

In Vitro and *In Vivo* Evaluation of Microspheres Loaded Topical Gel Delivery System of Ketoconazole in Male Rats against *Candida Glabrata*

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Abstract

Ketoconazole topical is an antifungal medication. Ketoconazole topical prevents fungus from growing on your skin. The aim of the present investigation was to design and formulate an appropriate microspheres loaded gel (MLG), and explore the parameters affecting the morphology and other characteristics of the resultant products employing scanning electron microscopy (SEM). Microspheres loaded topical gel has been shown that encapsulation and controlled release of ketoconazole could reduce the side effect while also reducing percutaneous absorption when administered to the skin. The particle size ranged from 30-120 μm and the microspheres loaded gel was found to be in the range 6999 to 18596 cps. The MLG formulations exhibited maximum gel strength was found to be (35 sec.), highest value of mucoadhesion force was found to be (26.72 dynes/cm²), and remarkable spreadability was found to be (4.44 gm.cm/sec). The microspheres were incorporated in various concentrations of gel formulations and the drug release from these formulations was up to 12 hours.

Keywords: Microspheres loaded gel, *In vivo* studies, SEM, Ketoconazole, RP-HPLC

INTRODUCTION

Candidiasis is the fungal infection that can manipulate the private parts. Genital infection is extremely more frequent in women than men, but when it does occur in males, thrush affects the head of the penis and the foreskin [1]. Efficacy of the formulation based on its pharmacological profile and type of formulation. The penile thrush is most common in men who are taking immunosuppressant, corticosteroids, antibiotics and suffering with metabolic syndrome such as diabetes and living with poor hygiene condition. A man is also recommended to avoid unprotected sex with a candidiasis infected female partner until the woman cleared her infection after the treatment. For the treatment of penile thrush creams and ointments are most commonly used and it's has limitation of duration of action. In addition, the application of topical drug delivery has numerous problems, such as, ointments that are often visually unpleasant, greasiness, stickiness, etc., that often ends in lack of patient compliance. Ketoconazole, a substituted Imidazole; it is having broad spectrum activity against systemic and superficial mycoses. It is readily but incompletely absorbed after oral dosing and it varies among individuals. [2] Drugs that are not soluble in water can be entrapped in microsponge pores, which are extremely small; thus, the drug functions as microscopic particles, producing a greater surface area and increasing the rate of solubilization.[3] Microspheres represent a polymeric matrix system containing the drug in

a state of uniform distribution throughout the matrix [4–6]. Cellulose based polymers such as ethylcellulose (EC) find wide application in the preparation of matrix-type microspheres of water-soluble drugs to control the dissolution rate of drugs from the dosage forms [7–9].The present work was envisaged to reduce the dosing frequency and improve patient compliance by designing and evaluating controlled release of ketoconazole microspheres loaded gel for Genital infection. The MLG have advantages such as efficient absorption and more drug retention time.

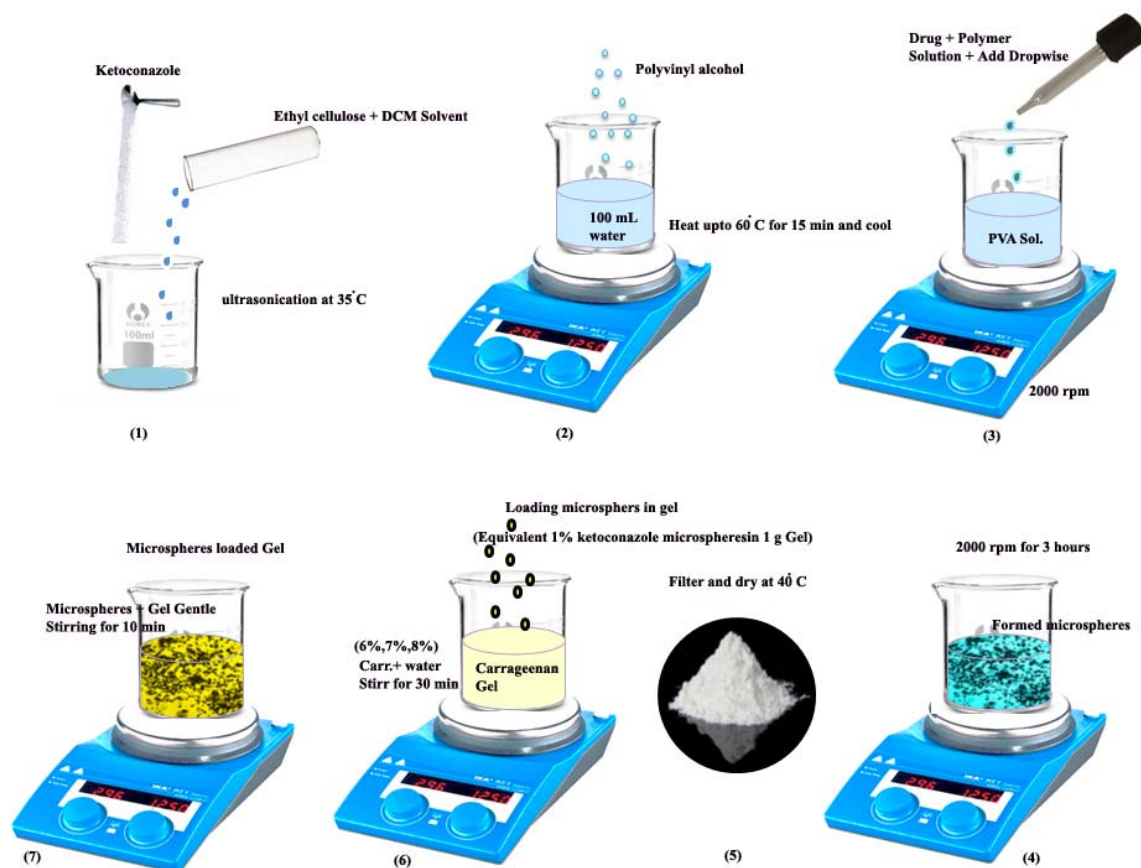
MATERIALS AND METHODS

Materials

Ketoconazole (SM Pharmaceuticals Sdn. Bhd. Sungai Petani, Malaysia), which is a water-in soluble drug, was chosen as a model drug. Polyvinyl alcohol (Esses, United Kingdom) was used as surfactant, which enhances the dispersibility of the polymer droplets. EC (Esses, United Kingdom) was used as a matrix-forming agent and Carrageenan (Esses, United Kingdom) was used as a gelling agent in this study. All other chemicals were of analytical or reagent grade and used without further purification.

Method

Figure 1: Flow diagram of preparation of microspheres loaded gel [10-12]



Particle size studies Size and Size distribution

The particle size of microspheres was determined using an optical microscopy method (Olympus 70g camera scope 9 DN-117M, USA). Approximately 100 microspheres were counted using calibrated microscope [13].

Scanning electron microscopy

The morphology and size of microspheres were observed by (Phenoworld) scanning electron microscopy. Prepared microspheres were coated with gold and studied under vacuum at room temperature [13]. The microspheres were viewed at an accelerating voltage of 4.8kV.

Determination of loading efficiency

A weighed quantity of microspheres were crushed into powder and added to 100 ml phosphate buffer (pH 7.4). The resulting mixture was kept under magnetic stirrer for 2 h. The solution was then filtered and determined by RP HPLC method. RP HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50 μ L loop volume. The LC solution version 1.25 was used for data collecting and processing (Shimadzu, Japan). The HPLC was carried out at a flow rate of 1.0 ml/min using a mobile that is phase constituted of acetonitrile, 10mm potassium dihydrogen phosphate buffer (pH 4.5) (65:35, v/v), and detection was made at 225 nm. The mobile phase was prepared daily, filtered through a 0.45 μ m membrane filter (Millipore) and sonicated before use. A Thermo C18 column (25cm \times 4.6mm i.d., 5 μ) was used for the separation. The drug

content was calculated from the calibration curve and expressed as loading efficiency [14].

Viscosity Studies

The rheological studies were conducted by using brookfield programmable DVII+ Model pro II type (USA). The viscosity of MLG was determined at different angular velocities (0.3, 0.6, 1.5, 3.0, 6.0, 12, 30 to 60 rpm) and means of two readings were used to estimate the viscosity [12].

Determination of mucoadhesive force

The mucoadhesive force of all the optimized batches was determined as follows, a section of the chicken mucosa fixed with mucosal side out onto each glass vial 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. Microspheres loaded gel 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 weight was kept rising in the pan until vials get detached. Mucoadhesive force was the minimum weight required to detach two vials. The chicken mucosa was changed for each measurement [15].

$$\text{Detachment stress (dynes/cm}^2\text{)} = m \text{ g/A}$$

Where m is the weight added to the balance in grams; g is the acceleration due to gravity taken as 980 cm/s²; and A is the area of tissue exposed.

Measurement of Gel Strength

A sample of 50 gm of microspheres loaded gel was placed in a 100 ml graduated. The apparatus for measuring gel strength (weighing 27 gm) was allowed to penetrate in gel.

The gel strength, which means the viscosity of the gels was determined by the time (seconds), the apparatus took to sink 5cm down through the prepared gel [15].

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 min. weight (50 g) was added to the pan. The time in which the upper glass slide moves over to the lower plate was taken as measure of spreadability [15].

$$S = ML/T$$

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 MLG formulations were studied using cellophane membrane using modified apparatus. The dissolution medium used was phosphate buffer, freshly prepared (pH 7.4). Cellophane membrane previously soaked overnight in the dissolution medium, was tied to one end of a specifically designed glass cylinder (open at both ends). One gram of MLG (equivalent to 1% w/w of ketoconazole) was accurately placed into this assembly. The cylinder was attached to stand and suspended in 200 ml of dissolution medium maintained at $37 \pm 1^\circ\text{C}$, the membrane just touching the receptor medium surface. The dissolution medium was stirred at 100 RPM speed using teflon coated magnetic bead. Aliquots, each of 5 ml volume were withdrawn periodically at predetermined time interval of 0.15, 0.30, 1.0, 2.0, 3.0, 4.0, 5.0 up to 12 hours and replaced by an equal volume of the receptor medium. The samples were appropriately diluted and measured by using RP HPLC method.

In vivo evaluation of therapeutic efficacy

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 microspheres loaded 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 [16].

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 microspheres 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 inoculated 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 μm . The sections were taken on a microscopic slide on which egg albumin (sticky substance) was applied and allowed for drying. Finally, Serial cross sections of the tissues were obtained and stained with periodic acid-Schiff (PAS) for fungal visualization [17].

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

Ethyl cellulose microspheres were prepared by an emulsion/solvent evaporation method, using water as non-solvent. Microencapsulated techniques have frequently been used for lipophilic drugs since hydrophilic drugs showed low loading efficiency [18]. Ketoconazole, due to its lipophilicity is likely to preferentially no partition out into the aqueous medium, leading to high loading efficiency.

The effects of numerous process and formulation limitations on the drug loading efficiency of microspheres are shown in Table 1. The highest (85.67%) entrapment loading was achieved with polymer-drug ratio (1:1) and further increase in polymer-drug ratio from 1:1 to 1:2 and 1:3 shown decrease in loading efficiency of ketoconazole. As the concentration of ethyl cellulose increased the viscosity of the solution increased resulting in the

Table 1. Effect of various parameters on entrapment efficiency and particle size

Formulation Code	Drug/polymer ratio (w/w)	Volume of processing medium (ml)	% of surfactant in processing medium (w/v)	Loading efficiency (%)*	Mean particle size (μm)*
E1	1:1	100	0.1	85.67 \pm 1.91	30.17
E2	2:1	100	0.1	81.32 \pm 1.02	34.63
E3	3:1	100	0.1	79.96 \pm 1.88	36.23
E4	1:2	100	0.1	42.50 \pm 1.75	98.20
E5	1:3	100	0.1	39.83 \pm 1.32	120.76
E6	1:1	150	0.1	61.24 \pm 1.67	66.47
E7	1:1	175	0.1	42.17 \pm 1.95	72.38
E8	1:1	100	0.2	41.15 \pm 1.62	68.52
E9	1:1	100	0.3	39.45 \pm 1.05	65.07

*mean \pm SD, n=3

Table 2. Characteristics of various microspheres loaded gel formulations

Formulation code	Mucoadhesive force (dynes/cm ²)	Spreadability (gm.cm/sec.)	Gel Strength sec.
MLG1	26.72	4.44	25
MLG2	26.91	3.13	28
MLG3	27.98	2.48	35

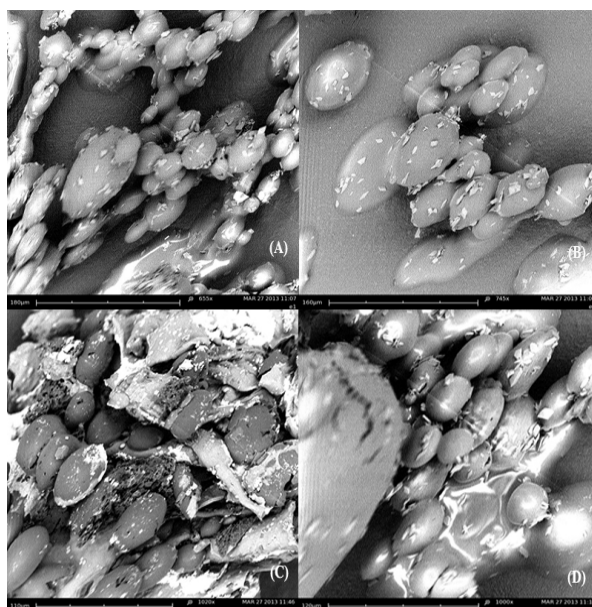


Figure 2. (A) and (B) showed the SEM photography of microspheres loaded with 200 and 300 mg of ketoconazole, (C) and (D) showed microspheres loaded gel with 100 and 200 mg of ketoconazole.

formation of bigger droplets. The bigger particles receipts much time for toughening, allowing time for drug diffusion out of the particles, which tends to decrease loading efficiency [19].

The polymer-drug ratio that shows the optimum drug encapsulation is kept constant and effect of other parameters on encapsulation efficiency was studied. Loading efficiency was decreased with increased concentration of surfactant. The volume of aqueous medium also significantly influenced the loading efficiency of the drug-loaded microspheres. As the volume of aqueous medium was increased from 100 ml to 150 ml and to 175 ml, the entrapment efficiency was decreased respectively. The reason may be the dilution of surfactant in aqueous medium, resulting in lower loading efficiency.

The average particle size of all the formulations were in the range of 30-120 μm and particle size was increased with increase in concentration of the polymer. The increase in viscosity of polymer solution with increase in polymer concentration produced larger particles in higher polymer formulations (figure 2).

The values of spreadability denote that the gel is easily spreadable by small amount of force. The spreadability of formulation MLG3 was found to be less as compared to MLG4 formulations; this indicates high viscosity of carrageenan gel rigid to spread on skin.

Gel strength is important because strong gels will support a much higher pressure than weak gels before they are washed out of the targeted site. The formulations exhibited moderate gel strength (Table 2), which may be due to concentration of carrageenan from (6 to 8%).

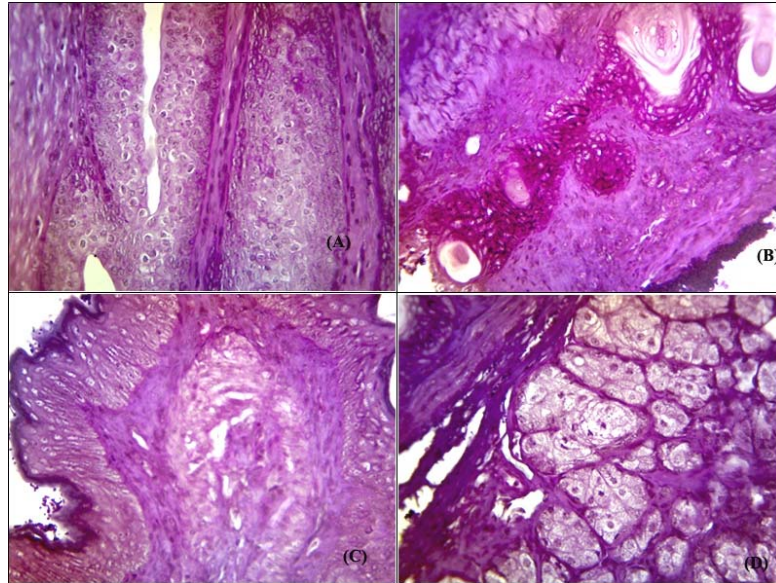


Figure 3. Histopathological analysis of the rat penal smooth muscle section from (A) control showed normal articheture and (B) showed *Candia* microorganism infection in smooth muscle surface (C) and (D) showed reduction in growth of *Candia* due to antifungal effect of standard and MLG, PAS, 400X

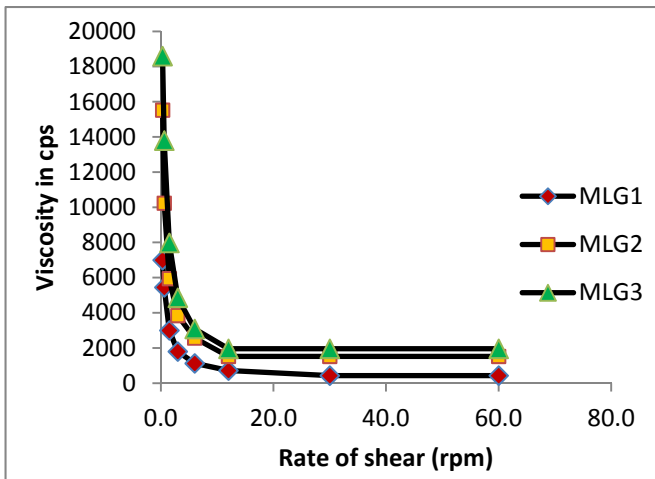


Figure 4. Showing the viscosity of MLG

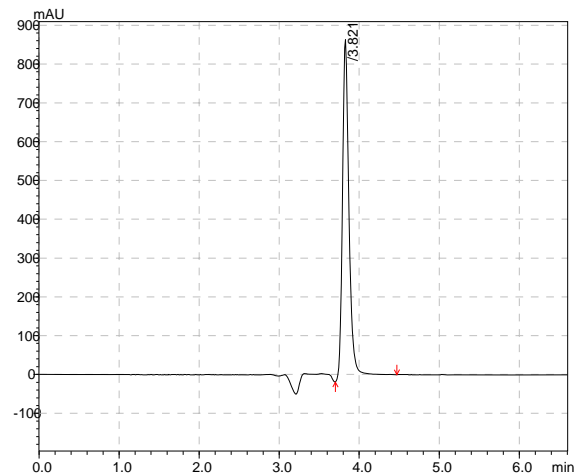


Figure 6: Typical chromatogram of ketoconazole

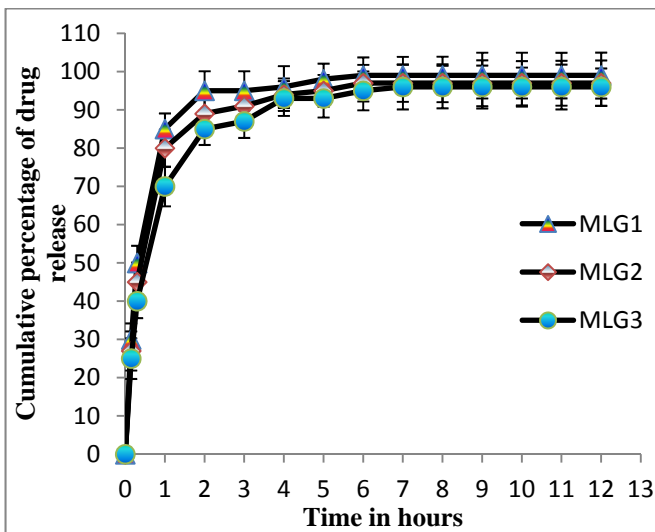


Figure 5. Showing the drug release of MLG

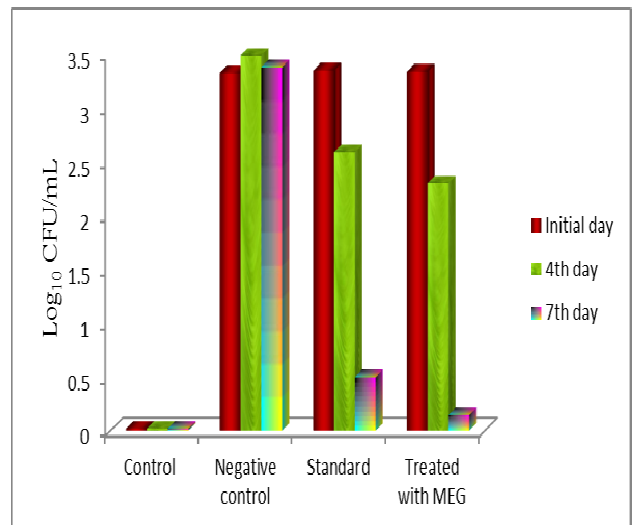


Figure 7: Quantitative microbiological analysis of the *Candida glabrata* in rat penal smooth muscle surface.

Mucoadhesive drug delivery system to mucosal membrane leads to an increase in the drug concentration at the absorption site and therefore improved bioavailability of systemic delivery drugs [20]. The mucoadhesive force is an important physico-chemical parameter for topical application. The formulations showed maximum mucoadhesive force, these may be due to increase in concentration of carrageenan in the formulations (Table 2).

All the MLG formulations were behaving as shear thinning systems (Figure 4). The formulation MLG 3 having the maximum concentration of carrageenan (8% w/v) showed maximum viscosity in gels forms and hence was not suitable for application.

The drug release was decreased with increase in concentration of the carrageenan as increase in the gel viscosity has effect on drug bust from the microspheres. It is understood that higher viscosity of gelling agent results in a longer diffusional path length, so drug release is extended for 12 hours (Figure 5-6).

The therapeutic efficacy of MLG was compared with *Candida glabrata* control by quantitative microbiological analysis and histopathological evaluations (Figure 3). MLG and standard marketed formulation treated animals showed significant reduction of CFU count on 4th day of the treatment onwards. The efficacy of the MLG is comparable with standard marketed formulation (Figure 7).

CONCLUSION

Microspheres loaded gel of ketoconazole was successfully prepared by emulsion solvent diffusion method using EC polymer and carrageenan as a gelling agent. The concentration of the EC influenced in all the evaluated parameters. The above findings revealed that Microspheres loaded gel of ketoconazole offer a convenient dosage form for achieving better release profiles and improved patient compliance. The *in vivo* animal studies conducted on adult wistar rats against *Candida glabrata* in penile candidiasis revealed that the MLG formulation made with ketoconazole were eradicating completely the fungal burden in comparison with standard.

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