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Research Article

ANTI-FUNGAL ACTIVITY OF MICROEMULSION BASED FLUCONAZOLE GEL FOR ONYCHOMYCOSIS AGAINST ASPERGILLUS NIGER

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ABSTRACT

Oral dosing of an antifungal agent could prove to be a cost-effective way to provide long-term treatment for fungal nail infections and less permeability of drug topical delivery. The endeavor of the present study was to evaluate microemulsion as a vehicle for topical drug delivery and to expand microemulsion-based gel of fluconazole for the treatment of onychomycosis. Experimental design was adopted to optimize the amount of oil (oleic acid), mixture of surfactant (tween 80), cosurfactant (propylene glycol) and water in the microemulsion. The optimized formulations of fluconazole microemulsion based gel were assessed for viscosity, zeta potential, spreadability, gel strength, mucoadhesive force, globule size, FTIR, and SEM. The viscosity of plain gel was found to be in the range 24995 to 61000 cps, whereas for the microemulsion loaded gel was up to 30000 cps. The maximum gel strength and mucoadhesion was found to be up to 145 seconds and 13141.51dynes/cm^2 respectively. Scanning electron microscopy showed that globules were spherical in shape. Drug containing microemulsion was converted into gel employing 1%, 1.5, 1.75% w/v of xanthan gum. The optimized gel showed better penetration and mucoadhesive properties as compared to the commercial gel. The cumulative percentage of drug release after 7 h for optimized MBGs was 72.23%. Fluconazole microemulsion based gel form showed better activity against *Aspergillus niger* than the commercial gel. It was concluded that drug-loaded gel could be a promising formulation for effective treatment of onychomycosis.

Keywords: *Aspergillus niger,* FTIR, MBGs, SEM, Spreadability, Xanthan gum

INTRODUCTION

Fungal nail infections are relatively less common in children than in adults because of faster nail plate growth in children and adolescents. Exposure to a large number of fungi from the environment, accompanied by predisposing factors, enhances the risk of dermatomycosis in this age group, however, nails candidiasis, being an endogenous infection, and depends primarily on the general immunity as well as on local damage to the nails[1-3]. Many recent reports have documented different opportunistic molds as possible aetiological agents of nail infections. The main molds involved in onychomycosis as primary pathogens include: *Scopulariopsis brevicaulis, Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scytalidium* spp.[4-8]. Fungal infections of the toenail are difficult to treat because of the unique properties of the nail unit [9,10]. A new generation of antifungal drug must penetrate the affected nail tissue and persist in high concentrations until the infecting pathogen is eradicated. Because topical antifungal agents fail to penetrate the hard keratin of the nail plate, they are usually ineffective in patients with onychomycosis[11] although pharmacokinetic data for the older oral antifungal agents griseofulvin and ketoconazole are scant, it is assumed that these drugs penetrate into the nail plate via the nail matrix [12]. Therefore therapy with these drugs must be given until the entire nail plate is clinically cured, which can take up to 18 months for toenails [13]. Microemulsions are clear, stable, isotropic mixtures of oil, water, and surfactant, frequently in combination with a cosurfactant. Fluconazole is a triazole, available in oral and parenteral forms, and is effective and well tolerated in the treatment of mucosal as well as invasive candidiasis[14-17]. The human nail forms a resistant barrier to the topical penetration of actives [18]. Thus, treatment of nail disorders, such as fungal infections, remains a challenge because of the difficulty encountered in achieving therapeutic concentrations of drugs at the site of infection, which is often under the nail [19]. Investigation of the scientific literature suggests, that a key to successful treatment of onychomycosis by a topical antifungal product lies in it, effectively, overcoming the nail barrier.

Creams and ointments are available to treat fungal nail infections. A nail lacquer called Penlac may be helpful for some people. There are a number of oral medications available. Sporanox and lamisil can be taken for several months. They have been found to be helpful and clear the fungus in up to half of cases of fungal nail infection. They do have a number of side effects. Many people are not able to take these medications because of other medications that they are taking, or medical problems such as liver or cardiac disease. Current topical treatments have limited effectiveness, possibly because they cannot sufficiently penetrate the nail plate to transport a therapeutically sufficient quantity of antifungal drug to the target sites to eradicate the infection of onychomycosis [20]. Microemulsions have been intensively studied during the last decades by many scientists and technologists because of their great potential in many food and pharmaceutical applications. The use of microemulsions is advantageous not only due to the facile and low cost preparation, but also because of the improved bioavailability. The increased absorption of drugs in topical applications is attributed to enhancement of penetration through the skin by the carrier.

MATERIALS

Fluconazole was kindly gifted by Fourt's India, Chennai, India. The entire chemicals were used AR grade such as propylene glycol, xanthan gum, tween 80, oleic acid, Sabaraud dextrose agar were received from R&M marketing, Essex, U.K. A commercial gel of fluconazole was purchased from local market. Double distilled water was used whenever required.

METHODS

Construction of pseudo-ternary phase diagrams

In order to find out the concentration range of components for the existing range of microemulsions, pseudo-ternary phase diagrams were constructed using H_2O titration method at ambient temperature (25°C). Oleic acid was selected as the oil phase. Tween 80 and Polyethylene glycol 200 were selected as surfactant and co-surfactant, respectively. Distilled water was used as an aqueous phase. Three phase diagrams were prepared with the 1:1, 2:1 and 3:1 weight ratios of tween 80 to Polyethylene glycol 200, respectively. For each phase diagram at a specific surfactant/co-surfactant weight ratio, the ratios of oil to the mixture of surfactant and co-surfactant were varied as 0.5:9.5, 1:9, 1.5:8.5, 2:8, 2.5:7.5, 3:7, 3.5:6.5, 4:6, 4.5: 5.5, 5:5, 5.5:4.5, 6:4, 6.5:3.5, 7:3, 7.5:2.5, 8:2, 8.5:1.5, 9:1, 9.5:0.5. The mixtures of oil, surfactant and co-surfactant at certain weight ratios were diluted with water drop wise, under moderate magnetic stirring. After being equilibrated, the mixtures were assessed visually and determined as being microemulsion, crude emulsions or gels. No attempt was made to distinguish between oil-in-water, water-in-oil or bicontinuous type

microemulsions. The phase diagram of S: CoS– Oil –water system as shown in Figure 1. The microemulsion for the further studies was selected from the phase diagram. The selected microemulsion was composed of 500 mg of fluconazole, oleic acid (12 ml), propylene glycol 200 (20 ml) , tween 80 (20 ml) and water (47.5 ml). The selection of the microemulsion composition was on the basis of its ability to solubilize desired amount of fluconazole.

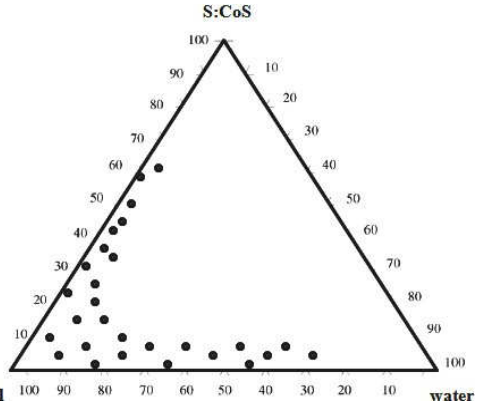


Fig. 1: The pseudo-ternary phase diagrams of the oil-surfactant: co surfactant mixture-water system of optimized formulations

Formulation of microemulsion based gel (MBGs)

Various ratio of gelling agent (1%, 1.5% and 1.75% w/v) namely, xanthan gums were added to form a microemulsion based gel. Gelling agent was dispersed slowly in 10 ml of the microemulsion with the help of magnetic stirrer. The suitable gelling agent was selected on the basis of compatibility with microemulsion structure, feel and ease of spreadability.

Evaluation of MBGs

Measurement of droplet size and zeta potential

The average droplet size and zeta potential of the microemulsions were measured using a Zetasizer Nano ZS (Malvern Instruments, UK). The measurement was done at 25°C.

Rheological studies

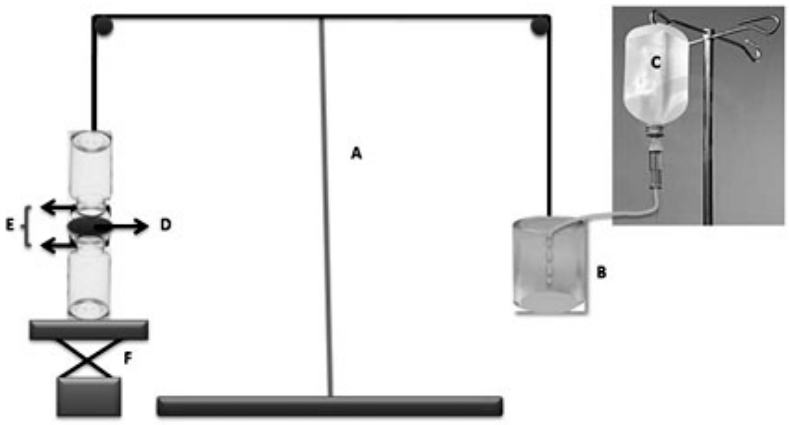
Brookefield programmable DVII+ Model pro II type viscometer was used for rheological studies. The prepared formulations were placed in a beaker and were allowed to equilibrate for 5 minutes before measuring the dial reading using spindle no. 63 for R4, R5 and R6. The viscosity of plain gel and MBGs were determined at different angular velocities (0.5, 10, 20, 30, 40 and 50 rpm) and average of two reading was used to calculate the viscosity.

Spreadability

The spreadability of the gel was determined using the following technique[21-23]: 0.5g gel was placed within a circle of 1 cm diameter premarked on a glass plate over which a second glass plate was placed. A weight of 1000 g was allowed to rest on the upper glass plate for 5minutes. The increase in the diameter due to spreading figure 3 of the gels was noted. All measurements were performed in triplicate (n = 3).

Determination of mucoadhesive Force

The experimental technique used for determining the bioadhesive force has been derived from a previously published method[24-25]. The experimental setup is presented in Figure 2. The mucoadhesive force of the formulations was determined as follows; a section of membrane was cut from the chicken and instantly fixed with mucosal side out onto each glass vial (E) using rubber band. The vial with chicken mucosa was connected to the balance in inverted position while first vial was placed on a height adjustable pan (A). Fluconazole MBGs was added onto the mucosa of first vial. Then the height of second vial was so adjusted that the mucosal surfaces of both vials come in intimate contact. Two minutes time of contact was given. Then, the switch (C) of the infusion apparatus was opened to make the water drop into the glass vial (B) with a constant flow rate of 5 ml/min. The weight of the water in the glass vial (B) kept increasing until the gel and the mucosal tissue were detached. Mucoadhesive force, the detachment stress (dyne/cm^2), was determined from the minimal weights that detached the gel. The chicken membrane pieces were changed for each measurement. All measurements were performed in triplicate (n = 3).



(A) Modified Balance (B) Glass Reservoir (C) Infusion Device (D) Membrane (E) Vial (F) Height Adjustment Pan.

Fig. 2: Mucoadhesive Force- measuring device

Measurement of Gel Strength

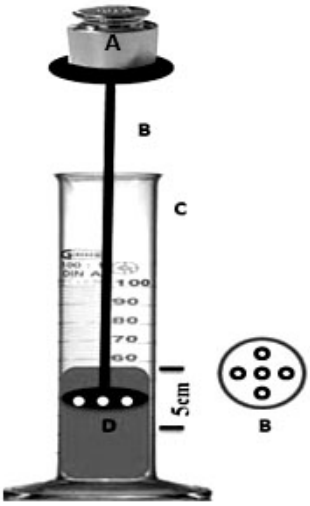
A sample of 50 g of gel was placed in a 100 ml graduated cylinder. The apparatus[24] for measuring gel strength was allowed to penetrate the gel as shown in Figure 3. Gel strength, *i.e.* the viscosity of the gels was determined by the time (in seconds) taken by the apparatus to sink down 5 cm through the prepared gel. All measurements were performed in triplicate (n = 3).

Antifungal activity

Antifungal activity of commercial gel and MBGs was evaluated against *Aspergillus niger* (ATCC 16404) by using a zone of inhibition by agar disc diffusion method. The mean zone of inhibition was recorded.

All the cultures were maintained of sabouraud dextrore agar medium and incubate at 30 °C. In order to prepare homogenous suspension of these fungi for disc assays, they were grown overnight in sabouraud broth, centrifuged to collect the pellet and resuspended in sterile phosphate buffered saline[26]. The fungal pellet was homogenized in sterile hand held homogenizer. This suspension was then plated on a sabouraud dextrose agar medium using a bacterial spreader to obtain an even growth. Sterile 6 mm whattmann filter paper disc were impregnated

with 100 mg/L of MBGs and commercial cream. These discs were then placed in the centre of quadrent of sabauroud dextrose agar medium plate. These plates and one control disc impregnated with 10 % DMSO in methanol. These plates were incubated at 30°C. Three replicates were used for MBGs as well as for commercial cream. After 48 hours the plates were removed and radii of inhibition zone were measured and the average calculated.



(A) Weights (B) Device (C) Measuring Cylinder (D) MBGs

Fig. 3: Gel Strength measuring device

Diffusion studies

The diffusion medium used was phosphate buffer pH 7.4. Assembly of diffusion cell for in–vitro diffusion studies the diffusion cell was designed as per the dimension given. Diffusion cell with an effective diffusion area of 3.14 $cm^2$was used for *in vitro* permeation studies. The diffusion cells were placed on the magnetic stirrers. The donor compartment consisting 0.5 % of microemulsion based gel containing fluconazole. The receptor compartment was filled with phosphate buffer. Then the chicken membrane was mounted on the cell carefully so as to avoid the entrapment of air bubble under the chicken membrane. Intimate contact of chicken membrane was ensured with receptor fluid by placing it tightly with clamp. The speed of the stirring was kept constant throughout the experiment. With the help of 1ml pipette 1ml of sample was withdrawn at a time intervals of 30 min from sampling port of receptor compartment and same volume was replaced with receptor fluid solution in order to maintain sink condition. The samples were appropriately diluted and the absorbance was measured at 261 nm using UV-VIS spectrophotometer.

Drug content

For determination of drug content about 1g of each microemulsion based gel formulation was weighed in a 100 ml volumetric flask and dissolved in methanol. It was diluted appropriately and analyzed spectrophotometrically at 261 nm.

FTIR spectra

The FT-IR spectra were recorded in KBr on Beckman Coulter DU 800 spectrophotometer.

SEM Analysis

The surface characteristics of microemulsion were performed on the formulation selected on the basis of particle size the formulation using scanning electron microscopy (Phenom World EMS 550X).

RESULTS AND DISCUSSION

Measurement of droplet size and zeta potential

The droplet sizes for the formulations are represented in Figure 4. The result shows that the droplet diameter decreases with increasing ratio of oil: surfactant/co-surfactant. Transmission electron microscopy showed that globules were spherical in shape. Average droplet size of optimized formulations ranged from 23 to 100 nm. Zeta potential of optimized formulation was (+29.3 ± 3.1 to +31.2 ± 1.2).

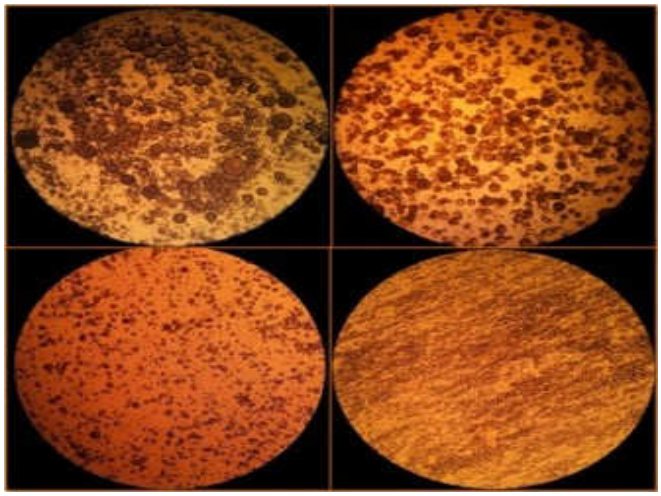


Fig. 4: Droplet size of microemulsion loaded gel (optical microscopy)

Rheological studies

The viscosity of 1.0 %, 1.5 and 1.75 % (w/v) of xanthan gum with that of the microemulsion based gel. The formulations were shear thinning and an increase in shear stress was observed with increase in angular velocity (pseudoplastic rheology) as shown in Figure.5 and 6.

Spreadability

The values of spreadability indicate that the gel is easily spreadable by small amount of shear. The spreadability plays an important role in patient compliance and helps in uniform application of gel to the skin. A good gel takes less time to spread and will have high spreadability. The spreadability of optimized formulations R4, R5 and R6 was found to be more as compared to other optimized formulations. This indicates spreadability of MBGs system containing gel having higher ratio of polymer was good as when compared with lower ratio as shown in Table.1

Measurement of gel strength

The gel strength is important because strong gels will support a much higher pressure than weak gels before they are washed out from the site of administration. The gel strength of formulation R4 and R6 (62.5 and 145.9 seconds) exhibited good gel strength between R code formulations as shown in (Table 1) which may due to increase in concentration of xanthan gum in the formulation.

Determination of mucoadhesive Force

The mucoadhesive force was significantly increased from 11578.75 dynes/cm^2 to 13141.51 dynes/cm^2 for the formula R4 and R6 which consists of 1% and 1.5% of xanthan gum, as the concentration of mucoadhesive polymer (xanthan gum) increased as shown in Table 1 . This also proved that xanthan gum has better mucoadhesive property.

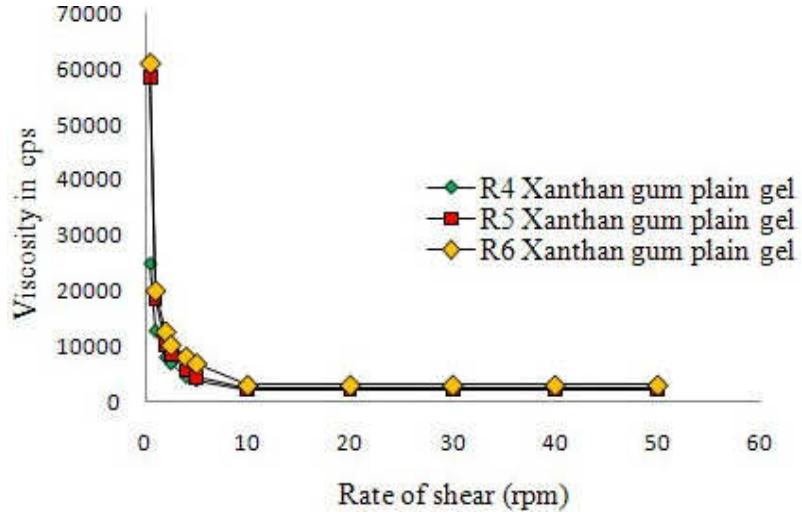


Fig. 5: showing the viscosity of plain gels of optimized formulation

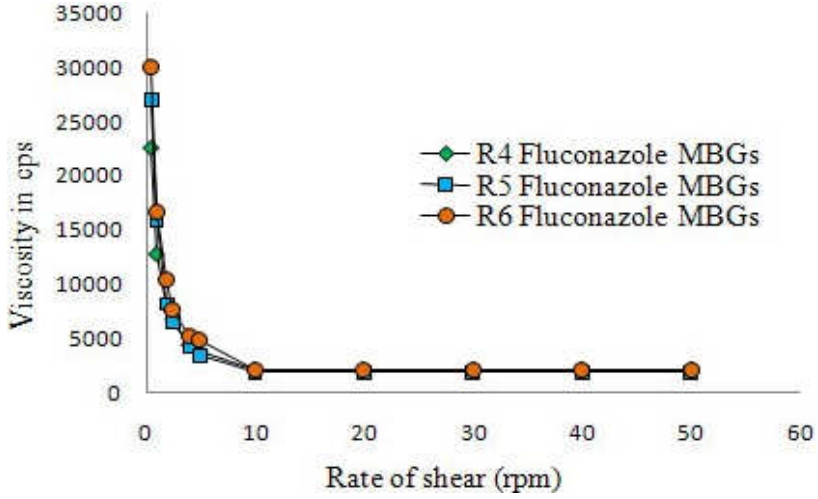


Fig. 6: Showing the viscosity of fluconazole MBGs of optimized formulation

Table 1: Characteristics of optimized formulation of MBGs

Formulation code	% Drug content (w/w)	Mucoadhesive force (dynes/cm^2)	Gel strength (seconds)	Spreadability (g.cm/sec.)
R4	89.21	11758.75 ± 0.44	62.0 ± 2	35.82 ± 0.23
R5	90.70	12214.33 ± 0.35	92.0 ± 1	30.42 ± 0.14
R6	90.44	13141.51 ± 0.21	145.0 ± 1	25.89 ± 0.24

*All the values are expressed as mean ±SD *(n=3)*

In-Vitro Diffusion studies (MBGs)

The formulations R4 and R5 released 70.1% and 72.2 % of drug respectively at 7th hour. The optimized formulations R4 and R5 containing higher concentration of xanthan gum (1.0 -1.75% w/v) .The *in vitro* drug release of the formulations R4 to R6 was 72.1% to 73.2 %, which leads to extended drug release the MBGS as shown in Figure 7.

***In vitro* antifungal activity**

It was observed from the results that the zone of inhibition for marketed cream was 1.1 cm, whereas it was 1.5 cm for the fluconazole MBGs as shown in Figure 8. It is clearly understood that the formulations showed better antifungal activity against A*spergillus niger* in comparison with marketed sample.

SEM Analysis

SEM is a powerful method for visualizing the structure of microemulsions (Figures 9, 10). Recently, we were able to show that this special preparation technique can be successfully used for the detection of plain gel and microemulsions based gel.

FTIR spectra:

The characteristic bands of pure fluconazole (3422.96, 3126.05, 2313.72, 1725.87, 1597.20, 1412.58, 1261.53, 1099.30, 833.56, 662.93 and 553.84 cm-1), The FTIR spectra of fluconazole and xanthan gum showed band at (3426.29, 2929.97, 2319.32, 1748.25, 1409.79, 1272.72, 1082.51, 827.97, 662.93 and 565.03 cm-1). Therefore fluconazole and xanthan gum can be used as excipients in the formulation of MBG gels as shown in Figure 11.

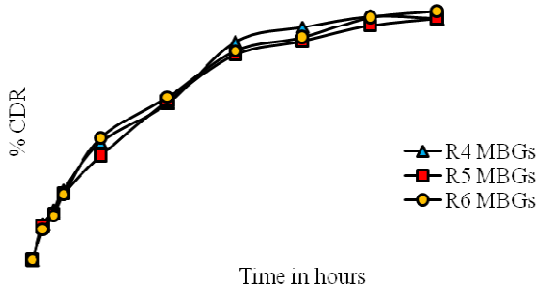


Fig. 7: Showing the drug release of R4, R5 and R6 formulations (MBGs)

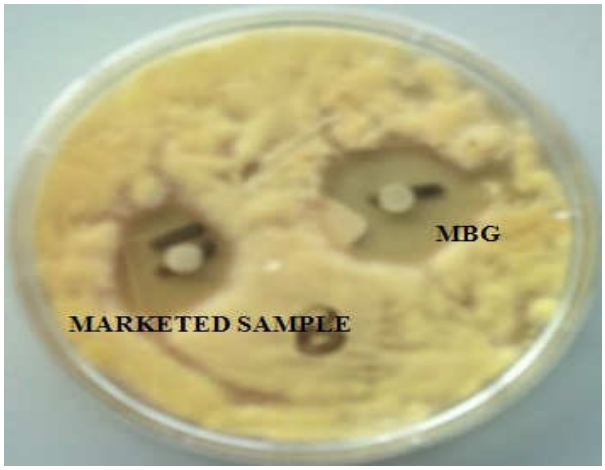


Fig. 8: zone of inhibition

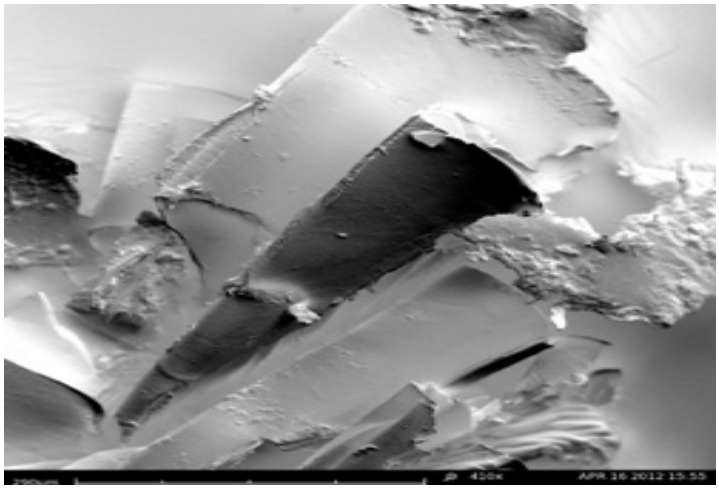


Fig. 9: SEM micrograph of the xanthan gum plain gel

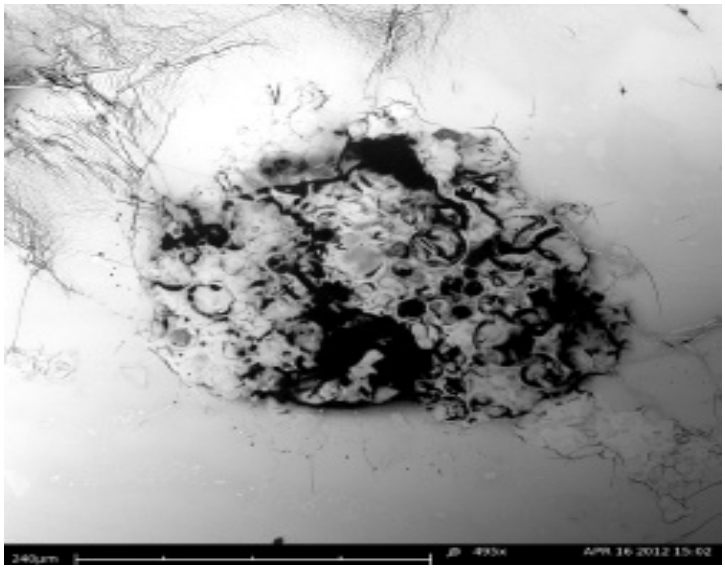


Fig. 10: SEM micrograph of the fluconazole MBGs

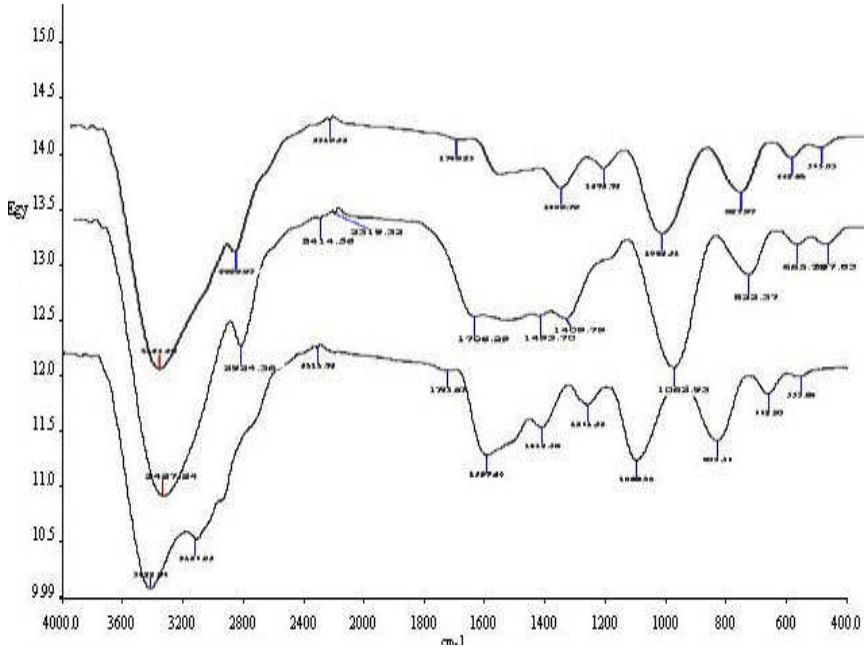


Fig. 11: FTIR spectra

CONCLUSION

The fluconazole MBGs could be effectively formulated for the topical treatment of onychomycosis. The optimized gel showed better penetration and mucoadhesive properties as compared to the commercial gel. The cumulative percentage of drug release after 7 h for optimized MBGs was 72.23%. Fluconazole microemulsion based gel form showed better activity against *Aspergillus niger* than the commercial gel. It was concluded that drug-loaded gel could be a promising formulation for effective treatment of onychomycosis. The *in-vitro* studies indicated that fluconazole MBGs could be a viable alternative to the current topical formulations available for the treatment of onychomycosis.

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