Development and optimization of ketoconazole loaded nano-transfersomal gel for vaginal delivery using Box-Behnken design: In vitro, ex vivo characterization and antimicrobial evaluation

Shalu Singha, Devina Vermaa, Mohd. Aamir Mirzb, Ayan Kumar Das c, Mridu dudeja c, Md. Khalid Anwer d, Yasmin Sultanaa, Sushama Talegaonkara, Zeenat Iqbal a, *

a Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard University, New Delhi 110062, India
b New Zealand Fulvic Limited, Mount Mounganui, Tauranga 3116, New Zealand
c Department of Microbiology, Hamdard Institute of Medical & Scientific Research, Jamia Hamdard, New Delhi 110062, India
d Department of Pharmaceutics, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-kharj 11942, Saudi Arabia

ARTICLE INFO

Article history:
Received 2 January 2017
Received in revised form 26 February 2017
Accepted 8 March 2017
Available online 11 March 2017

Keywords:
Ketoconazole
Transfersome
Nanogel
Permeation
Antimicrobial studies

ABSTRACT

In the present study, Ketoconazole loaded transfersomal formulation was developed using 3-factor, 3-level Box–Behnken design to find out the best formulation. Optimized transfersomal formulation was prepared by solvent evaporation method and evaluated in vitro for vesicle size (126.9 ± 5.45 nm) and entrapment efficiency (82.6%). Transfersomal gel were developed by incorporation of optimized transfersomal formulation into 1% carbopol gel base and characterized by physical evaluation and rheological studies. The cumulative release of drug were shown at 72 h approximately 74% and 97% of ketoconazole from suspension and transfersomal gels respectively. The flux for transfersomal gel was found to be about 3 times that of drug suspension gel. Results of the histopathological studies of gel treated skin indicated negligible sign of toxicity and irritation. The prepared transfersomal gel showed antimicrobial activity against Candida albicans with MIC 4.57–4.6 mg/mL and better zone of inhibition as compared to reference standard. The developed gel showed promising antimicrobial activity. These overall findings suggested that transfersomal gel holds an excellent potential for ketoconazole delivery.

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1. Introduction

Ketoconazole, an antifungal drug, is used to treat serious fungal or yeast infections, such as candidiasis, blastomycosis, coccidiomycosis, histoplasmosis, chromoblastomycosis. This medicine works by killing the fungus or yeast, or preventing its growth [1–3]. Vulvovaginal candidiasis (VVC) is a common gynecological pathology occurring when there is a yeast infection that affects nearly 75% of all adult women [4,5]. Classically, VVC is described by a white “cheese” release alongside vulva and vaginal irritation. The most widely recognized reason for contagious contaminations is Candida species, prompting a scope of intrinsically life-threatening to non-life-unthreatening mucocutaneous disease. Candida albicans is a demographic yeast that colonizes the skin, reproductive and the gastrointestinal tract [6]. The pathogenesis and prognosis of candidial contaminations are influenced by the host immune status furthermore vary enormously as indicated by infection presentations. Subsequently, diagnosis, managment, and treatment choice changes and ought to be considered in the general setting of the affected human host [7]. The treatment methods of Vaginal Candidiasis primarily include topical treatment and long term treatment (3–5 months) may be most effective. The long term use of drug increases the chances of missing the dose and increasing the side effects. Therefore, it is better to develop a formulation which reduces the frequency of administration and which retains for long period of time [8].

Transfersomes are novel ultra deformable vesicular carrier system primarily composed of phospholipid, surfactant, and water. Because of their self-optimized and ultra-flexible membrane properties, they can deliver the drug into the vagina through the vaginal mucosa [9]. The vesicular transfersomes are more elastic than the conventional liposomes because of the presence of the
edge activators in the vesicular membrane and are thus well suited for delivering drugs through the vaginal mucosa as compared to the rigid liposomes [10,11]. This enhanced penetration of Transfersomes is associated by its ability to squeezing themselves along the intracellular sealing lipid of the stratum corneum. The extremely high flexibility of their membrane permits transfersomes to squeeze themselves even through pores much smaller than their own diameter. This is due to high flexibility of the transfersome membrane and is achieved by judiciously combining at least two lipophilic/amphiphilic components (phospholipids plus bio surfactant) with sufficiently different packing characteristics into single bilayer. Transfersomes has the potential of overcoming the skin barrier and have been reported to enhance the permeability of drug [12,13].

2. Material and method

2.1. Materials

Different phospholipids phospholipon 90 G and Lipoid S100 were obtained from Lipoid® GmbH (Germany). Tween 80 was purchased from Thomas Bakers (Mumbai, India). Ethanol was purchased from Jiangsu Huaxi International Trade, Co. Ltd., China. Ketoconazole was obtained as a generous gift from Sigma Aldrich (Germany). Water and other solvents used in the HPLC analytical method were of HPLC grade. All other reagents were of analytical grade and were purchased from Merck, Mumbai, India.

2.2. Experimental design for optimization of transfersomes using factorial design

A 3 factor, 3-level factorial design was used to explore the quadratic response surfaces and for constructing second for second order polynomial models using Design-Expert® (Version 10, Stat-Ease Inc., Minneapolis, MN). A design matrix comprising of 17 experimental runs constructed, for which the non-linear computer generated quadratic model is defined as: 

\[ Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \]

where Y is the measured response associated with each factor level combination; b0 is constant; b1, b2, b3 are linear coefficients, b12, b13, b23, are interaction coefficients between the three factors, b11, b22, b33, are quadratic coefficients computed from the observed experimental values of Y from experimental runs; and X1, X2 and X3 (i = 1, 2, 3) represent the interaction and quadratic terms, respectively. The independent variables were selected as amount of Lipoid S 100 (X1), Tween 80 (X2) and ethanol (X3). The dependent variables were particle size (Y1), entrapment efficiency (Y2), and poly dispersive index (PDI) (Y3) with constraints applied on the formulation of transfersomes. The concentration range of independent variables under study is mentioned in the Table 1 along with their low and high levels.

2.3. Development of optimized transfersomes

Transfersomes were prepared by conventional solvent evaporation method. Optimized batch of transfersomes was prepared using surfactant, phospholipids and drug. Accurately weighed ketoconazole was dissolved in ethanolic solution of Lipoid S 100 in a beakers organic phase. This organic phase were added slowly to aqueous phase containing surfactant dropwise with the help of syringe on continuous stirring and is kept for almost 6–7 h for solvent to evaporate. Then this mixture is sonicated for 2–3mins to obtain nano transfersomes particles [14].

2.4. Characterization of optimized ketoconazole transfersomal formulation

The in vitro behavior of the prepared formulation would be depicted through its characterization.

2.4.1. Scanning Electron Microscopy (SEM)

The surface morphology of optimized transfersome was examined by SEM. 1–2 drops of vesicular dispersion was mounted on a glass and paste over grid by using double-sided carbon adhesive tape and sputter-coated with conductive gold-palladium. A round coverslip was gently placed over the stub to enable uniform conductivity and a silver paint lining was applied to the edges of the coverslip to fill the narrow spacing between the stub and coverslip. They were viewed with an EVO LS 10 (Carl Zeiss, Brighton, Germany) scanning electron microscope operating at an accelerating voltage of 200 kV under high vacuum. The particles were examined for surface characteristics like shape, size, pores, pits and presence of aggregation [15].

2.4.2. Transmission Electron Microscopy (TEM)

Morphology and structure of the transfersome was studied using TEM (Morgagni 268 D FEI Company 155, Netherland). The samples were treated on copper grids (Polysciences, Warrington, PA, USA) without salt of heavy metal viz. 1% phosphotungstic acid for negative staining, followed by sample drying. They were then analyzed by TEM at an accelerating voltage of 200 kV and data acquisition was done on the AMT Image Engine [16].

2.4.3. Vesicle size determination

The vesicles size and size distribution were determined by Dynamic Light Scattering (DLS) technique using a computerized inspection system (Malvern Zetasizer, Nano-ZS, Malvern, U.K.) with DTS (nano) software® [16]. Optimized vesicular suspension was diluted with distilled water and placed in quartz cuvette and then subjected to size analysis and the measurements were conducted in triplicate.

2.5. Determination of percent entrapment efficiency (PDE)

Entrapped transfersomal vesicle were separated from untrapped ketoconazole by centrifugation at 10000 rpm for 45 min. The concentration of ketoconazole in supernatant were measured by UV spectrophotometer. Percent drug entrapment was calculated by using following equation [1]:

\[ PDE = \frac{W - w}{W} \times 100 \]

where W = theoretical amount of Ketoconazole; w = observed amount of Ketoconazole in supernatant.

Table 1

| Considered variables and responses along with their levels and constraints. |
|------------------|--------|--------|
| **Factor**       | **Levels** |
| **Independent variables** | Low | High |
| X1 = Lipoid S 100 (mg) | 30 | 90 |
| X2 = Surfactant (mg) | 20 | 60 |
| X3 = Solvent (ml) | 1 | 3 |
| **Dependent variables** | | |
| Y1 = Particle size (nm) | | |
| Y2 = Entrapment efficiency (SEE) | | |
| Y3 = Polydispersity index (PDI) | | |
2.6. Development of KET loaded transfersomal gel

The optimized ketoconazole transfersome was incorporated in gel by dispersing the 1% Carbopol 974P in a sufficient quantity of distilled water with continuous stirring for 10 min. After complete dispersion, the 1% Carbopol 974P solution was left overnight for complete swelling and then sonicated for 15 min to remove air bubbles. Triethanolamine was then added to it, until transparent gel was formed.

2.7. Evaluation of KET loaded transfersomal gel

2.7.1. Homogeneity

Developed gels were tested for homogeneity by visual inspection after the gels have been settled in the container. They were observed for their appearance and presence of any aggregates [17].

2.7.2. Rheology

The viscosity of formulated gel was determined using Brookfield viscometer with spindle number C-50-1 and the determined terminations were carried out in triplicate [17].

2.7.3. pH evaluation

The apparent pH of the gel was measured by pH meter (Accu- mentAB 15, Fisher scientific, USA) in triplicate at 25±1°C.

2.7.4. Extrudability

A simple method was adopted for determination of extrudability in terms of weight in gram required to extrude a 0.5 cm ribbon of gel in 10 s from a collapsible tube [17]. A closed collapsible tube containing gel was pressed firmly at the crimped end. When the cap was removed gel extruded until pressure was released. More quantity extruded better is extrudability.

2.7.5. In-vitro release study of ketoconazole loaded transfersomal gel

In-vitro release study was carried out in 100 mL of Phosphate buffer of pH 4.5 at 100 rpm and 37±0.5°C. 1 mL of transfersomal gel formulation was placed in treated dialysis bag (cut off molecular weight of 12000 g/mol) and 3 mL sample withdrawn at regular time interval (0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16 & 24 h) and filtered through 0.45 μm membrane filter the samples were then analyzed for drug content by UV spectrophotometry and cumulative percentage drug release was determined [19]. The flux (µg/cm²/h) and Permeability coefficient (Kp) were calculated using following equations:

\[ \text{Flux} = \frac{\text{cumulative amount of drug permeated}}{\text{v/s time}}. \]

\[ \text{Permeability coefficient (Kp)} = \frac{\text{Flux}}{\text{Drug concentration in donor compartment}} \]

2.7.6. Ex-vivo permeation studies

Ex-vivo permeation was performed using a Franz Diffusion cell with an effective diffusion surface area and receiver chamber capacity using excised, defatted goat vaginal tissues. The tissue was stored in the deep freezer at -21°C. During experiment, it was brought to room temperature and mounted between donor and receiver compartment of the Franz diffusion cell. The lumen side of the vaginal tissue was facing the donor compartment and the opposite side was facing the receiver compartment. The vaginal tissue was initially stabilized with stimulated vaginal fluid (SVF) (pH-4.2) before initiation of the experiment. Receiver chamber was filled with SVF and stirred with a magnetic rotor at a speed of 100 rpm in the incubator shaker maintaining temperature at 37°C ± 1°C. The whole media was replaced with the fresh buffer after every 30 min to stabilize. After running six cycles of stabilization, 1 mL of the sample (ketoconazole transfersomal gel containing 0.5% w/v) was placed into the donor compartment with 0.75 mL of SVF to mimic the condition of vaginal milieu. 20 mL phosphate buffer (pH 4.5) were added to the receptor compartment. The samples were withdrawn at regular interval (0.5,1,2,3,4,6,8,10, 12, 14, 16 & 24 h) and filtered through 0.45 μm membrane filter the samples were then analyzed for drug content by UV spectrophotometry and cumulative percentage drug release was determined [19].

2.8. Histopathology

The gel was then subjected to the histopathological using goat vaginal skin section. The vaginal skin section samples was removed and fixed in 10% neutral buffered formalin. After processing, skin were sectioned into 5 mm thick and stained with hematoxylin and eosin and examined by light microscopy for morphological changes [20].

2.9. Stability study as per ICH Q1A (R2)

Stability study of the Transfersomal formulation was carried out as per the guidelines given in the ICH Q1A (R2) (ICH topic Q1 (R2), 2009). Three batches of ketoconazole transfersomal formulation (CTF) was stored for 3 months at 25°C ± 2°C/65% ± 5% and 40 ± 2°C/75% ± 5% RH. At the end of 0, 30, 60, 90 days, samples were withdrawn and evaluated for particle size distribution, precipitation and phase separation. For evaluation of drug content, sample were diluted with solvent and analyzed using HPLC method. The logarithmic percentage of drug remaining in the formulation was plotted against time (days). Slopes of the straight lines for each temperature were obtained and degradation rate constants (K) were calculated [21] using the following equation:

\[ \text{Slope} = -K/2.303 \]

where, K is degradation rate constant.

The shelf life of the transfersomal formulation at 25°C was determined by calculating the time required for degradation of 10% drug in the formulation from the following equation:
where, $t_{10^9} = \frac{2.303}{K} \times \log\left(\frac{100}{90}\right)$

where, $t_{10^9}$ is the time required for degradation of 10% drug in the transfersomal formulation.

2.10. Antimicrobial studies

An isolated culture of Candida albicans were suspended into a tube containing sabouraud dextrose broth and suspension was vortexed, then incubated at 37 °C for 24 h. The turbidity of growth was adjusted using 0.5 Mcfarland standard. One mL of the inoculum was blended with the liquefied Mueller-Hinton agar, then filled a sterile petri dish (15 cm, diameter), and permitted to harden. Wells were cut by punching a stainless steel chamber onto the plate and expelling the agar by a Pasteur pipette to frame a well. Eight different concentration of ketoconazole were filled in each well of agar plate and incubated at 37 °C for 24 h. Zone of inhibition diameter were measured at different time interval (0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 18 h and 24 h). Minimum inhibitory concentration (MIC) of ketoconazole in transfersomal gel was also determined [22].

3. Results & discussion

3.1. Optimization and characterization of KTZ transfersomal formulation

3.1.1. Optimization of transfersomal formulation

The optimum Ketoconazole Transfersomal formulation systems was selected based on the particle size and entrapment efficiency; minimizing the vesicle size by applying point prediction method of the Design Expert software [23]. Upon exploring the various response variables and comprehensive evaluation of feasibility search exhaustive grid search, the composition shown in Table 1 was selected.

Based on the 17 different transfersomal formulations designed by the Design Expert® software, particles with a wide average size range from 126.4 nm to 392 nm were obtained (Table 2). Among these formulation, Formulation (F2) composition with Lipoid S 100 (60 mg), Tween 80 (40 mg), and ethanol (2 mL) and 10 mg of ketoconazole was found to fulfill requisites of an optimum formulation. F2 was found optimized with particle size, entrapment efficiency (%EE) and PDI, 126.9 ± 5.45 nm, 82.6% and 0.255 respectively. The size and morphology of the optimized formulation evaluated using TEM, SEM and further incorporated in gel. Response surface 3D plots were generated by Design Expert software showing effect of independent variables on Vesicle size, Entrapment efficiency and PDI of KTF. These plots were utilized to study about the impacts of two independent variables on the responses while holding the third variable at a constant level. The graphs obtained for responses Y1, Y2 and Y3 are shown in Figs. 1–3.

3.2. Characterization of optimized ketoconazole transfersomal formulation

3.2.1. Vesicular shape, morphology and PDE

The micrographs obtained from SEM and TEM reveals the presence of irregular shape and smooth and even surface of the transfersome (Fig. 4). The size as depicted in the TEM ranged from 140 to 250 nm which was found close with the result obtained by using Malvern zetasizer (126.9 ± 5.45 nm). The experimental value (82.6%) of entrapment efficiency of optimized formulation was also very close to the predicted value (89.4%) Design Expert® software (Table 3).

3.3. Evaluation of ketoconazole gel formulation

Ketoconazole Transfersomal gel prepared with 1% Carbopol 974P was evaluated for crucial parameters listed in Table 4. The gel showed good homogeneity, sufficient skin retention due to permissible viscosity, high spreadability and extrudability for the formulation.

Apparent viscosity values as a function of increasing shear rate for gel was obtained as shown in Fig. 5. The rheogram for gel demonstrated characteristic of pseudoplastic flow, which indicates that their viscosity decreases as the shear rate increases [24]. The gel was found uniform with pourable viscosity.

3.4. In- vitro release study of ketoconazole loaded transfersomal gel

In vitro release studies were carried out for drug suspension and

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<th>Runs</th>
<th>Factors</th>
<th>Responses</th>
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<tr>
<td></td>
<td>Lipid (mg)</td>
<td>Surfactant (mg)</td>
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<tr>
<td>1</td>
<td>80</td>
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Fig. 1. 3D-Response surface plot showing effect of independent variables on particle size of ketoconazole Transfersomal formulation (KTF).

Fig. 2. 3D-Response surface plot showing effect of independent variables on entrapment efficiency of Ketoconazole transfersomal formulation (KTF).
**Fig. 3.** 3D-Response surface plot showing effect of independent variables on PDI of Ketoconazole Transfersomal formulation (KTF).

**Fig. 4.** TEM and SEM images of Ketoconazole Transfersomal formulation.

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<th>Table 3</th>
<th>Composition of optimized ketoconazole Transfersomal formulation.</th>
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<td>Composition</td>
<td>Optimized level</td>
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<td>Lipoid S 100 (mg)</td>
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<tr>
<td>Tween 80 (mg)</td>
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<td>Ethanol (ml)</td>
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<th>Table 4</th>
<th>Evaluation of Ketoconazole Transfersomal gel formulation (KTGF).</th>
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<td>Homogeneity</td>
<td>Mean Viscosity (Pa.s)±S.D.</td>
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<td>Good</td>
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transfersomal gel in phosphate buffer pH 4.5. The % cumulative drug release from the formulations were presented in Fig. 6. Initially, formulations shows fast drug release with burst effect followed by slow and consistent release for the remains of time of study confirming a controlled release pattern. The initial burst release of medication is valuable as it help to achieve therapeutic concentration in negligible time duration after that it maintain steady release to keep up managed and control concentration of drug [25]. The cumulative release of ketoconazole from suspension and transfersomal gels were initially fast i.e, 27.35% and 40.67% after 6 h respectively. Maximum cumulative release were shown at 72 h approximately 74% and 97% of ketoconazole from suspension and transfersomal gels respectively.

The correlation coefficient was determined for transfersomal gel by kinetic model (Table 5). The result of in vitro release study of Ketoconazole Transfersomal gel formulation was fitted to different releases models, the results however showed that the release profile of KTFG followed Higuchi kinetics (values of $R^2$ 0.9874). The Higuichi model was developed to depict the release of low soluble drugs incorporated in the semisolid and solid matrices.

3.5. Ex-vivo permeation studies

Flux values of the gels i.e. transfersomal gel and drug suspension gel was found in the range of $0.017 \pm 0.01 \text{mg/cm}^2/\text{h}$ and $0.006 \pm 0.0004 \text{mg/cm}^2/\text{h}$ respectively (Table 6.). The flux value for transfersomal gel was significantly higher than drug suspension gel. This can be attributed to the presence of polymer which play a major role in drug diffusion and permeation. Another reason of enhanced permeation of transfersomal gel could be the nanosized ultratransformable nature of the transfersomal vesicles and increase in the interfacial area which influences transportation of the drug [19].

3.6. Histopathology

Results of the histopathological studies showed that the intensity of dye in the animal skin treated with the optimized transfersomal gel was much higher than control. This indicated the bio-adhesive potential of the formulation and hence better retention shown in Fig. 7.

The prolonged retention of the gel can be attributed to the fact that the basic components of the mucus layers are mucin glycoproteins with oligosaccharide chains. Since the pKa of sialic acid is 2.6, the mucin network carries a substantial negative charge at physiological vaginal pH of pH 4–5. This charged portion interacts with the ionic components of the gel resulting in bioadhesion. It was concluded that optimized gel of ketoconazole did not show any

![Fig. 5. Rheogram of ketoconazole transfersomal gel.](image)

![Fig. 6. Comparative release kinetic for transfersome gel formulation and drug suspension.](image)

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<th>Table 5</th>
<th>Drug release kinetics.</th>
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<td>Zero order</td>
<td>First order</td>
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<td>$R^2$</td>
<td>$R^2$</td>
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<td>0.8392</td>
<td>0.8510</td>
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<th>Table 6</th>
<th>Ex-vivo permeation profile of Transfersomal gel and drug suspension gel. Mean ± SD was calculated from the slope of the linear portion of graph. $K_p$ was calculated by dividing Flux with the concentration of the drug in donor cell. Standard deviation in case of $K_p$ has not been given, considering the small values and bulkiness of the table.</th>
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<td>Samples</td>
<td>Flux (mg/cm²/h)</td>
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<td>Transfersomal Gel</td>
<td>$0.017 \pm 0.01$</td>
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<tr>
<td>Drug suspension Gel</td>
<td>$0.006 \pm 0.0004$</td>
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sign of toxicity. On a 0–4 scale basis, it was given the score zero, considering the negligible signs of irritation.

3.7. Stability study as per ICH Q1A (R2) and determination of shelf life

At predetermined time intervals, sample were kept for stability studies and were evaluated for the particle size distribution, formation of precipitate, phase separation and drug content. It was found that transfersomes at the end of 90 days showed no precipitation or, phase separation and particle size of 189 ± 2.98 (Table 7). Slopes and degradation rate constants (K) were calculated and presented in Table 8 [21]. The shelf life of formulation transfersomes was found to be 452 days i. e 1.252 years at 25 °C.

3.8. Antimicrobial studies

The Fig. 8 showed the zone of inhibition of developed transfersomal gel was significant higher as compared to reference standard of KET. The MIC was found to be 4.57–4.6 μg/mL and the aliquots were found to be effective throughout the time period which suggest that the concentration of drug was above MIC throughout the release and was efficacious [22].
4. Conclusion

In summary, Ketoconazole loaded transfersomes were optimized using 3-factor, 3-level Box–Behnken design. Optimized transfersome was prepared by conventional solvent evaporation method. The optimized formulation had a suitable vesicle shape, size, and maximum percent entrapment efficiency for penetration through the skin. The said transfersomal formulation was finally incorporated into a carbopol gel matrix and suitably characterized in terms homogeneity, spreadability, pH, and viscosity, in vitro release and ex vivo permeation studies. The developed gel was further evaluated for histopathology and microbial studies. The developed formulations were apparently free from any skin irritation potential and were effective in inhibiting microbial growth as well. Hence, it was concluded that the developed formulation benefits from its nano size and promises better therapeutic efficacy. Thus, the proposed study indicates that the potential of use of ultra-deformable transfersomal system in treatment of Vaginal Candidiasis.

Conflict of interest statement

Authors report no conflict of interest of this work. Author is responsible for content and the writing of the paper.

Acknowledgement

Authors are thankful to Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard University, New Delhi, India, for providing essential facilities in this research.

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