

Anticandidal Activity Of Ethosomal Gel Containing Miconazole Nitrate In Male Sprague Dawley Rat

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Abstract

The purpose of the present study was to formulate miconazole nitrate ethosomal gel and study the possibility of enhanced properties of ethosomal system through in-vitro and in-vivo evaluations. The ethosomes were successfully prepared and formulated into gel. Compositions of the formulations were manipulated to investigate their effects on viscosity, gel strength, spreadability, diffusion, drug release kinetics and in-vivo therapeutic efficacy. In addition, the formulations were characterized for morphology and compatibility with microscope and Fourier Transform Infra-Red studies. The formulated gels were clear and transparent in appearance. The maximum gel strength exhibited by the miconazole nitrate gel formulations was 95.7 sec. while the spreadability was established in the range of 17.75-54.19 gm.c/sec. The drug release..... Animal studies.....

Keywords: Ethosome, Miconazole nitrate, Cholesterol, Carbopol 940, Gel, Transdermal drug delivery system

INTRODUCTION

Fungal infections are one of the most concerning skin infections caused by various species of pathogenic fungi, affecting more than 25% of world populations. Fungal infections can affect the skin, mucus membranes, nails, mouth, intestine and vagina of a person. These infections can progress very rapidly especially in immunity compromised patients. Antifungal drugs are thus needed to be prescribed for the patients to treat the infections. Miconazole nitrate, an imidazole derivative, is one of the effective broad-spectrum antifungal drugs in treating candidiasis, protozoal and vaginal anaerobic bacterial infections. Miconazole nitrate is obtainable commercially as tablets and injections. Despite the broad-spectrum action of miconazole nitrate, the systemic efficacy of miconazole nitrate is low due to intensive hepatic first pass metabolism effect and poor water solubility.(1) Systemic administration of the drug also can lead to some of the well-known adverse drug effects like bloating, abdominal discomfort, nausea and vomiting in the patients. In order to overcome these limitations, miconazole nitrate is proposed to be formulated into topical dosage form.(2) However, conventional topical formulations of miconazole nitrate has a poor skin penetration, which necessitates higher dose of drug to be administered to treat the infections. This problem remains the concern of researchers until ethosomes come into sight.

Ethosomes are novel drug delivery system which widely employed for topical drugs. Ethosomes are soft and flexible vesicular carrier that composed of a relatively high concentration of ethanol, phospholipids and water. The size of ethosomes can be ranged from microns to nanometres. The interest of applying ethosomes in topical drug delivery system is increasing because ethosomes can offer a greater drug permeation through the skin to deeper tissues and enhanced entrapment efficiency as compared to other vesicular carriers like liposomes and niosomes.(3) The enhanced drug delivery capacity is owing to the small size and capability of the ethosomes to deform the skin barrier and then permeate intact across the narrow intercellular passage in the skin layer. Ethanol is an efficient permeation enhancer.(4) The high concentration of ethanol allows the ethosomes to disturb the lipid bilayer, thus contributing to the deformability of the skin and facilitating drug absorption. Along with the deformability of ethosomes, high concentration of ethanol in the composition of ethosomes also allow the lipid membrane to arrange less packed than conventional vesicles without compromising the stability and flexibility of the structures. Cholesterol can also be incorporated into ethosomes to improve the entrapment efficiency of drugs and stability.(5) Furthermore, the compositions of ethosome are safe, non-invasive and being approved for pharmaceutical purposes. The enhanced ethosomal drug permeation capability has been established in numerous researches by comparing the efficiency of ethosomes with other drug delivery systems. For instance,

Horwitz et al. conducted a research to compare the efficacy of acyclovir in ethosome and acyclovir in cream. The results proved that the ethosome preparation was more effective in treating herpetic infection..

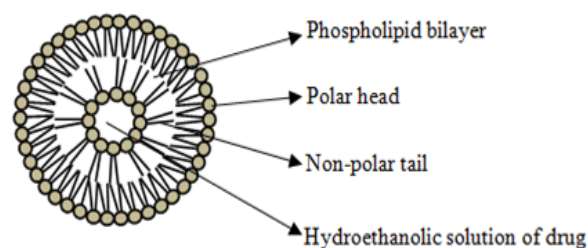


Figure 1: Structure of ethosome.(6)

A wide arrays of hydrophilic, lipophilic and amphiphilic drugs, proteins, peptides and other active pharmaceutical ingredients can be entrapped in ethosomes.(7) These drug-entrapped ethosomes can be administered as semisolid dosage forms such as cream and gel. Topical treatments are preferred than systemic treatment in fungal infections. Ethosomes are found to be offering a promising beneficial for delivery of antifungal drug for local skin fungal infections as topical and transdermal preparations. Transdermal preparations enable the drug to be delivered direct to site of local infections in controlled drug delivery profile. The drug is not absorb into portal circulation from the gut, thus it can by-pass hepatic first pass metabolism of the drug. Patient compliance also can be improved by offering a simpler route of drug administration.(8) Nevertheless, there are still some limitations on ethosomes, including poor production yield, limited to potent drug, and percutaneous drug absorption is depended on molecular size of the formulations

In this paper, ethosomes were synthesized to entrap the antifungal miconazole nitrate, which were further formulated into nanogel. The purpose of this work was to study the possibility of enhanced properties of miconazole nitrate ethosomal nanogel and its efficiency through in-vitro and animal evaluations. The present work emphasized on the development of an efficient ethosomal system to be incorporated into an appropriate gel for effective therapeutic benefits. Carbopol and hydroxypropyl methylcellulose were commonly used gelling agents for ethosomal systems. They were found to be compatible with ethosomal systems. While in this study, various concentrations of carbopol 940 was employed as gelling agent to prepare miconazole nitrate ethosomal gel. The formulations were then characterized for morphology, FTIR, viscosity, spreadability, gel strength, drug release kinetics and in vivo therapeutic efficacy study.

MATERIALS AND METHODS

Materials:

Miconazole nitrate obtained from Sigma-Aldrich Co., Cholesterol obtained from Sigma-Aldrich Co., Poloxamer 407, Ethanol, Sodium chloride, Potassium bromide obtained from Nacalai Tesque, Distilled water from MDL-4 Lab, AIMST University. All other materials and chemicals were of analytical grade.

Methods:

Preparation of Miconazole Nitrate loaded ethosomal:

Miconazole Nitrate ethosomes were prepared by using different proportions of drug and polymers. The polymer used in this preparation of Miconazole Nitrate ethosomes was Cholesterol. Accurately weighed Miconazole Nitrate and cholesterol were first dissolved in 5ml of ethanol respectively. They were then mixed thoroughly and added into Poloxamer 407 in normal saline solution. The mixture was sonicated for 10 mins at 60 mV to obtain uniform size of ethosomes. It was followed with stirring at 1200 rpm using magnetic stirrer for 1 hour for solvent evaporation.

Table 1: Composition of Miconazole Nitrate loaded ethosomes.

Cod e	Miconazole Nitrate (mg)	Cholesterol (mg)	Ethanol (ml)	Normal saline solution (ml)	Poloxamer 407 (mg)
M1	100	50	10	10	100
M2	100	100	10	10	100
M3	100	150	10	10	100
M4	100	200	10	10	100

Preparation of Miconazole Nitrate Ethosome Carbopol Gel:

Carbopol 940 gel of different concentration were prepared by dissolving required quantities of carbopol 940 powder in 250 ml respectively under heat. Small quantities of triethanolamine was added with stirring until a transparent clear gel was obtained. Miconazole nitrate ethosome was loaded into the carbopol gel. The pH of gel was adjusted to about neutral with triethanolamine.

Table 2: Formulation of Miconazole Nitrate loaded ethosomal gel.

Formulation code	Carbopol 940 concentration (%)	Volume of distilled water (ml)	Weight of carbopol 940 powder needed (g)	pH
M1	0.8	250	2.0	7.18
M2	1.0	250	2.5	6.97
M3	1.2	250	3.0	7.15
M4	1.4	250	3.5	7.19

Microscopic Analysis of Miconazole Nitrate Ethosome:

Miconazole Nitrate ethosomes were examined under microscope.

Fourier Transforms Infrared Spectroscopy:

FT-IR study of drug, polymers, and their physical mixture were performed to identify any interaction or incompatibility between the drug and polymer used. FT-IR spectra were performed employing KBr pellet technique at room temperature. The IR spectra were recorded using Perkin Elmer Spectrum GX FT-IR, measured over a range 4000-400 cm^{-1} .

FTIR of the following were obtained:

- Pure miconazole nitrate
- Pure cholesterol
- Pure poloxamer 407
- Mixture of cholesterol and poloxamer 407
- Mixture of miconazole nitrate, cholesterol and poloxamer 407

Determination of Viscosity of Miconazole Nitrate Ethosome Loaded Carbopol Gel:

The Viscosity of Miconazole Ethosome Loaded Carbopol Gels were measured by Brookfield programmable DVII +Model pro II type (USA). The viscosity was noted in Centipoise.

Determination of Gel Strength:

Gel strength was measured by placing 50g of formulation into a 100 ml graduated cylinder and gelled at 37°C using thermostat. A piston of weight 25g was placed onto the gelled solution and allowed to penetrate 4cm in the gel. Time taken by weight to sink 4cm was measured.(9)

Determination of Spreadability:

For the determination of spreadability, excess of sample was applied in between two glass slides and was compressed to uniform thickness by placing 1000g weight for 5 minutes. 75.87g weight was added to the pan. The time in which the upper glass slide moves over to the lower plate for 5cm was taken as measure of spreadability.(9)

$$S = ML/T$$

Where, M= weight tide to upper slide (g)

L= length moved on the glass slide (cm)

T= time taken (sec)

Diffusion studies:

The in vitro release of miconazole nitrate formulations were studied by using modified apparatus with cellophane membrane. Freshly prepared phosphate buffer with pH 7.4 was employed as dissolution medium. Cellophane membrane was soaked overnight in the phosphate buffer and then attached to one end of glass cylinder which specifically designed to be opened at both ends. 1% w/w of miconazole nitrate ethosomal gel was placed into the cylinder. The cylinder was suspended in 200ml of dissolution medium maintained at a temperature of $37 \pm 1^\circ\text{C}$. 5 ml of the dissolution medium was withdrawn at predetermined interval. Fresh dissolution medium was added to replace the removed aliquots. The amount of miconazole nitrate in each aliquot was measured and noted.(10)

Drug Release Kinetics:

To analyze the in vitro release data various kinetic models were used to describe the release kinetics. The zero order rate Eq. (1) describes the systems where the drug release rate is independent of its concentration (Hadjiioannou et al., 1993). The first order Eq. (2) describes the release from system where release rate is concentration dependent (Bourne, 2002). Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion Eq. (3).

$$C = k^0 t \quad (1)$$

Where, k^0 is zero-order rate constant expressed in units of concentration/time and t is the time.

$$\text{Log } C = \text{Log } C_0 - kt / 2.303 \quad (2)$$

Where, C_0 is the initial concentration of drug and k is first order constant.

$$Q = Kt^{1/2} \quad (3)$$

The following plots were made: cumulative % drug release vs. time (zero order kinetic model); log cumulative of % drug remaining vs. time (first order kinetic model); cumulative % drug release vs. square root of time (Higuchi model) log cumulative % drug release vs. log time (Korsmeyer model).

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Bourne DW. Pharmacokinetics In: Banker GS, Rhodes CT, eds. Modern Pharmaceutics. 4th ed, New York, NY: Marcel Dekker Inc, 2002;67-92

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In vivo evaluation of therapeutic efficacy of microsponges enriched gel:

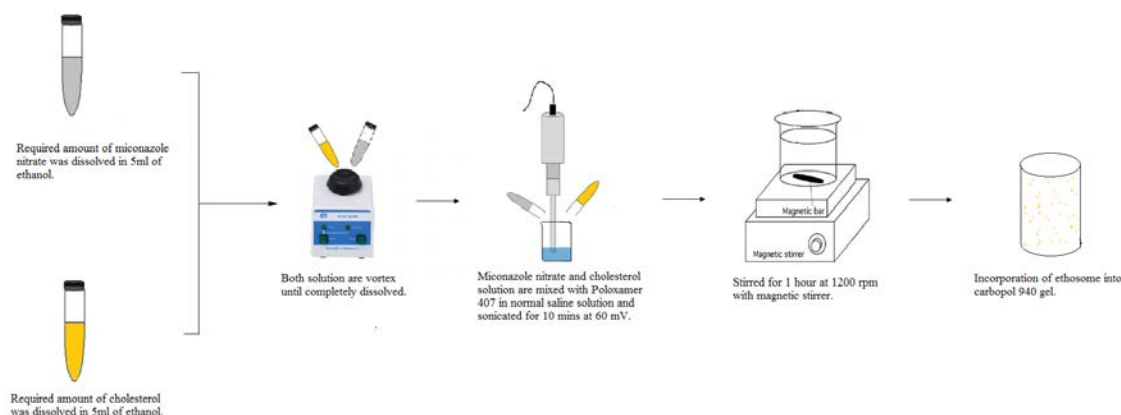


Figure 2: Method of preparation of miconazole nitrate loaded ethosomal gel.

Animals: Adult Wistar rats (280 ± 10 g) of either gender were obtained from SCS College of pharmacy, Harapanahalli. The animals were housed in large, spacious polyacrylic cages at an ambient room temperature with 12-h light/12-h dark cycle. Rats had free access to water and rodent pellets diet. The study was approved by the Institute Animal Ethics Committee of the SCS College of pharmacy, Harapanahalli and all the animal experiments were carried out according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Ministry of Environment and Forests, Government of India.

Acute toxicity testing: The female rats were used for the acute toxicity testing. Hair present in the dorsal surface of the animal (2 X 2 cm) was removed by applying hair remover and cleaned with alcohol. The screening area was marked (1 X 1 cm) and 0.5 g of a microsp sponge enriched gel was applied to the surface of an animal's skin. During the observation period (14 days), signs such as erythema and edema were assessed [13]

Evaluation of therapeutic efficacy: The male rats were used for the experiment. The rats were divided into the four groups viz., normal control (group I), *Candida glabrata* control (group II), standard treatment group (group III) and microsponges enriched gel treatment group (group IV). Group II to IV animals were changed with intravenous methylprednisolone (5mg/kg) for 3 days for induction and maintenance of cell-mediated immunosuppression (Organisms from stock isolates were stored in nutrient agar at 27°C, streaked onto nutrient broth, and incubated at 37°C for 24 h and included culture was used for further experiment).

Candida glabrata culture was diluted with PBS and swabbed in smooth muscle of rat pennies and allowed to grow for 3 days until the growth of *Candida* was observed on ischiocavernosus smooth muscle. The colony growth was confirmed by counting colony-forming-unit. The animals which as cfu value of more than 3 cfu/ml were included in the study. The animals were treated for week period and visually observed its physical changes. The swab culture was collected on initial day, 4th and 7th day of the experiment for microscopical evaluation. End of the experiment the animals were sacrificed and ischiocavernosus smooth muscle was collected from all the experimental animals and preserved in 10% formalin.

Microscopical evaluation: The colony was collected in sterile cotton swab and transferred into 0.5 ml sterile phosphate buffer saline (PBS). The mixture was diluted 10 fold and inculcated in nutrient agar media, incubated for 48 h at 37°C. The yeast count was expressed as log 10 of cfu/ml of PBS.

Histopathologic analysis: The liver and pancreas were dehydrated with alcohol for 12 h each and cleaned with xylene for 15-20 min. The tissue blocks were prepared and the blocks were cut using microtome to get sections of thickness 5 μ m. The sections were taken on a microscopic slide on which egg albumin (sticky substance) was applied and allowed for drying. Finally, the sections were stained with eosin (acidic stain) and hemotoxylin (basic stain) [14].

Statistical analysis

All the data were expressed as mean \pm SEM. Statistical significance between the groups were tested using one-way analysis of variance (ANOVA) followed by Dunnett's^t-test post-hoc test. A P less than 0.5 were considered significant.

RESULTS AND DISCUSSION

In present work, different miconazole nitrate ethosomal gels were developed and characterized for pH, viscosity, spreadability, gel strength, compatibility, drug release kinetic and in vivo therapeutic efficacy of antifungal effect.

All the gel formulations were clear and opaque in appearance. The pH of all formulations ranged from 6.97 – 7.19. Microscopic analysis provided a visual and descriptive information of a portion of the miconazole nitrate ethosomes. Figure 2 showed the microscopic images of the four ethosome formulations observed with microscope.

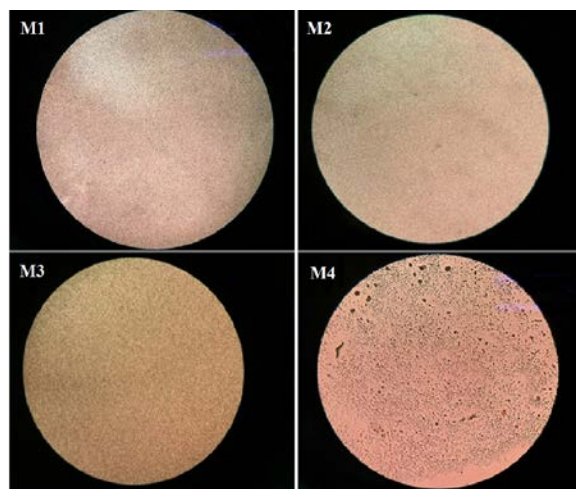


Figure 3: Microscopic images of Miconazole Nitrate ethosomes.



Figure 4: Appearance of miconazole nitrate ethosomal gel.

The FTIR spectral analysis of miconazole nitrate showed the principal peaks observed at 3892.96, 3749.94, 3556.15, 3353.82, 3199.29, 2995.37, 2644.39, 2518.19, 2181.47, 2035.65, 1935.33, 1749.75, 1598.04, 1483.94, 1189.11, 1054.58, 957.54, 839.32, 71688 and 588.91 (unit cm^{-1}). The spectral analysis of cholesterol showed peaks at 3969.40, 3770.36, 3545.76, 3413.22, 3381.95, 3194.94, 3074.60, 2876.15, 2760.14, 2623.32, 2446.52, 2314.95, 2234.85, 2059.64, 1984.13, 1880.02, 1756.89, 1635.71, 1594.21, 1412.66, 1389.60, 1372.20, 1236.41, 1067.35, 973.95, 949.73, 924.77, 815.07, 718.43 and 625.60 (unit cm^{-1}). The spectral analysis of poloxamer 407 showed peaks at 3937.76, 3790.13, 3431.27, 3136.64, 2960.79, 2729.35, 2642.90, 2557.10, 2433.28, 2236.08, 2076.35, 1880.75, 1678.04, 1507.93, 1388.81, 1234.90, 993.23, 847.24, 730.93 and 584.49 (unit cm^{-1}).

The spectrum of physical mixture of cholesterol and poloxamer 407 showed the peaks at 3786.94, 3650.37, 3505.11, 3260.34, 3211.65, 2958.35, 2725.35, 2628.01, 2448.05, 2217.45, 2078.31, 1706.04, 1504.23, 1234.30, 1022.13, 846.18, 587.40 and 429.77 (unit in cm^{-1}). The spectrum of physical mixture of miconazole nitrate, cholesterol and poloxamer 407 showed the peaks at 3918.88, 3673.92, 3547.03, 3432.41, 3181.13, 3055.90, 2966.21, 2727.31, 2623.51, 2369.73, 2193.57, 2160.44, 1979.34, 1772.15, 1682.90, 1504.36, 1234.61, 1000.40, 838.34, 714.88, 584.48 and 554.85 (unit in cm^{-1}).

There was no shift of peak in any of the spectra, establishing the compatibility miconazole nitrate with cholesterol and poloxamer 407. Therefore, miconazole nitrate can be formulated into ethosomes with cholesterol and poloxamer 407 without compromising their functional groups. The FTIR spectrums were shown in Figure 3(A), 3(B), 3(C), 3(D) and 3(E).

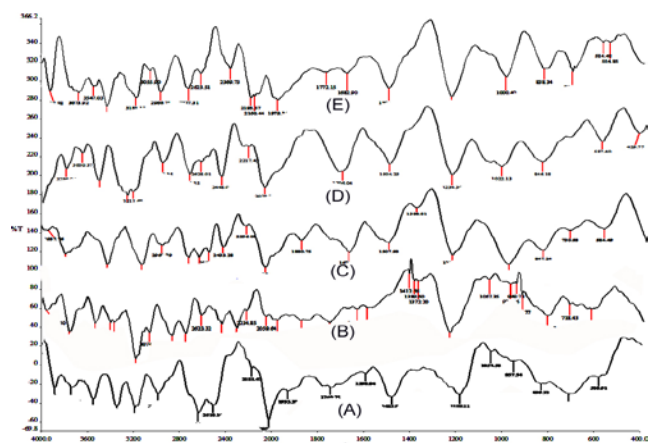


Figure 5: (A) Miconazole, (B) Cholesterol, (C) Poloxamer 407, (D) Cholesterol + Poloxamer 407, (E) Miconazole + Cholesterol + Poloxamer 407

The rheological properties of the four ethosomal gel formulations were determined at various shear rates. The viscosity of the gel was important to be determined because viscosity of a gel was

directly linked to the drug release properties of the formulations. When the rate of shear increased, the viscosity of the gel formulations decreased. The result of the viscosity studies also showed that M4 with the greatest concentration of carbopol 940 (1.4%) was established with highest viscosity when compared with the other formulations. All formulations established carbopol 940 concentration-dependent viscosity.

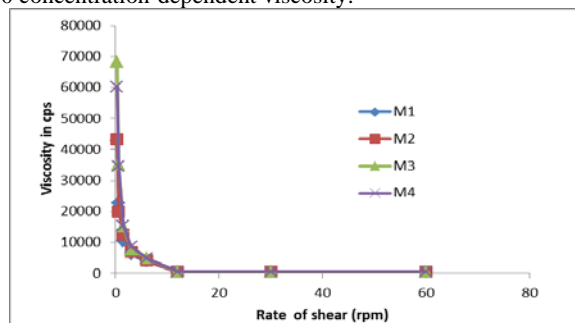


Figure 6: Rheological properties of miconazole nitrate ethosomal gel formulations.

The gel strength for each formulation was determined as a strong gel can withstand higher pressure before being washed out from the application site than weak gel. M4 formulation was having a greatest gel strength while M1 formulation was having the lowest gel strength. This indicated the increasing concentration carbopol 940 can increase the gel strength of the final gel also.

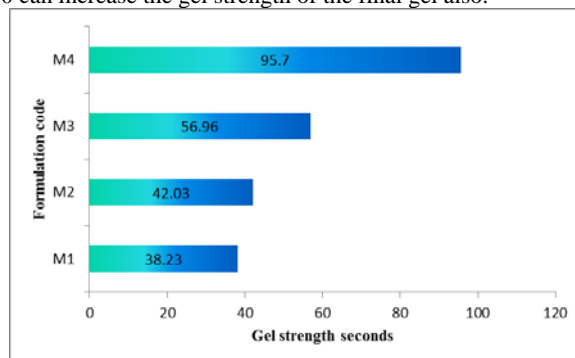


Figure 7: Gel strength of miconazole nitrate ethosomal gel formulations.

Spreadability of the gel was expressed in term of time required for two slides to slip off from the gel for a fixed distance with a constant weight attached (unit gm.cm/sec). Spreadability of the formulations is inversely proportional to the viscosity. The lesser the time taken for the slides to slip off from the gel, the better the spreadability of the gel. A greater spreadability indicated a smaller shear is needed to spread the gel. The spreadability of M1 was found to be greater than the other formulations.

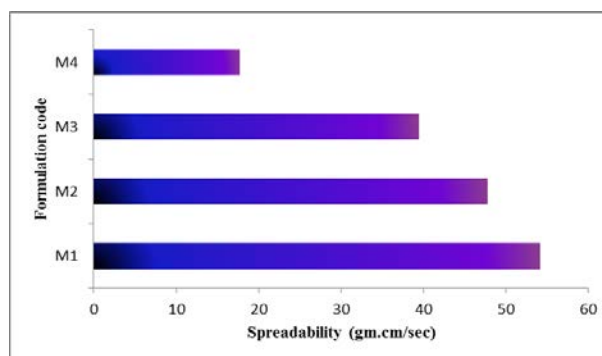


Figure 8: Spreadability of miconazole nitrate ethosomal gel formulations.

Table 3: Characteristics of various miconazole nitrate ethosomal gel.

Formula code	SSpreadability (gm.cm/sec.)	Gel Strength (seconds)
M1	54.19	38.23
M2	47.84	42.03
M3	39.52	56.96
M4	17.75	95.7

The *in vitro* diffusion profile of fluconazole from formulations M1, M2, M3 and M4 were conducted in dissolution medium of phosphate buffer (pH 7.4). The formulations R3 and M4 showed 83.21% and 80.31% respectively at 9 hours (Fig. 5). Typical chromatogram of miconazole sample was shown in Fig.6.

The results from *in vitro* diffusion study suggested the enhanced penetration capability of ethosomal system can improve miconazole nitrate delivery across the skin barrier under non-occlusive situation. Interaction of ethanol as permeation enhancer with skin, increasing the lipid fluidity and impairing the barrier function of skin layers, thus increasing the permeability and delivery of drug transdermally.

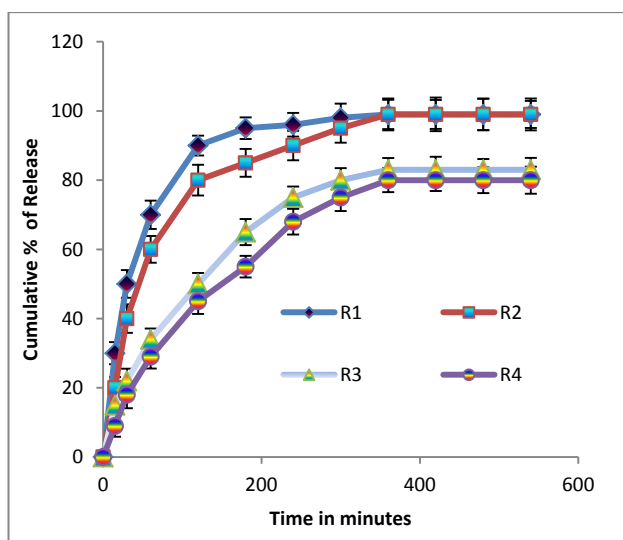


Figure 5: Showing the Diffusion of optimized formulation

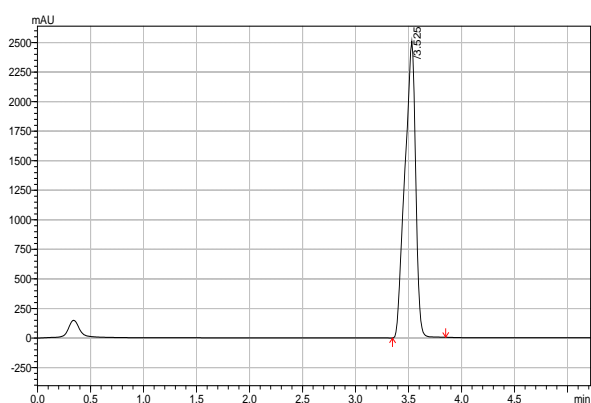


Figure 6: Typical chromatogram of drug sample

Drug Release Kinetics

The drug release from miconazole nitrate gel formulation was followed diffusion controlled zero order mechanism from the *in situ* gel, as the value of 'r' for zero order kinetics was 0.990 and also found to be more than that of first order which ranged from 0.983 to 0.986. The value of 'r' for Higuchi kinetics which ranged from 0.989 to 0.990. All the formulations were subjected to PCP

DISSO software analysis. All the formulations K1, K6 and K7 exhibited good *in vitro* release kinetics with fickian type of diffusion mechanism. More over to comprehend the drug release mechanism the data were fitted in to korsmeyer -peppas exponential model where the 'n' values were in the range of 0.055 to 0.057. It was understood that K code formulation were following predominantly zero order and fickian diffusion mechanism of drug release from its formulations. (Table 5)

Order of process	K1	K6	K7
Zero order	R ² 0.990	0.99	0.99
	M 6.155	6.53	6.795
	C -0.480	-0.211	0.476
First order	R ² 0.986	0.985	0.983
	M -0.048	-0.052	-0.062
	C 2.057	2.058	2.086
Higuchi	R ² 0.990	0.99	0.989
	M 6.155	6.53	6.795
	C -0.480	-0.211	0.476
korsmeyer	R ² 0.968	0.944	0.96
	M 0.056	0.057	0.055
	C 1.262	1.28	1.317

The therapeutic efficacy of microspunge enriched gel was compared with *Candida glabrata* control by quantitative microbiological analysis and histopathological evaluations (Table -5; Figure - 7). MEG and standard marketed formulation treated animals showed significant reduction of CFU count on 4th day of the treatment onwards. The efficacy of the MFG is comparable with standard marketed formulation.

Table- 5: Quantitative microbiological analysis of the *Candida glabrata* in rat penal smooth muscle surface.

Mean	Initial day	4th day	7th day
Control	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02
Negative control	3.33 ± 0.05	3.49 ± 0.07	3.38 ± 0.11
Standard	3.35 ± 0.04	2.59 ± 0.11**	0.50 ± 0.16***
Treated with MEG	3.34 ± 0.09	2.30 ± 0.05***	0.15 ± 0.04***

P < 0.05, ** P < 0.01 and ***P < 0.001 compare to control group (One-way ANOVA followed by tukey's multiple comparison test)

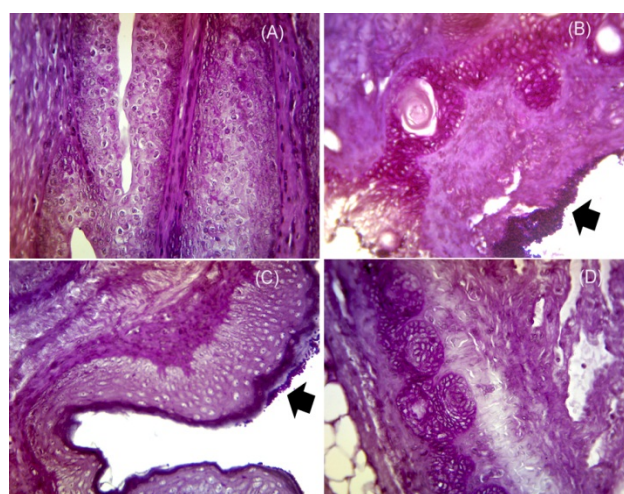


Figure 7. Histopathological analysis of the rat penal smooth muscle section from (A) control showed normal articheture and (B) showed *Candia* microorganism infection in smooth muscle surface (C) and (D) showed reduction in growth of *Candia* due to antifungal effect of standard and MLG, PAS, 400X

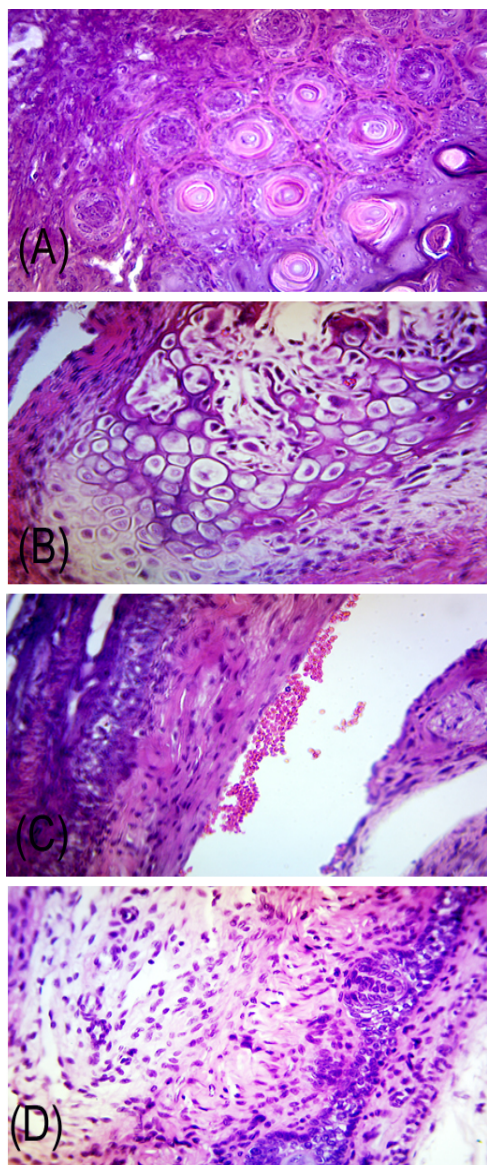


Figure 3. Histopathological analysis of the rat penile smooth muscle section from (A) control showed normal architecture and (B) showed *Candida* microorganism infection in smooth muscle surface (C) and (D) showed reduction in growth of *Candida* due to antifungal effect of standard and MLG, PAS, 400X

CONCLUSION

Ethosomes of miconazole nitrate were prepared successfully by using different concentrations of cholesterol as well as the incorporation of the ethosomes into carbopol 940 base gel to obtain ethosomal gel formulations. The prepared formulations were characterized for various properties. The compositions of ethosomes and gels were manipulated to investigate their effects on the characteristics of final formulations. The miconazole nitrate ethosomal gels established significant therapeutic efficacy against cutaneous candidiasis caused by *C. albicans*. From the results of the study, it can be concluded that miconazole nitrate ethosomes can be integrated as transdermal drug delivery systems with enhanced drug delivery efficiency and therapeutic efficacy, suggesting an approach to overcome the higher dose of miconazole nitrate required in conventional topical administration, frequent application and systemic adverse effects.

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