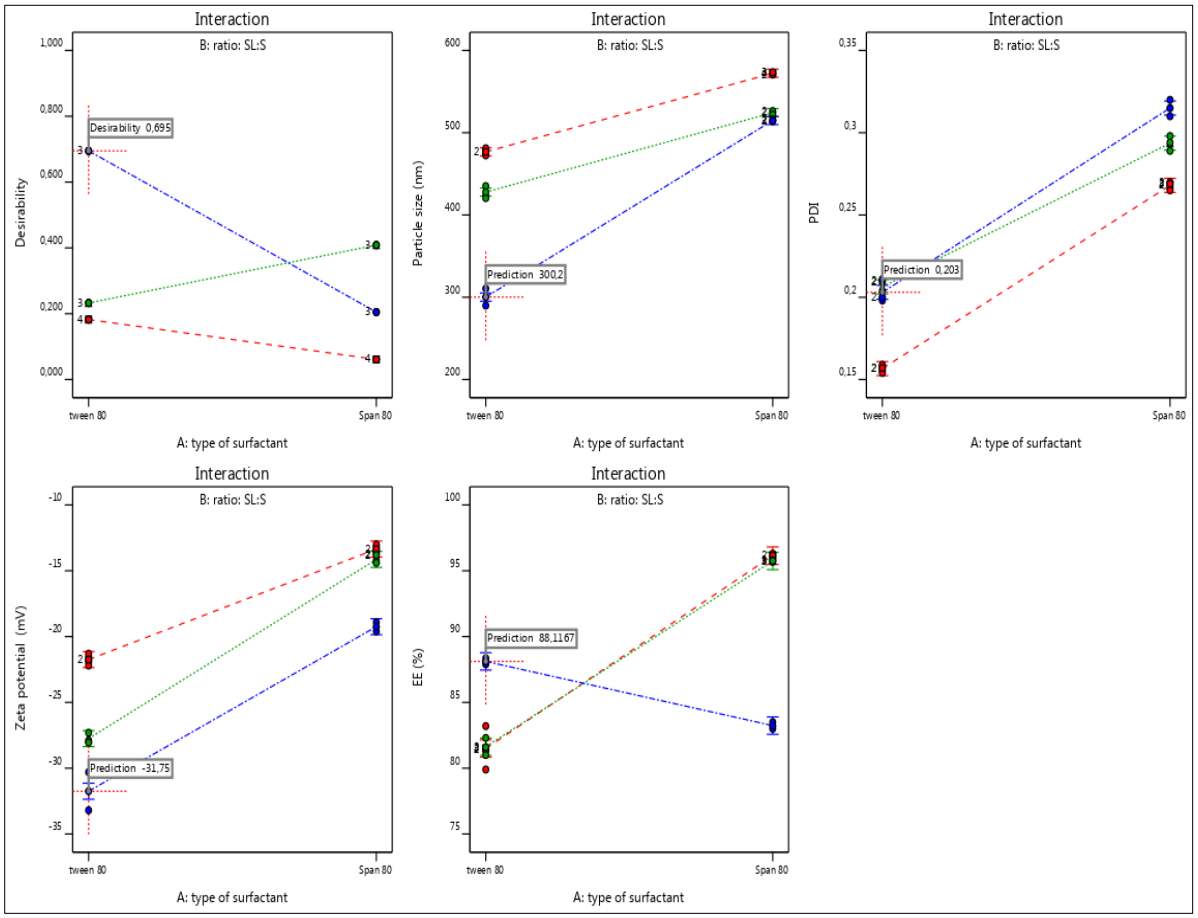

Figure 5 Predicted results for the optimal formulation obtained by the Design Expert® software

3.3. Characterization's results of the optimal formulation

The optimal formulation obtained by the software was prepared and characterized by measuring vesicle size, PDI, zeta potential and % EE. To confirm the experimental results of the optimal formulation (Figure 6), they were statistically compared with the predicted ones (Table 4).

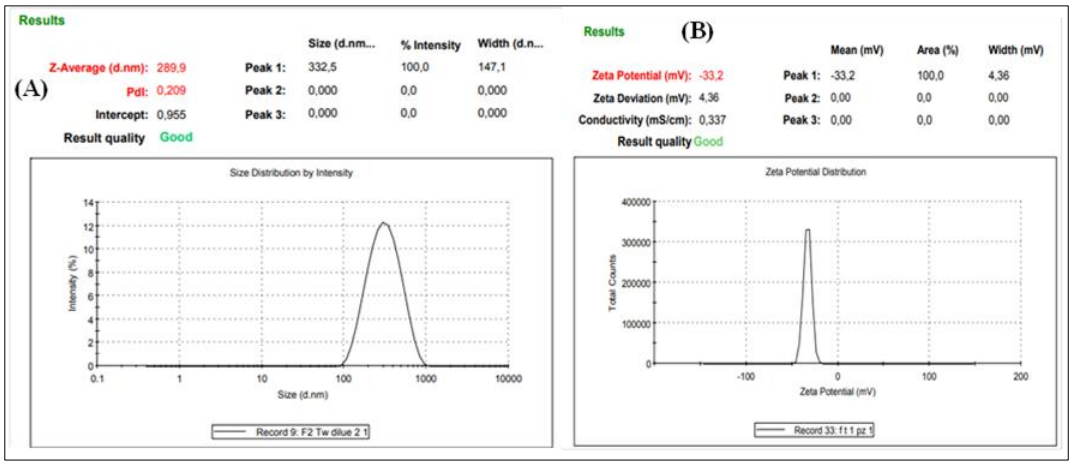

Figure 6 Particle Size (A) and Zeta Potential (B) values of the Optimal Formulation

Table 4 Actual and predicted results of the optimal formulation

Optimal formulation	Predicted average	Observed results	Standard deviation	Standard error	*95% PI lower	**95% PI upper
Particle size (nm)	300.2	289.9	5.5696	6.43122	286.188	314.212
PDI	0.203	0.209	0.004795	0.005537	0.190934	0.215066
Zeta potential (mV)	-31.75	-33.2	0.683232	0.788928	-33.4689	-30.0311
EE (%)	88.1167	88.35	0.74096	0.855587	86.2525	89.9808

*95% PI Lower: The lower bound of the 95% prediction interval. **95% PI Upper: The upper bound of the 95% prediction interval.

According to the values provided by the full-factorial design, the optimal Fluconazole transethosomal formulation was carefully developed based on parameters aimed at achieving the minimal particle size, the minimal PDI with a high absolute zeta potential and the highest % EE. As shown in Table 4, it was noted that the observed values were consistent with the prediction ones, indicating the validity of the model for preparing Fluconazole-loaded transethosomes and, consequently, the reproducibility and accuracy of the preparation method. Thus, the optimal formulation was selected for further research studies.

4. Conclusion

To advance the investigation of transethosomes as carriers for Fluconazole for topical administration, eighteen formulations were successfully prepared and characterized in this study using a full factorial design 2^1, 3^1. The optimal formulation, with a small particle size, low PDI, satisfactory zeta potential and high % EE, was selected based on the high desirability values obtained from the experimental design. Furthermore, this study enabled the in-depth identification and optimization of the key factors involved in the experimental process, such as the type of surfactant used and the soybean lecithin-to-surfactant ratio. To ensure safety and effectiveness in use, the work presented in this article provides the perspective of continuing the study by evaluating the antifungal activity, long-term stability, and safety of Fluconazole-loaded transethosomes.

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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