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QbD Approach in the Formulation and Evaluation of Miconazole Nitrate Loaded Ethosomal Cream-o-gel

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Abstract

The present study aims to prepare Miconazole Nitrate loaded Ethosomes using QbD principle and formulating them as a suitable dermatological cream-o-gel for enhanced antifungal activity, skin permeation and bioavailability in the treatment of Candidiasis. Central composite 32 factorial design was used for investigating the effects of two variables, concentration of soya lecithin (X1) and ethanol (X2) on the responses of particle size and % entrapment efficiency (% EE) which were taken as critical quality attributes (CQAs). Optimization with response surface method clarified the relationship between X1, X2 and CQAs, and design space was established based on the constraints set on the responses. The optimized batch with concentration of soya lecithin 2gm and ethanol 30ml was subjected to ultrasonication using Probe Sonicator to form nanoparticulate ethosomal dispersion. This Ethosomal vesicles showed a mean particle size of 63.78 nm, zeta potential -41.2 mV and %EE 81.54%. SEM studies revealed 3-Dimensional nature of Ethosomes with smooth surface. DSC, results exhibited entrapment of Miconazole Nitrate in Ethosomes. Ethosomes were loaded into cream-o-gel (CG1-CG5) using concentration 2.5%, 3%, 3.5%, 4%, 4.5% w/v of polymer Simulgel INS 100. CG3 formulation showed enhanced % cumulative drug release (67.95±0.25%) in comparison to plain drug cream-o-gel (35.91±0.47%) after 6 hrs. Texture analysis results showed the formulation to be less sticky and having good spread ability. It showed potential antifungal activity against Calbicans in comparison to marketed cream and did not cause any skin irritation on healthy wistar rats. Results suggested ethosomes asefficient carriers for Miconazole Nitrate topical delivery.

Keywords

QbD, Ethosomes, Miconazole Nitrate, Cream-o-gel, Candidiasis.

1. Introduction

Candidiasis is a superficial fungal infection of skin, nails, or mucous membrane with the yeast form of fungus Candida causing mild inflammation. It has emerged as a significant medical problem due to

advances in modern medicine owing to indiscriminate long-term use of antibiotics, immunosuppressive and cytotoxic therapies, immune-defects and more recently in AIDS patients **(1).** It usually occurs in warm, moist, and creased area, such as axillary folds, inguinal or intergluteal areas **(2).** Since most of the fungal infections are appearing over the skin, the treatment regimen of these infections always comprises external application formulations such as creams, ointments, lotions. However, skin tends to be the strongest barrier for the entry of drug entities and hence it is essential to design a drug delivery system that would deliver the medicament into the skin layers (cutaneous delivery), or through the skin and into the systemic circulation (percutaneous absorption) **(3).** Miconazole nitrate is a broad-spectrum antifungal agent of the imidazole group and is used primarily in the treatment of Candidiasis **(4).** It acts by means of a combination of two mechanisms: ergosterol biosynthesis inhibition, which causes lysis of fungal cell membranes because of the changes in both membrane integrity and fluidity, and direct membrane damage of the fungal cells **(5).** The limited solubility of miconazole nitrate and its intensive hepatic transformation results in poor oral drug bioavailability which hinders its use for systemic treatment via gastro intestinal tract. Its poor skin-penetration capability presents a problem in the treatment of cutaneous diseases by topical application. Its use in topical formulation is not efficacious because deep ereated fungal infections are difficult to treat with conventional topical formulation. The dose in conventional formulation is given at a higher level due to its low permeability. For effective treatment, the drug must be delivered in sufficient concentration to the site of infection. To design of effective formulations for Miconazole Nitrate has long been a major challenge. The use of lipid vesicles as drug delivery systems for skin treatment has attracted increasing attention in recent years. The interest of both the pharmaceutical and cosmetic industry for skin delivery has prompted the development and investigation of a wide variety of vesicular systems. A novel approach has been recently developed by Touitou et al to address the limitations of conventional topical drug delivery systems; in the form, of Ethosomes which are soft malleable vesicles composed mainly of phospholipids, ethanol (relatively high concentration), and water **(6).** The size of the ethosomes vesicles can be modulated from tens of nanometres to microns. Ethosomes permeate through the skin layers more rapidly and possess significantly higher transdermal flux **(7).** The high concentration of ethanol makes the ethosomes unique, as ethanol is known for its disturbance of skin lipid bilayer organization; therefore, when integrated into a vesicle membrane, it gives that vesicle the ability to penetrate the stratum corneum **(8).** Quality by Design (QbD) is the modern approach for quality of pharmaceuticals. QbD describes a pharmaceutical development approach referring to formulation design and development and manufacturing processes to maintain the prescribed product quality **(9).** In view of this, Ethosomes of Miconazole Nitrate were formulated by employing Quality by design (QbD) principles to gain an understanding of Ethosomes formulation. In the present research work, ethosomal approach was selected to enhance the permeability of Miconazole Nitrate that increase bioavailability, reduce the side effects, reduce large doses, and increase the therapeutic efficacy. This

study also focuses on the design of pharmaceutically acceptable formulation for topical application by incorporating the Miconazole Nitrate loaded ethosomes into cream-o-gel **(10,11)**.

2. Materials and Methods

2.1. Materials

Miconazole nitrate was generously gifted by Bliss Pharmaceuticals, Mumbai. Soya lecithin (Phospholipid) and Cholesterol were purchased from Himedia Laboratories Pvt. Ltd, Mumbai. Ethanol was purchased from Sterling Chemicals Pvt. Ltd, Pune. Simulgel INS 100 and Sepicide HB were obtained as gift samples from Seppic Kreglinger supplier, Belgium, Europe and other reagents and solvents were purchased from Research Lab., Fine Chem Industries, Mumbai.

2.2. Methods

- Preformulation Studies
- Spectroscopic Studies

2.2.1. Spectroscopic Studies

(i) Determination of λ max of Miconazole Nitrate in methanol and PBS pH 6.8 : methanol system (7:3)

The ultraviolet absorption spectrum of a solution of Miconazole Nitrate in the two solvent systems was obtained by scanning for maximum absorbance between 200 to 400 nm on Double-beam UV spectrophotometer J V-530 Jasco Corporation, Tokyo, Japan.

(ii)Calibration curve of Miconazole Nitrate in methanol and PBS pH 6.8 : methanol system (7:3)

The calibration curve of Miconazole Nitrate was plotted by measuring the absorbance on Double-beam UV spectrophotometer J V-530 Jasco Corporation, Tokyo, Japan of 10µg/ml to 60µg/ml solution prepared from the stock solution of Miconazole Nitrate in the two solvent systems at the λ max 272 nm and 270 nm respectively, taking absorbance on Y-axis and concentration on the X-axis. Average of triplicate readings was taken and tabulated.

(iii)Differential Scanning Calorimetry (DSC) of Miconazole Nitrate

Differential scanning calorimetric studies of Miconazole Nitrate was performed by using Differential Scanning Calorimeter (DSC; Mettler Toledo Star system). 10 mg of sample and alumina filled in aluminium pan was placed in sample and control compartment of furnace **(12)**. Samples were heated from 30 to 250C, increased at 10ºC / min under constant flushing with nitrogen gas, flow rate was adjusted to 50 ml/min.

(iv)Drug and Excipients Compatibility Studies

Drug–excipient interaction was studied by FTIR spectroscopy **(13, 14)**. The spectra were recorded for pure Miconazole Nitrate and excipient (Soya Lecithin) mixture using FTIR Spectrophotometer (Model No. FTIR4100 Jasco Corporation Tokyo, Japan). The scanning range was 400-4000cm^{-1} and the resolution of 1cm^{-1}.

(v)Screening of Miconazole Nitrate for Antifungal Activity

Minimum Inhibitory Concentration (MIC) of Miconazole Nitrate was determined by a) Broth dilution method and b) Cup plate diffusion method **(15, 16)**. Stock solution of Miconazole Nitrate was prepared in DMSO (dimethyl sulfoxide).

Broth dilution method: Cultures of *Candida albicans* were prepared separately. The medium (Sabouraud dextrose broth) was poured in the test tubes and sterilized by autoclave using 15 lb pressure at 121°C for 30 min. Using sterile pipettes exact amount of drug solution was added as indicated in the Table 1 to obtain a final volume of 10 ml. The tubes were then inoculated with 0.05 ml of the standardized culture and further incubated at 28°C for specified period of time and observed for any microbial growth in form of turbidity. The test procedure was repeated to check the reproducibility of the results. The lower concentration that inhibited the microbial growth was taken as the Minimum Inhibitory Concentration (MIC). Fluconazole was used as a reference standard (100µg/ml) solution.

Table (1): Protocol for Evaluation of MIC by Broth Dilution Method

Sr no.	Amount of drug solution per (ml)	Amount of medium (ml)	Total volume of solution (ml)	Conc. Of drug in final solution (µg/ml)	Result
1	0.1	9.9	10	1	+
2	0.2	9.8	10	2	+
3	0.3	9.7	10	3	+
4	0.4	9.6	10	4	+
5	0.5	9.5	10	5	-

6	0.6	9.4	10	6	-
7	0.7	9.3	10	7	-
8	0.8	9.2	10	8	-
9	0.9	9.1	10	9	-
10	1	9	10	10	-
control (+ve) [with culture]	0	10	10	0	+
control (-ve) [without culture]	5ml DMSO	5	10	0	
Standard (Fluconazole)	10	9	10	10	-

(**+**: turbidity, **-**: no turbidity)

Cup plate diffusion method: Anti-fungal susceptibility was done by cup plate diffusion method. The cup plate diffusion method involves sterilization of Petri plates, seeding of medium, inoculation and incubation. The plates were sterilized by dry heat in an oven at 160°C for one hour. Sabouraud dextrose agar was prepared and sterilized by autoclaving. 20ml of Sabouraud dextrose agar was added in sterile Petri plates aseptically and kept to solidify. Using the micropipette, solution of *Candida albicans* culture (in Sabouraud dextrose broth) was spread over the surface of an agar plate using the sterile hockey stick. After solidifying, wells of 8 mm were bored aseptically using sterile cork borer. The agar plugs were taken out carefully so as not to disturb the surrounding medium. The holes were filled completely with Miconazole Nitrate solution (1,3,5,7,9 µg/ml). The plates were kept in an incubator at 28°C for specified time. After this Petri plates were observed for the antifungal activity.

3. Application of Quality by design (QbD)

The concentration of Phospholipid and Ethanol have been identified as a key source for inter and intra batch variability in the formulation of Ethosomes **(9,17).** Quality by design(QbD) principles were applied to gain an understanding of formulation of Ethosomes.

3.1. Critical Quality Attribute (CQA)

A critical quality attribute as defined by ICH Q8 (R2) is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. Particle size and % Entrapment efficiency were identified as Critical Quality Attributes (CQA's) for evaluation of Ethosomes.

3.2. Critical Process Parameters (CPP)

A critical process parameter (CPP) is any measurable input (input material attribute or operating parameter) or output (process state variable or output material attribute) of a process step that must be controlled to achieve the desired product quality and process consistency. Concentration of Soya lecithin and Ethanol were selected as critical process parameters (material attributes).

3.3. Quality Risk Assessment

A key objective of risk assessment is to identify which material attributes and process parameters affect the drug product CQAs, that is, to understand and predict sources of variability in the manufacturing process so that an appropriate control strategy can be implemented to ensure that the CQAs are within the desired requirements. As discussed above the CQA's are Particle size and % Entrapment efficiency and the CPP's are Soya Lecithin and Ethanol. As the concentration of Soya Lecithin and Ethanol were changed in formulation of Miconazole Nitrate loaded Ethosomes, the variability were found in the product performance as this are the critical parameters for the Miconazole Nitrate loaded Ethosomes. These parameters were found to directly affect the Particle size and % entrapment efficiency. The variability in the product performance will be studied by using the factorial design.

4. Factorial Formulations of Ethosomes of Miconazole Nitrate

The factorial batches of Miconazole Nitrate Loaded Ethosomes were prepared by applying 3^2 Factorial Design with factor X1(Soya Lecithin Concentration) and X2(Ethanol concentration) as two independent variables at three levels –1, 0 and + 1 **(18)**.

Table (2): 3^2 Factorial Batches of Miconazole Nitrate Loaded Ethosomes

Factorial Batch	**Coded form**		**Actual form**	
	X1	**X2**	**Soya Lecithin(gm)**	**Ethanol(ml)**
F1	-1	-1	1	20
F2	0	-1	2	20
F3	+1	-1	3	20
F4	-1	0	1	30
F5	0	0	2	30
F6	+1	0	3	30
F7	-1	+1	1	40
F8	0	+1	2	40
F9	+1	+1	3	40

The different dependent responses were Y1 (Particle size) and Y2 (Entrapment Efficiency). The coded levels and the actual values of the variables used in this factorial design have been shown in Table 2**.** Accordingly, total nine formulations were designed.

4.1. Method of preparation

Factorial batches of Ethosomes were prepared by Hot method utilized for the preparation of ethosomal formulation using soya lecithin as phospholipid, cholesterol as stabilising agent and ethanol in the concentration as depicted in the Table 2. In all the formulations, the amount of Miconazole Nitrate (drug) was kept constant **(19)**. In this method, accurately weighed 200 mg of Miconazole Nitrate was dissolved in ethanol and propylene glycol was added to it and heated to 40 °C. In a separate vessel, Soya Lecithin and Cholesterol were dispersed in distilled water by heating on a water bath at 40 °C until a colloidal solution was obtained. Once both the mixtures reached at 40°C, the drug solution was added to the soya lecithin dispersion of water in a closed vessel under stirring at 3000 rpm using Ultra Turrex homogenizer IKA T25, Remi Motors Ltd, RM-12C Mumbai. After adding, mixing was continued for another 10 minutes to get microparticulate dispersion. Finally, the formulations were stored under refrigeration (2-8°C) until further use. Formula of the Factorial batches of Ethosomes have been depicted in Table 3**.**

Table (3): Formulation of the Factorial Batches of Miconazole Nitrate Loaded Ethosomes

sr no.	**Ingredients**	**Formulation Codes**								
		F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
1	Miconazole Nitrate (mg)	200	200	200	200	200	200	200	200	200
2	Soya lecithin (gm)	1	2	3	1	2	3	1	2	3
3	Ethanol (ml)	20	20	20	30	30	30	30	40	40
4	Cholesterol (gm)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
5	Propylene glycol (ml)	10	10	10	10	10	10	10	10	10
6	Water q.s (ml)	100	100	100	100	100	100	100	100	100

5. Evaluation of Factorial Batches of Ethosomal Formulation

5.1. Visual Observation

The prepared batches were observed visually for phase separation after 24 hrs storage at 2-8°C.

5.2. Droplet size determination

Droplet size of the prepared dispersion was determined by using Motic Digital Microscope. The formulation was placed on the slide and observed under 40X magnification **(20).**

5.3. % Entrapment Efficiency (%EE)

Miconazole Nitrate entrapped within the ethosomes was determined by centrifugation method. A volume of 10 ml of ethosomal dispersion was withdrawn and transferred to Eppendorf tube. The unentrapped drug was separated from the ethosomes by subjecting the dispersion to centrifugation in a cooling centrifuge (Remi Instrument Ltd, Mumbai) at 15,000 rpm at a temperature of 4°C for 60 minutes, where upon the pellets of ethosomes and the supernatant containing free drug were obtained. The supernatant was anal yzed for the drug content after suitable dilution with methanol by measuring absorbance at 272 nm using UV-Vis spectrophotometer. All tests were performed in triplicate **(21-23).** The % Entrapment efficiency was expressed as percentage of total drug entrapped using the following formula:

$$\% \text{ Entrapment Efficiency} = \frac{A_1 - A_2}{A_1} \times 100$$

A1= Amount of drug added initially

A2= Amount of drug determined in the supernatant by spectrophotometry,

A1-A2= Represents the amount of drug entrapped in the formulation.

6. Optimization

Central composite design was used to develop design space in order to optimize and evaluate the effects of the CPPs (material attributes) on the CQAs. A 3^2 (three-level, two-factor) randomized full factorial design was utilized in the present study **(18, 24)**. The different independent variables include Soya Lecithin Concentration (X1) & Ethanol Concentration (X2). The batches were evaluated and the effect of individual variable was studied according to the response surface methodology. The

dependent responses include Particle Size (Y1) and Entrapment Efficiency (Y2). To describe the response surface curvature, the design was evaluated by quadratic model, which bears the form of equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_{12} + \beta_{22} X_{22}$$

The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X_1X_2) show how the response changes when two factors are simultaneously changed. The polynomial terms (X_{12} and X_{22}) are included to investigate non-linearity. Statistical validity of the model was established on the basis of Analysis of variance (ANOVA) and the 2D contour plot and 3D response graphs were constructed using Design-Expert software (Version 9.0.4.1).

6.1. Formulation of Optimized Batch

The effect of method variables on the responses were statically evaluated by applying one-way ANOVA, using the commercially available software package Design-Expert version 9.0.4.1 in order to optimize the formulation parameters **(25)**. On the basis of the results obtained optimized batch was formulated in the similar manner as the factorial batches with concentration of 2 gm of soya lecithin and 30 ml ethanol respectively. This microparticulate ethosomal dispersion was subjected to ultrasonication using Probe Sonicator, PS 150, Orchid Scientifics, and Innovative India Pvt. Ltd. at 4°C in 3 cycles of 2 minutes each with 5-minutes rest between the cycles to form nanoparticulate ethosomal dispersion. Finally, the formulations were stored under refrigeration (2-8ºC) until further use.

6.2. Evaluation of Optimised batch

Various parameters were evaluated for optimised batch which include Appearance, % Entrapment Efficiency, Particle Size Analysis, Zeta Potential, Scanning Electron Microscopy, Differential Scanning Calorimetry, and Stability Studies.

6.2.1. Appearance

The optimized formulation was evaluated for its final appearance.

6.2.2. % Entrapment Efficiency

It was carried out in same manner as that of the factorial batches.

6.2.3. Particle Size Analysis

Mean particle size and size distribution of optimized batch of Ethosomes was determined by dynamic light scattering using Zetasizer Ver. 6.34 (Malvern instrument Ltd., Malvern, UK) at 25°C **(26,27)**.

6.2.4. Zeta Potential

Charge on drug loaded droplet surface was determined using Zetasizer Ver. 6.34 (Malvern Instruments Ltd., Malvern, UK). Analysis time was kept for 60s and average ZP, charge and mobility of optimized batch of ethosomes was determined at 25°C **(28,29)**.

6.2.5. Scanning Electron Microscopy (SEM)

SEM analysis of the prepared formulations was carried out to understand the morphology and 3-Dimensional surface properties of Ethosomes. It was done by drop casting method on silicon wafer. A drop of ethosomal dispersion was used as sample for SEM analysis. Sample was analysed under Scanning electron microscope (SEM, Nova NanoSEM) at an accelerating voltage of 18.00 kV **(30)**.

6.2.6. Differential Scanning Calorimetry (DSC)

The melting behaviour of Ethosomes was studied using DSC (model series: DSC823, METTLER TOLEDO), 10 mg of lyophilized sample of ethosomes formulation and alumina filled in aluminium pan was placed in sample compartment and control compartment of furnace. Sample was heated from 30ºC to 300ºC, increased at 10ºC / min under constant flushing with nitrogen gas, flow rate was adjusted to 50 ml/min **(12)**.

6.2.7. Stability Studies

Stability studies of Ethosome dispersion was conducted for 1 month due to lack of time. The Ethosomes dispersion was stored at refrigerated condition (2-8ºC) for 1 month and was evaluated for %EE and particle size after one month (30 days) of storage. The formulation was also examined visually for the evidence of any caking and discoloration **(31)**.

6.2.8. Formulation of Miconazole Nitrate Loaded Ethosomal Cream-O-Gel

The optimized ethosomal vesicle suspension was incorporated into cream-o-gel (2.5%, 3%, 3.5%, 4%, 4.5% w/v) as presented in Table 4. To the mixture of specified amount of Simulgel INS 100 and glycerine, ethosomal vesicle suspension was dispersed slowly with gentle stirring for 2 min followed by addition of Sepicide HB (preservative) with continuous stirring **(32,33)**.

Table (4): Formulation of Miconazole Nitrate Loaded Ethosomal Cream-O-Gel

Ingredients	CG1	CG2	CG3	CG4	CG5
Ethosomal Suspension (ml)	100	100	100	100	100
Simulgel INS 100 (gm)	2.5	3	3.5	4	4.5
Glycerin (gm)	3	3	3	3	3
Sepicide HB (gm)	0.2	0.2	0.2	0.2	0.2

7. Evaluation of Batches of Miconazole Nitrate Loaded Ethosomal Cream-O-Gel (34-39)

7.1. Appearance

The prepared cream-o-gels were inspected visually for clarity, colour, texture and homogeneity. The test is important regarding patient compliance.

7.2. pH

The pH of each formulation was determined by using digital pH meter (model 802, systronic). The pH meter was calibrated before each use with standard pH 4, 7 and 10 buffer solutions. 1 gm of formulation was stirred in distilled water till forms a uniform suspension. The volume is made upto 100 ml and pH of the dispersion was measured using pH meter. The measurement of pH of each formulation was performed in triplicate and mean values were calculated.

7.3. Viscosity

A plate and cone Brookfield viscometer (Model CAP 2000+2) along with assembly was used to determine viscosity. The measurement was done at a speed of 5 rpm for 10 sec run time. The viscosity was determined at 37°C.

7.4. Spreadability

Spreadability of each formulation was determined by an apparatus, which was suitably modified in the laboratory and used for the study. It consists of a wooden block and provided with a pulley at one end. A rectangular ground glass plate was fixed on the wooden block. Excess of cream-o-gel (about 1gm) under study was placed on this ground plate, and then the cream-o-gel was sandwiched between this plate and another glass plate having the dimensions of the ground plate attached with a hook. A 100gm weight was placed on the top of the two plates for 5 minutes to expel air and to provide a uniform film of the cream-o-gel between the plates. Excess of the cream-o-gel was scrapped off from the edges. The top plate was then subjected to a pull of 20gm with the help of a string attached to the hook and the time (in seconds) required by the top plate to cover a distance of 7.5 cm was noted. The spreadability was calculated using the formula:

$$S = \frac{M \times L}{T}$$

Where S = Spreadability

M = Weight tied to upper slide

L = length of glass slides

T = time taken to separate the slides.

7.5. Centrifugation Study

In this study, each ethosomal cream-o-gel formulation was subjected to centrifugation at 3000 rpm for 30 min at room temperature (RT) and was inspected for creaming, flocculation and phase separation.

7.6. Drug Content

5 g of each cream-o-gel (equivalent 10 mg of drug) formulation were taken in 100 ml volumetric flask containing 50 ml methanol and stirred for 30 minutes and allowed to stand for 24 hours. The volume was made up to 100 ml and 1ml of the above solution was further diluted to 10 ml with methanol. The drug content was determined by measuring the absorbance at 272 nm using Double-beam UV spectrophotometer J V-530 Jasco Corporation, Tokyo, Japan.

7.7. In-vitro drug release study

The in-vitro drug release studies of the Miconazole Nitrate loaded ethosomal cream-o-gel formulations were carried out in Franz Diffusion cell using Dialysis membrane (Himedia laboratories Pvt Ltd, flat width: 42.44 mm; inflated diameter: 25.4 mm). The membrane was soaked in Methanolic phosphate buffer (3:7) (pH 6.8) for overnight before experiment. The membrane was positioned between the two cell halves of a glass chamber. The two compartments were held together with a clamp. The receiver compartment contained methanolic phosphate buffer (3:7) (pH 6.8) and in the upper donor compartment 1 gm of formulation was spread evenly on the membrane. The receptor phase methanolic phosphate buffer (3:7) (pH 6.8) was continuously stirred with help of magnetic stirrer and was maintained at temperature of 37 ± 10°C during the experiments. One ml of the sample was withdrawn from the receiver compartment at time intervals 0, 30, 60, 120, 180, 240, 300, 360 minutes and the same amount of fresh buffer solution was added to maintain the sink condition in receiver compartment. Care was taken to ensure that no air bubbles were lodged underneath the diffusion membrane during the experiments. The samples were diluted up to 10 ml with methanolic phosphate buffer (3:7) (pH 6.8) and were analysed spectrophotometrically at a wavelength of 270 nm. By determining the amount of Miconazole Nitrate released at various time intervals, the Cumulative % drug release (Miconazole Nitrate) versus time was plotted. This experiment was carried out for a

period of 6 hours.

8. Evaluation of Miconazole Nitrate loaded Ethosomal Cream-o-gel formulation (CG3)

8.1. In-Vitro Drug Release Study of Miconazole Nitrate Loaded Ethosomal Cream-O-Gel (CG3) and Plain Drug Cream-O-Gel Formulation

It was carried out in same manner as that of the above

8.2. In-vitro anti-fungal activity

Antimicrobial efficiency studies were carried out to ascertain the biological activity of developed cream-o-gel formulation against microorganisms. This was determined in the agar diffusion medium employing Cup plate technique. Marketed cream (Micogel cream) was used as standard. The standard and the developed formulation (test) was taken into cups bored into sterile sabouraud dextrose agar previously seeded with Candida albicans after allowing diffusion of formulation for two hours. The plate was incubated for 24 hours at 28°C. The zone of inhibition was compared with that of the standard **(16)**.

9. Texture Analysis

Texture analysis of the Miconazole Nitrate loaded Ethosomal cream-o-gel formulation (CG3) was performed by evaluating two different parameters i.e. Adhesiveness and spreadability. These mechanical properties have been assessed using the texture analyser TA.XT plus (Stable Micro Systems, Godalming, UK). Data acquisition and mathematical analysis have been performed using a computer equipped with the Texture Expert software **(40-42)**.

9.1. Spreadability test

Spreadability test was performed by using the texture analyser TA.XT plus (Stable Micro Systems, Goldalming, UK) in Compression mode. A cone analytical probe (60°) was forced down into each sample at a defined rate (1.00 mm/s) and to a defined depth (23.0 mm). The test was performed and results were observed. When a trigger force of 5 g has been achieved, the probe proceeds to penetrate the sample at a test speed of 3.00 mm/s to a depth of 23 mm. During this time, the force to penetrate

the sample increases. When the specified penetration distance has been reached, the probe withdraws from the sample at the post-test speed of 10.00 mm/s. The maximum force value on the graph is a measure of the firmness of the

sample at the specified depth. The area under the positive curve is a measure of the energy required to deform the sample to the defined distance (Hardness Work Done). Research has shown that the firmness and energy required deforming a sample to a defined depth grades samples in order of spreadability. A higher peak load (firmness) and hardness work done value indicate a less spreadable sample. Conversely, a lower peak load (firmness) value coupled with a lower hardness work done value indicates a more spreadable sample.

9.2. Adhesion Test

Adhesion test was performed by using the texture analyser TA.XT Plus (Stable Micro Systems, Goldalming, UK) in Compression mode. The analytical probe was forced down into each sample at a defined rate (1.50 mm/s) and to a defined depth (5.0 mm). The test was performed and results were observed. When a trigger force of 2.5 g has been achieved, the probe proceeds to penetrate the sample at a test speed of 2.00 mm/s to a depth of 5mm. During this time, the force to penetrate the sample increases. When the specified penetration distance has been reached, the probe withdraws from the sample at the post-test speed of 10.00 mm/s.

10. Skin Irritation Study

Skin irritation test was performed on 6 Healthy Wistar rats. The developed Cream-o-gel formulation was applied topically and was monitored for any erythema and oedema followed by photographic imaging of skin after subsequent application at 24 hr, 48 hr and 72 hr respectively and were compared with images taken at 0 hr (just prior to first application). The experiment was carried out as per OECD test guideline number 404 updated latest by 2002 and the protocol (registration number-296/CPCSEA/2000) and the research protocol number

ACP/IAEC/2015/02 was duly approved by Institutional Animal Ethics Committee (IAEC) following CPCSEA (Govt. of India) guidelines **(43-45)**.

10.1. Procedure

The acute dermal irritation of the developed topical antifungal formulation will be performed according to OECD guidelines 404. The animals were divided in two groups Control (6 rats) and Test (6 rats). The area on the back of each rat was shaved 24 hrs prior to the experiment. A dose of 0.5 g of the developed

topical antifungal formulation as a test substance, was applied to the shaved area of approximately 6 $cm^2$of skin of respective animals. The treated sites for formulation on respective rats were covered by gauze and the back of the rat was wrapped with a non-occlusive bandage. The animals were then returned to their cages. At the end of the exposure period, residual test substance should be removed, where practicable, using water or an appropriate solvent without altering the existing response or the integrity of the epidermis. Observation of the sites was done at 24 hours after application, and repeated at 48 and 72 hrs thereafter. The reactions, defined as erythema and edema, were evaluated according to the scoring system for skin reactions as presented in Table 5A. Control animals were prepared in the same manner and 0.5 gram of the cream base (without drug) were applied to the control animals and observations were made similar to the test animals

Table (5A): Grading of Skin Reactions

Reaction	**Score**
Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4
Oedema Formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by defnite raising)	2
Moderate oedema (raised approximately 1mm)	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure)	4

10.2. Primary Irritation Index (PII)

The score of Primary Irritation (SPI) was calculated for each rat as the following, Scores for erythema and edema at 24, 48 and 72 hours were summed and divided by the number of the observations for the treated sites. The SPI for the control sites were calculated in the same fashion as test.

SPI for each rat = ∑ Erythema and Edema grade at 24, 48 and 72 hrs / Number of observations

The difference between the summation of SPI scores of the 6 animals from the treated site and 6 animals from the control site were calculated and were used for Primary Irritation Index

determination. The Primary Irritation Index (PII) was calculated as the arithmetical mean of these SPI values of the 6 rats. The irritation degree was categorized as negligible, or slight, moderate or severe irritation based on the PII as presented in Table 5B.

$$PII = \Sigma SPI\ (Test) - \Sigma SPI\ (Base) / \text{Number of animals}$$

Table (5B): Response Categories of Irritation in Rat

Category	**Primary Irritation Index**
Negligible	0-0.4
Slight irritation	0.5-1.9
Moderate irritation	2-4.9
Severe irritation	5-8

10.3. Accelerated stability study

Optimized ethosomal cream-o-gel formulation was subjected to stability studies for 1 month due to lack of time. The formulation was filled in wide-mouth glass bottles and were stored at ambient temperature (25 ± 2°C and 60 ± 5% RH) and elevated temperature (40 ± 1°C and 75 ± 5% RH). These samples were evaluated for appearance, pH, viscosity, drug content, spreadability and in-vitro drug release study.

11. Results

11.1. Preformulation

The preformulation studies were carried out in order to determine certain fundamental properties of the drug molecule and the excipients. λ max value of Miconazole Nitrate in Methanol and PBS pH 6.8-Methanol System (7:3) was found to be at 272nm and 270nm respectively as depicted in Figure 1A and 1B. The calibration curve of Miconazole Nitrate in Methanol and PBS pH 6.8-Methanol System (7:3) have been depicted in Figure 2A and 2B. The melting behaviour of pure Miconazole Nitrate powder was studied using DSC which showed sharp endothermic peak at 185.59°C as depicted in Figure 3. FTIR studies revealed characteristics peaks of Miconazole Nitrate and compatibility with excipient (Soya Lecithin) was evaluated by FTIR peak matching method. As depicted in Figure 4 the FTIR spectrum of Miconazole Nitrate was characterized by bands at 3181 cm^{-1}(Imidazole -C-N stretch), 3107 cm^{-1} (Aromatic -C-H stretch), 2963 cm^{-1}(Aliphatic -C-H stretch), 1547 cm^{-1} (-C-C aromatic), 1474 (-CH2 bending), 1328 cm^{-1} (-C-N stretch), 1170 cm^{-1} (-C-O), 827 cm^{-1} (Aromatic [out-of-plane bend]), 637 cm^{-1}

(-C-Cl). After careful inspection of the spectra of the physical mixture of Miconazole Nitrate with Soya Lecithin, the activity of the who

Figure (1A): λ max of Miconazole Nitrate in Methanol

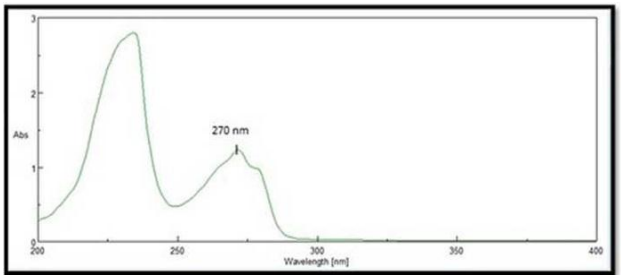


Figure (1B): λ max of Miconazole Nitrate in PBS pH 6.8-Methanol Solvent System (7:3)

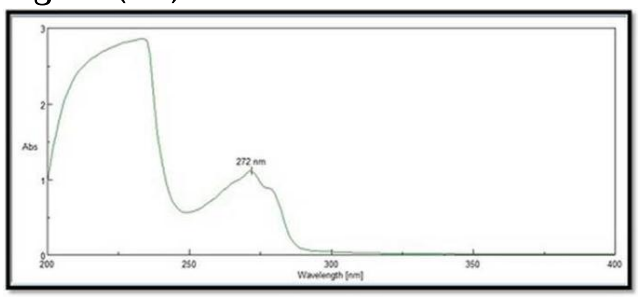


Figure (2A): Calibration curve of Miconazole Nitrate in Methanol

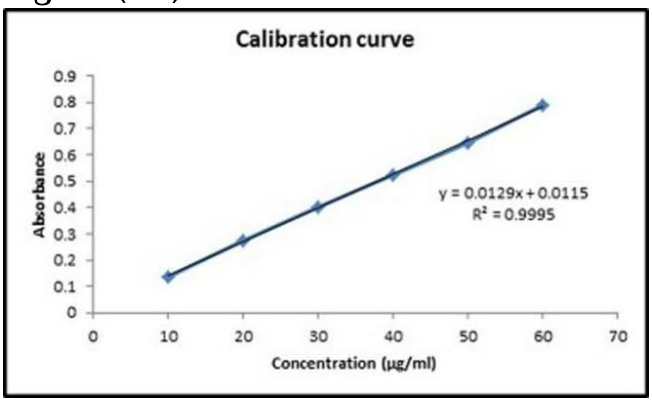


Figure (2B): Calibration curve of Miconazole Nitrate in PBS pH 6.8-Methanol system (7:3)

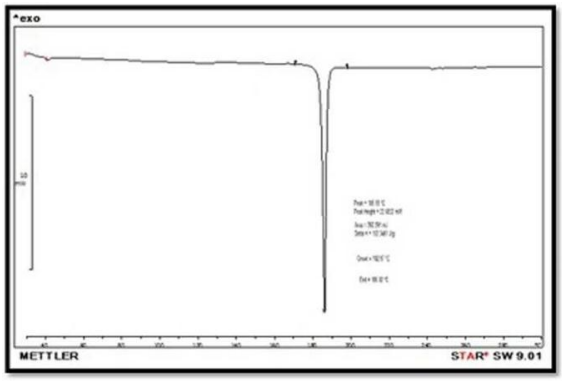


Figure (3): Differential Scanning Calorimetry Thermogram of Miconazole Nitrate

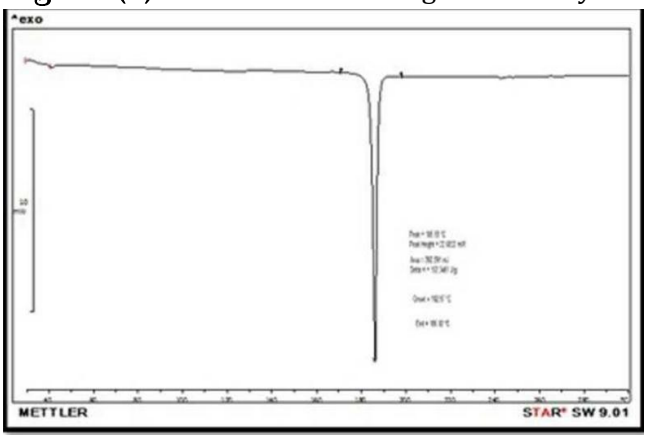


Figure (4): Drug and Excipient Compatibility (A= Drug Miconazole Nitrate, B= Soya Lecithin, C= Drug + Soya Lecithin)

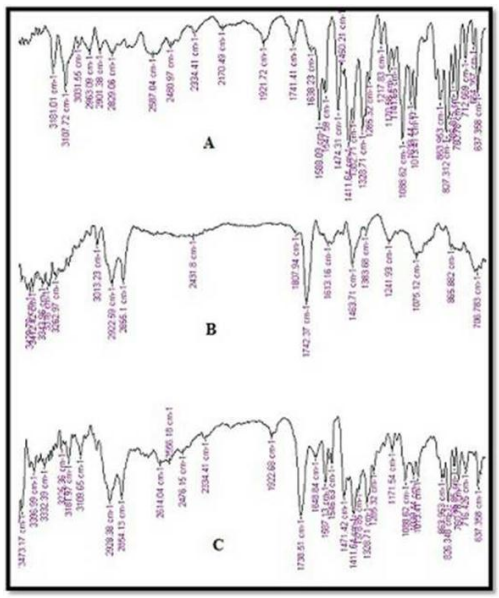


Figure (5): MIC of Miconazole Nitrate by cup plate diffusion method

11.2. Quality by design (QbD)

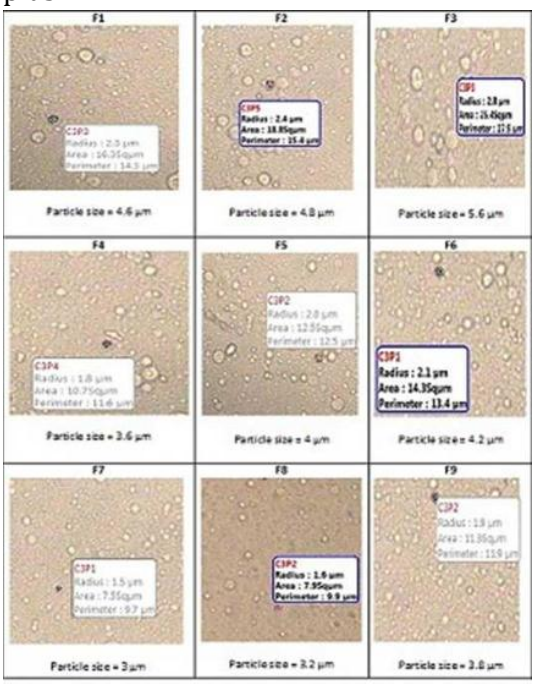
Ethosomes of Miconazole Nitrate were successfully formulated by employing Quality by design (QbD) approach to gain an understanding of Ethosomes formulation. Design of experiment (DOE) i.e Central composite design was used to understand the relationship between the Independent and dependent variables. Soya Lecithin and Ethanol were identified as Critical process parameters and Particle size and % Entrapment efficiency were identified as Critical quality attributes. The 3^2 Factorial batches of Ethosomes of Miconazole Nitrate were prepared by Hot method using soya lecithin as phospholipid, cholesterol as stabilising agent and ethanol. The concentration of phospholipid and ethanol have been identified as a key source of inter and intra batch variability in the formulation of Ethosomes. Changes in phospholipid and ethanol concentration resulted in significant changes in the responses, Particle size and % EE.

Table (6): Particle size and %Entrapment Efficiency of Ethosomal Factorial Batches F1-F9

Batch No.	**Particle size (µm)**	**% Entrapment Efficiency**
F1	4.6	76.80%
F2	4.8	84.30%
F3	5.6	86.80%
F4	3.6	74.60%
F5	4	82.20%
F6	4.2	80.90%
F7	3	71.50%
F8	3.2	72.80%
F6	3.8	73.90%

Based on the outcome from DOE study, a design space was defined in terms of Critical process parameters resulting in optimum particle size and % Entrapment efficiency. The results of Particle size and % EE for the factorial batches have been depicted in Figure 6 and Table 6.

Figure (6): Droplet size determination of Ethosomal factorial batches F1-F9 by motic image plus



11.3. Optimization

For optimization of factorial batches, Design expert software (Version 9.0.4.1) was used to fit polynomial equation with added interaction terms to correlate the study responses, particle size and entrapment efficiency. The 2D contour plot and 3D surface response plot was plotted for understanding the variables and responses. The predicted and experimental values were critically compared from 2D counter plot and 3D surface response plot.

11.3.1. Regression Analyses of Particle size

Quadratic model was significant with model f-value of 36.10. The "Pred R-Squared" of 0.8489 was in reasonable agreement with the "Adj R-Squared" of 0.9564; i.e. the difference was less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio of 17.661 indicates an adequate signal. This model can be used to navigate the design space. The details of Analysis of variance (ANOVA) for measured response have been presented in Table 7. The quadratic equation generated by software is as follows:

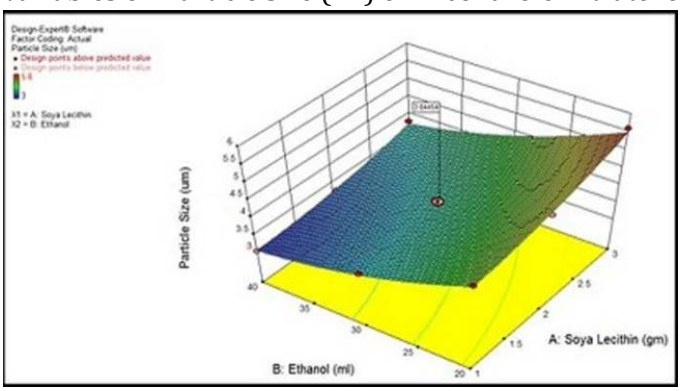
$$Y_1 = +3.84 + 0.40 * X_1 - 0.83 * X_2 - 0.050 * X_1X_2 + 0.13 * X_1^2 + 0.23 * X_2^2$$

Table (7): Data of ANOVA Study for Dependent Variable (Particle Size) from 3^2 Factorial Design

Source	Sum of squares	D.F	Mean square	F-value	Probability	
Response (Y1) = Particle size						
X1-Soya Lecithin	0.96	1	0.96	32.81	0.0106	Significant
X2-Ethanol	4.17	1	4.17	142.41	0.0013	
X1X2	0.010	1	0.010	0.34	0.5999	
$X1^2$	0.036	1	0.036	1.22	0.3508	
$X2^2$	0.11	1	0.11	3.72	0.1493	

The equation presents a positive effect of Soya Lecithin (Factor X_1) concentration and a negative effect of Ethanol (Factor X_2) concentration on particle size, indicating an increase in particle size as the concentration of soya lecithin increases and decrease in particle size as the concentration of ethanol increases. The combined effect of X_1 & X_2 indicates significant effect on particle size. The combined effect of factors X_1 and X_2 can further be elucidated with the help of response surface plots as shown in Figure 7A and B which demonstrated that Particle size increases, as the concentration of Soya Lecithin increases, and decreases as the concentration of Ethanol increases. It indicates that for 4µm Particle size, the concentration of soya lecithin and ethanol was 2 gm and 30 ml respectively. Figure 7C shows a linear relationship between the observed response values and the predicted values indicating the correctness of the model. For 4 µm Particle Size, the predicted value was 3.844 µm indicating correlation between the actual and predicted values. Hence, the concentration of batch F5 was selected.

Figure (7A): Response surface plot (3D surface plot) showing effect of formulation variables on Particle size (Y1) of Miconazole Nitrate loaded Ethosomes



Figure(7B): Response surface plot (Contour plot) showing effect of formulation variables on Particle Size (Y1) of Miconazole Nitrate loaded Ethosomes

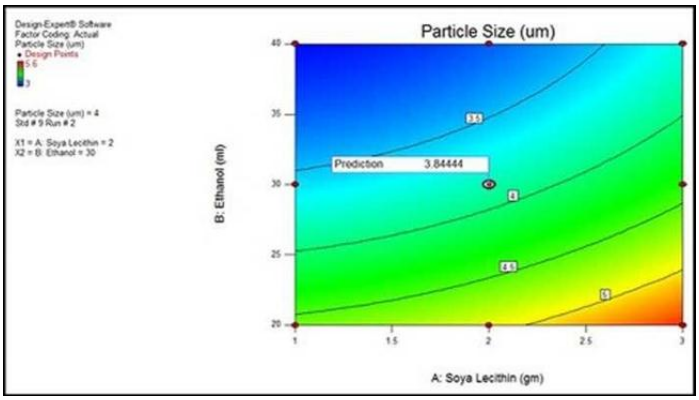
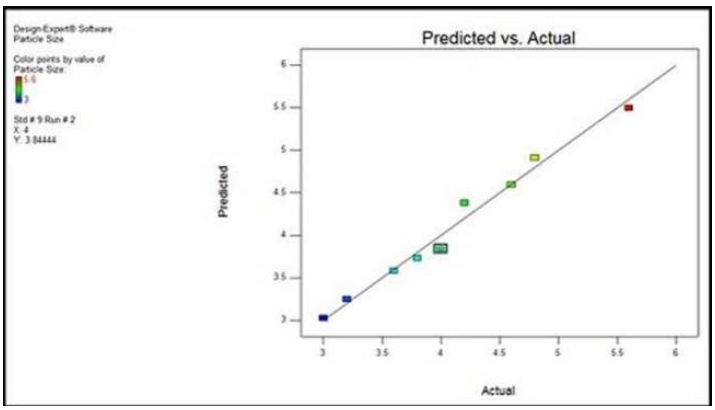


Figure (7C): Correlation between actual and predicted values for Particle size (Y1) of Miconazole Nitrate loaded Ethosomes.



11.3.2. Regression Analyses of % Entrapment Efficiency

Quadratic model was significant with model f-value of 22.32. The "Pred R-Squared" of 0.7675 was in reasonable agreement with the "Adj R-Squared" of 0.9302; i.e. the difference was less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio of 13.599 indicates an adequate signal. This model can be used to navigate the design space. The details of Analysis of variance (ANOVA) for measured response have been presented in Table 8**.** The quadratic equation generated by software is as follows:

$$Y_2 = +80.80 + 3.12 * X_1 - 4.95 * X_2 - 1.90 * X_1X_2 - 2.35 * X_1^2 - 1.55 * X_2^2$$

Table (8): Data of ANOVA Study for Dependent Variable (% Entrapment Efficiency) from 3^2 Factorial Design

Source	**Sum of squares**	**D.F**	**Mean square**	**F-value**	**Probability**	
Response (Y2) = % Entrapment Efficiency						
X_1-Soya Lecithin	58.28	1	58.28	27.61	0.0134	Significant
X_2-Ethanol	147.01	1	147.01	69.64	0.0036	
X_1X_2	14.44	1	14.44	6.84	0.0793	
X_1^2	11.05	1	11.05	5.23	0.1062	
X_2^2	4.81	1	4.81	2.28	0.2285	

The equation presents a positive effect of Soya Lecithin (Factor X_1) concentration and a negative effect of Ethanol (Factor X_2) concentration on entrapment efficiency, indicating an increase in entrapment efficiency as the concentration of soya lecithin increases and decrease in entrapment efficiency as the concentration of ethanol increases. The combined effect of X_1 & X_2 indicates significant effect on Entrapment Efficiency. The combined effect of factors X_1 and X_2 can further be elucidated with the help of response surface plots as shown in Figure 8A and B which demonstrated that % Entrapment Efficiency increases, as the concentration of Soya Lecithin increases, and decreases as the concentration of Ethanol increases. It indicates that for 82.2% Entrapment Efficiency, the concentration of soya lecithin and ethanol was 2 gm and 30 ml respectively. Figure 8C shows a linear relationship between the observed response values and the predicted values indicating the correctness of the model. For 82.2% Entrapment Efficiency, the predicted value was 80.8% indicating correlation between the actual and predicted values. Hence, the concentration of batch F5 was selected. The responses indicate that the optimized batch was F5. The surface response plot and contour plot revealed that F5 batch was the optimised batch with 4µm particle size and 82.2% entrapment efficiency. This optimized batch (F5 batch) was subjected to ultrasonication using Probe Sonicator, PS 150, Orchid Scientifics and Innovatives India Pvt. Ltd. at 4° C in 3 cycles of 2 minutes each with 5-minutes rest between the cycles to form nanoparticulate ethosomal dispersion.

Figure (8A): Response surface plot (3D surface plot) showing effect of formulation variables on Entrapment Efficiency (Y2) of Miconazole Nitrate loaded Ethosomes

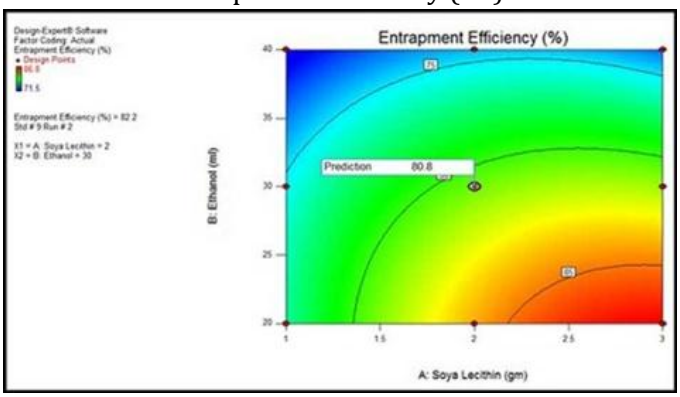


Figure (8B): Response surface plot (Contour plot) showing effect of formulation variables on Entrapment Efficiency (Y2) of Miconazole Nitrate loaded Ethosomes

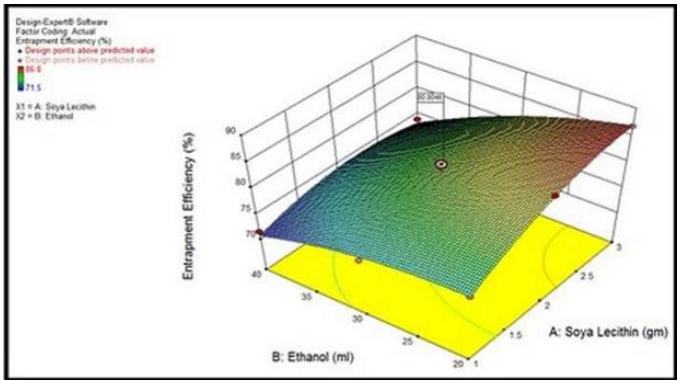
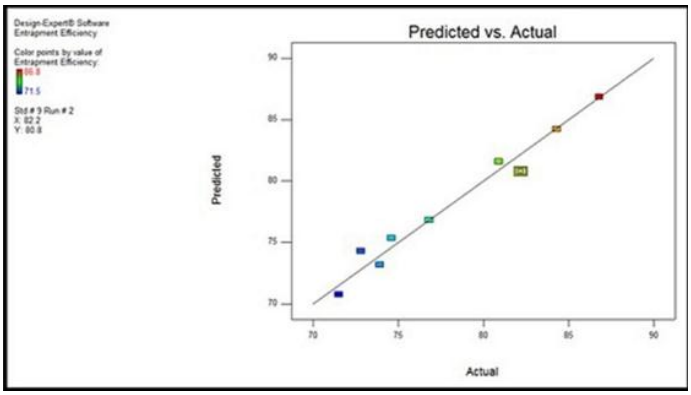


Figure (8C): Correlation between actual and predicted values for % Entrapment Efficiency (Y2) of Miconazole Nitrate loaded Ethosomes



11.3.3. Evaluation of optimized batch

The optimized ultrasonicated batch was further evaluated for % EE, particle size, zeta potential, SEM, and DSC. The % EE was found to be 81.54%. Ethosomes showed a mean particle size of 63.78 nm and zeta potential -41.2 mV as depicted in Figure 9 and 10**.** SEM studies revealed more or less spherical

vesicular structures of ethosomes and three dimensional structure with a smooth surface as seen in Figure 11A, B and C. DSC results exhibit the entrapment of Miconazole Nitrate in Ethosomes. The thermal curves of ethosomal formulation of Miconazole Nitrate are shown in the Figure 12 which showed a small peak at 152.42°C and a major peak at 162.30°C. The stability study carried out for 1 month by storage at refrigerated condition (2-8°C) showed no significant change in % EE which was found to be 80.93 ±0.032 %. This result confirms that there was no drug expulsion from Ethosomes.

Figure (9): Particle Size Distribution by Intensity of Optimized Batch of Ethosomes

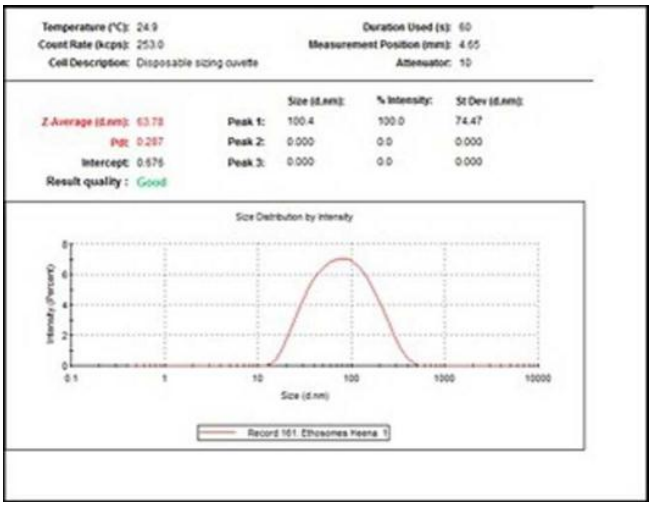


Figure (10): Zeta Potential of Optimized Batch of Ethosomes

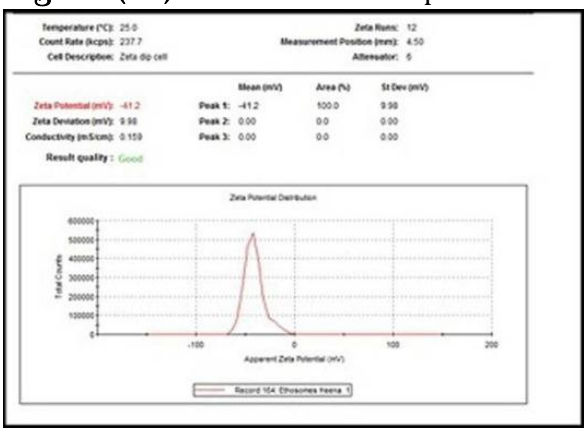


Figure (11A): Scanning Electron Microscopy of Miconazole Nitrate Loaded Ethosomes

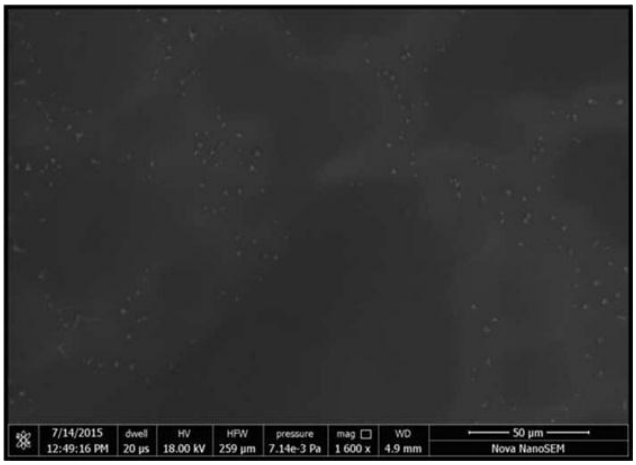


Figure (11B): Scanning Electron Microscopy of Miconazole Nitrate Loaded Ethosomes

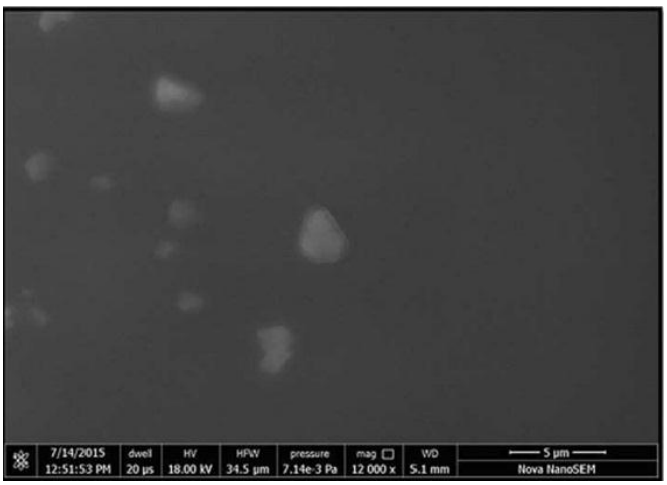


Figure (11C): Scanning Electron Microscopy of Miconazole Nitrate Loaded Ethosomes

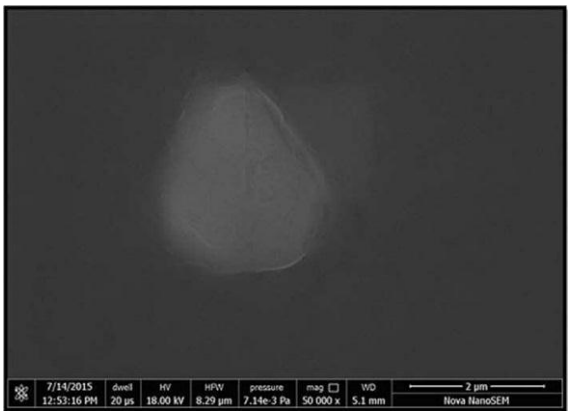
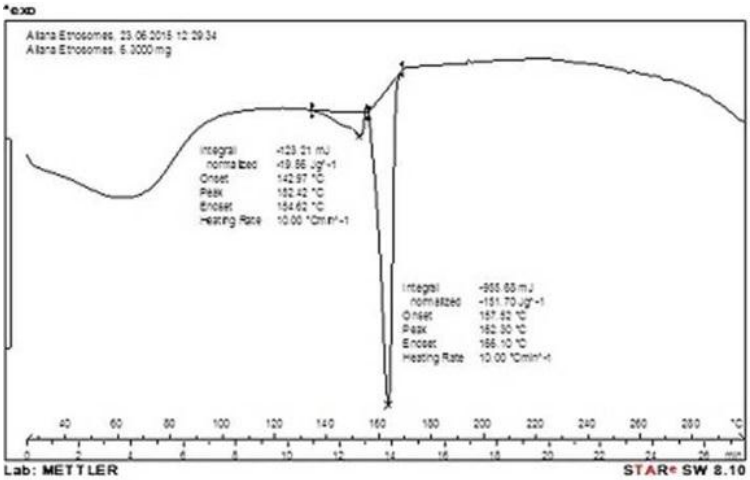


Figure (12): DSC Thermogram of Miconazole Nitrate Loaded Ethosomes



11.3.4. Miconazole Nitrate loaded ethosomal cream-o-gel

The Miconazole Nitrate loaded ethosomal formulation was made more pharmaceutically acceptable for topical application by incorporating it into the cream-o-gel. The Miconazole Nitrate loaded ethosomal cream-o-gel (CG1 to CG5) was formulated with concentration 2.5%, 3%, 3.5%, 4%, 4.5% w/v of polymer Simulgel INS 100 respectively and was evaluated for appearance, pH, viscosity, spreadability, centrifugation study, drug content and in-vitro drug release study and these results are presented in the Table 9. The prepared Miconazole Nitrate loaded ethosomal

cream-o-gel formulations were off-white viscous creamy preparations with a smooth and homogeneous appearance. The pH values of all formulations was found to lie in the normal pH range of the skin. In the centrifugation study, all the formulations showed stability i.e. there was no creaming, flocculation, and phase separation. As seen from the Figure 13, in-vitro drug release study showed that as the concentration of Simulgel INS 100 increases, drug requires more time to get diffused from the cream-o-gel network and thus drug release retards. Based on this results, CG3 formulation (3.5% w/v Simulgel INS 100) having good viscosity, acceptable consistency and drug release was selected for further evaluations. A comparative drug release study of the selected Miconazole Nitrate loaded ethosomal cream-o-gel (CG3) formulation and the plain drug cream-o-gel formulation as depicted in Figure 14, showed significant differences. % cumulative drug release of Miconazole Nitrate was 67.95±0.25% from ethosomal cream-o-gel formulation (CG3) and 35.91 ± 0.47% from plain drug cream-o-gel formulation after a period of 6 hrs. In-vitro anti-fungal study results as depicted in Figure 15A and B showed that the zone of inhibition of marketed cream (Micogel cream) and ethosomal cream-o-gel formulation (CG3) was found to be 28.5 mm and 27 mm respectively. Texture analysis of the Miconazole Nitrate loaded Ethosomal cream-o-gel formulation (CG3) was performed by evaluating two different parameters i.e. Adhesiveness and Spreadability using the texture analyser TA.XT plus. From the results of spreadability study as depicted in Figure 16, it was found that the formulation had firmness value of 77.778g and the peak positive distance was found to be 28.071mm and the separation distance was 0.051 respectively. Hence the formulation had good spreadability. From the results of adhesion study as depicted in Figure 17, it was found that the formulation had good hardness (6.712g)

and the stickiness (adhesion) value was found to be -6.256 g, which indicates that the formulation was less sticky (adhesive) and hence it had good patient compliance. Skin irritation study results have been depicted in Table 10 and Figure 18 A and B. The Primary irritation index (PII) was found to be 0, which falls under negligible irritation category. Hence the developed Miconazole Nitrate loaded Ethosomal cream-o-gel formulation was found to be free from skin irritation when tested on the skin of animals (healthy Wistar rats). During the stability study, Miconazole Nitrate loaded Ethosomal cream-o-gel formulation (CG3) was found to be stable for the period of one month at 25±2°C and accelerated temperature of 40 ± 2^0 C.

Figure (13): In vitro % cumulative drug release from Miconazole Nitrate loaded Ethosomal Cream-o-gel formulation batches

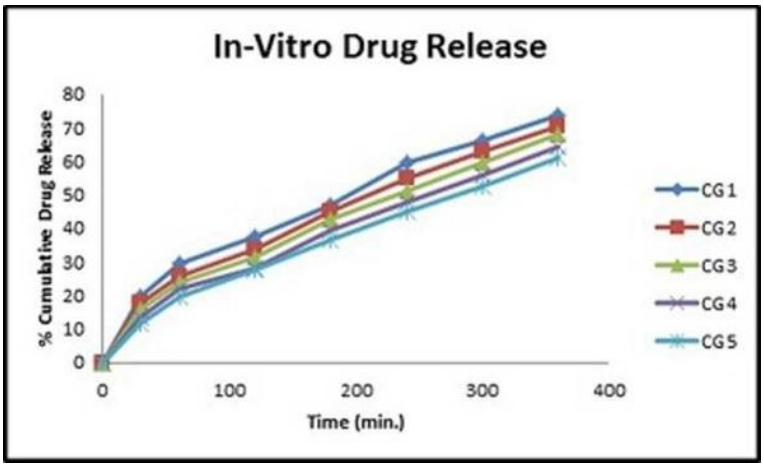


Figure (14): In-vitro drug release of Miconazole Nitrate loaded Ethosomal Cream-o-gel formulation (CG3) and Plain Drug Cream-O-Gel Formulation

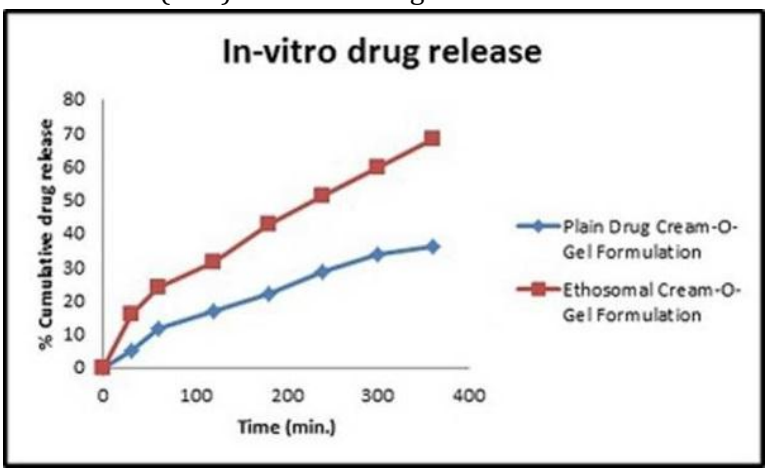


Figure (15A): Zone of inhibition of marketed cream (Micogel cream)

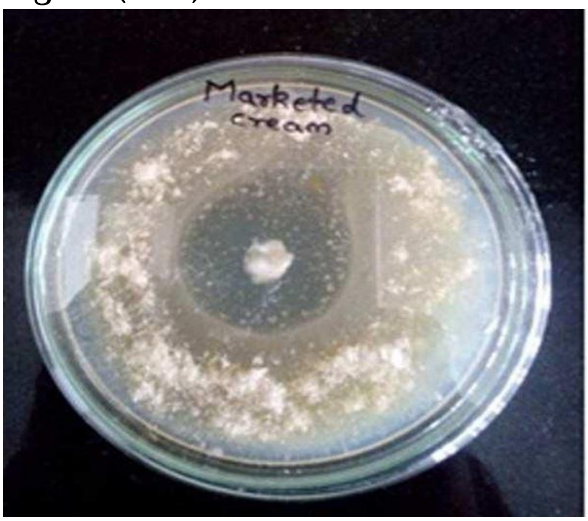


Figure (15B): Zone of inhibition of Miconazole Nitrate loaded ethosomal cream-o-gel

Figure (16): Spreadability of Miconazole Nitrate loaded Ethosomal cream-o-gel

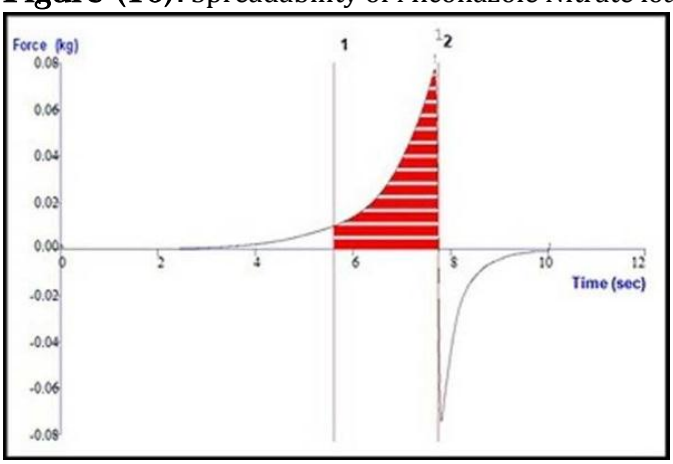


Figure (17): Adhesion of Miconazole Nitrate loaded Ethosomal cream-o-gel

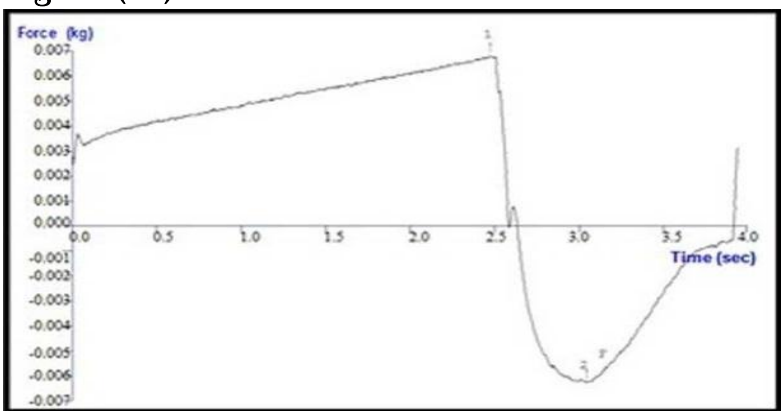


Figure (18A): Skin Irritation Study of Animals applied with Cream-o-gel base

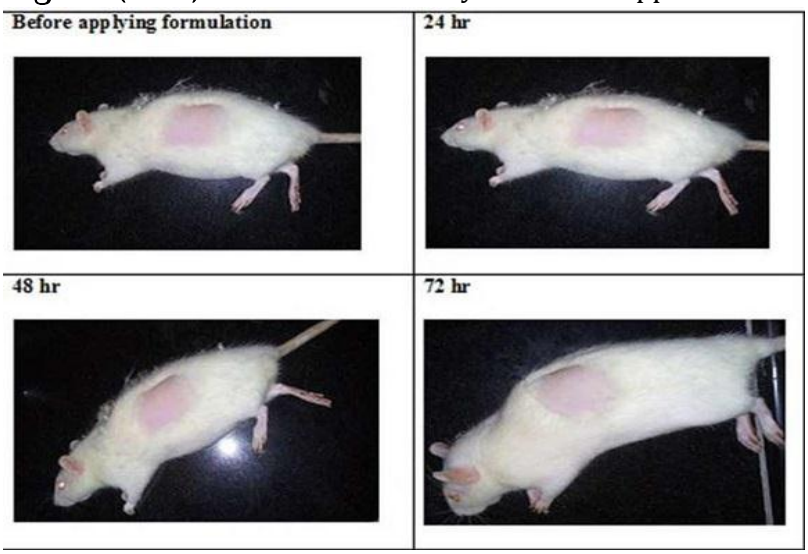


Figure (18B): Skin Irritation Study of Animals applied with Miconazole Nitrate loaded Ethosomal Cream-o-gel formulation (CG3)

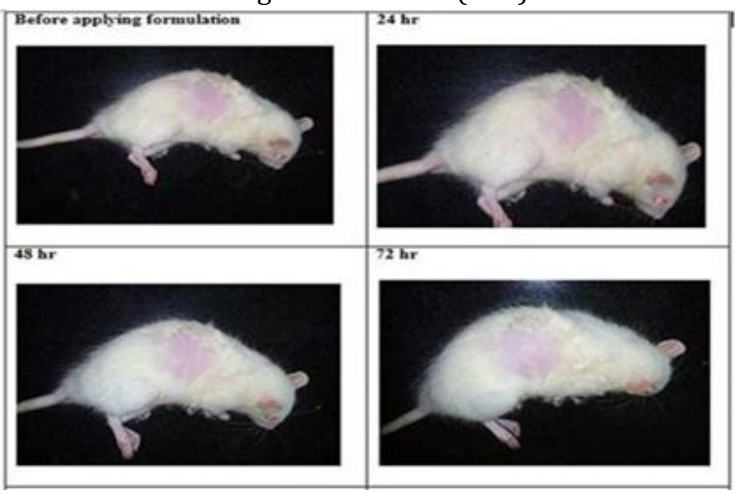


Table (9): pH, Viscosity, Spreadability, Centrifugation Study and Drug Content of Miconazole Nitrate Loaded Ethosomal Cream-O-Gel Formulations.

Sr no	Formulations	pH	Viscosity (Pa/s) (mean SD ± n=3)	Spreadability (gm.cm/s) (mean ± SD,n=3)	Percentage Drug Content
1	CG1	6.18 ± 0.52	2.215 ± 0.43	32.60 ± 0.15	96.62%
2	CG2	6.32 ± 0.15	3.514 ± 0.25	30.61 ± 0.46	97.81%
3	CG3	6.59 ± 0.25	4.917 ± 0.14	28.30 ± 0.25	98.02%
4	CG4	6.65 ± 0.41	5.214 ± 0.18	26.78 ± 0.61	97.19%
5	CG5	6.84 ± 0.16	6.452 ± 0.54	24.59 ± 0.32	96.32%

12. Discussion

QbD is concerned with the achievement of certain predictable quality with desired and predetermined specifications through relating the critical process parameters (CPP) to the critical quality attributes (CQAs) of drug product. It uses multivariate experiments to understand product and process and to establish a design space through design of experiments(DOE). The Particle size distribution of ethosomes was known to be influenced by the composition of ethosomes. The size of the vesicles decreased when the ethanol concentration was increased from 20% to 40%. This significant difference in the size of ethosomal formulations was because of the presence of different concentrations of ethanol. Probably, ethanol causes a modification in net charge of the system and confers it some degree of stearic stabilization that leads to decrease in mean vesicle size. Also, it was observed that the size of the vesicles increase with the increasing soya lecithin concentration. The concentration of ethanol and soya lecithin, used for ethosomes preparation, were found to have influenced the entrapment efficiency. The entrapment efficiency of ethosomes was found to have decreased with the increase in ethanol

concentration. The reason for this could be the increase in the fluidity and presence of thinner membrane of the ethosomal vesicles with the higher ethanol concentration.

Table (10): Score of Skin Irritation Test after Application of Cream-O-Gel Base and Miconazole Nitrate Loaded Ethosomal Cream-O-Gel

Cream-o-gel base							
		Score for skin reaction					
		Erythema			**Edema**		
Sr No.	Rats	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
1	1	0	0	0	0	0	0
2	2	0	0	0	0	0	0
3	3	1	0	0	0	0	0
4	4	0	0	0	0	0	0
5	5	0	1	0	0	0	0
6	6	0	0	0	0	0	0
Miconazole Nitrate loaded Ethosomal Cream-o-gel formulation (CG3)							
1	1	0	0	0	0	0	0
2	2	1	0	0	0	0	0
3	3	0	0	0	0	0	0
4	4	0	0	0	0	0	0
5	5	0	1	0	0	0	0
6	6	0	0	0	0	0	0

The entrapment efficiency increased with the increase in the concentration of soya lecithin. Mean particle size and size distribution of optimised batch of Ethosomes was determined by dynamic light scattering using Zetasizer Ver. 6.34 (Malvern instrument Ltd., Malvern, UK) at room temperature. Photon correlation spectroscopy (PCS) is a technique used to determine the mean particle size diameter (mean PCS diameter)/ Z-average and the width of the particle size distribution expressed as Polydispersity Index (PDI). The measurement using PCS is based on the light scattering phenomena in which the statistical intensity fluctuations of the scattered light from the particles in the measuring cell are measured and the Particle Size Distribution is represented as Intensity. Figure 9 indicates that 100% nanoparticles are of 100.4 nm size. The mean particle size diameter/Z-average was 63.78 nm size. PDI shows the particle size distribution. This value helps to determine whether the suspension is mono-dispersed or poly-dispersed. Generally, if the PDI value lies between 0.00-0.5. the suspension is considered mono-dispersed, while PDI values greater than 0.5 indicates poly-dispersed suspension. PDI of 0.287 was obtained which indicates that 570 the ethosomal suspensions are mono-dispersed. Charge on drug loaded droplet surface was determined using Zetasizer Ver. 6.34 (Malvern Instruments Ltd., Malvern, UK). In colloidal systems, according to electric double layer theory, there is net balance of

attractive as well as repulsive forces. Zeta potential imparts positive or negative charge on surface of colloidal particle so one of these two forces may play its role. Zeta potential is an important parameter that affects the aggregation of vesicles and depicts the physical stability of vesicular systems. Zeta potential of optimized formulation was found to be (-41.2 mV) as seen in Figure 10, which indicated good stability. Formulations having zeta potential value negative than −30 mV are normally considered stable for colloidal dispersion and this range of zeta potential prevents the aggregation between vesicles and hence, enhances its physical stability. It has been investigated that high zeta potential in Ethosomes increase the interbilayer distance owing to electrostatic repulsion. The high negative charge on ethosomes is dependent on two factors: (i) ethanol, which provides a net negative surface charge thus avoiding aggregation of vesicles due to electrostatic repulsion; and (ii) lecithin, which provides a greater rigidity to the layers and reduced likelihood of vesicles fusion, as well as a greater resistance to the high rotational energy exerted by sonication, resulting in a high negative charge. The SEM morphology showed the formation of uniform, more or less spherical vesicular structures of ethosomes. Encapsulation of Miconazole Nitrate can be clearly observed within the vesicles. It confirms the ethosomes to be three dimensional with a smooth surface. The thermal curves of ethosomal formulation of Miconazole Nitrate are shown in the Figure 12 which showed a small peak at 152.42°C and a major peak at 162.30°C. But the DSC thermogram of Miconazole Nitrate loaded ethosomes did not show the melting thermogram of Miconazole Nitrate i.e.185.59°C, in fact the thermogram was observed at 152.42°C and 162.30°C. This signifies that incorporated Miconazole Nitrate has interacted well with the phospholipid. The absence of Miconazole Nitrate melting thermogram also proves the enhanced entrapment of drug within the vesicles. The Miconazole Nitrate loaded ethosomal formulation was made more pharmaceutically acceptable for topical application by incorporating it into the cream-o-gel (CG1 to CG5) which was formulated with concentration 2.5%, 3%, 3.5%, 4%, 4.5% w/v of polymer Simulgel INS 100 respectively. It was concluded that, as the concentration of Simulgel INS 100 increases gradually, viscosity of the cream-o-gel increases and the Spreadability was found to be decreased. The higher drug release was observed with formulation CG1. The progressive increase in the amount of drug diffusion through membrane from formulation was attributed to gradual decrease in the concentration of polymer. It has been concluded that, if the concentration of polymer increases, the diffusion of drug through the membrane decreases. As the concentration of Simulgel INS 100 increases, viscosity of the cream-o-gel increases causing thickening of the cream-o-gel. Drug requires more time to get diffused from the cream-o-gel network and thus drug release retards. A comparative drug release study of the selected Miconazole Nitrate loaded ethosomal cream-o-gel (CG3) formulation and the plain drug cream-o-gel formulation showed significant differences. % cumulative drug release of Miconazole

Nitrate was more from ethosomal cream-o-gel formulation (CG3) than plain drug cream-o-gel formulation after a period of 6 hrs. Hence proving the effectiveness of the Ethosomal formulation to achieve high permeability and ultimately increase the bioavailability of the drug. In-vitro antifungal activity results depicts that the zone of inhibition of Miconazole Nitrate loaded ethosomal cream-o-gel is very near to that of Marketed cream (Micogel cream 2%). The developed formulation showed comparable in-vitro antifungal activity with the marketed cream (2%), even though the conc. of Miconazole Nitrate was less in the developed formulation. This can be attributed to the extra potential of ethanol to kill organisms by denaturing their proteins and dissolving their lipids, apart from skin fluidization and penetration. From this analysis, we can assume that ethosomes containing formulation will prove as a better carrier system and a therapeutically promising candidate for the efficient treatment of fungal infection over the conventional formulations. Hence the developed Miconazole Nitrate loaded ethosomal cream-o-gel formulation has potential antifungal activity against *C.albicans.*

13. Conclusion

Together the results indicate that, with the aid of QbD approach, Miconazole Nitrate loaded ethosomes had been successfully developed and the formulation was made pharmaceutically acceptable for topical application by incorporating it into the cream-o-gel, and the hypothesis of Ethosomal formulation to achieve high permeability and ultimately increase the bioavailability of the drug possessing potential antifungal activity was achieved.

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