IJPSR (2020), Volume 11, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 21 January 2019; received in revised form, 05 July 2019; accepted, 05 November 2019; published 01 January 2020

ANTIFUNGAL ACTIVITY OF AMPHOTERICIN-B ETHOSOMAL GEL AGAINST *CANDIDA ALBICANS*: A COMPARATIVE STUDY

Chanpreet Kaur [*1] and Poonam Maurya [2]

Department of Pharmaceutics [1], School of Pharmaceutical Sciences, Jaipur National University, Jaipur - 302017, Rajasthan, India.
Department of Pharmaceutics [2], Shri Rawatpura Sarkar Institute of Pharmacy, Datia - 475661, Madhya Pradesh, India.

Keywords:

Amphotericin B, Ethosomal gel, Antifungal activity, *Candida albicans*, Dermatomycoses, Carbopol 930

Correspondence to Author:
Chanpreet Kaur

Department of Pharmaceutics, School of Pharmaceutical Sciences, Jaipur National University, Jaipur - 302017, Rajasthan, India.

E-mail: chanpreetkaur25@gmail.com

ABSTRACT: Amphotericin B is among the gold standard antifungal agents used for the treatment of the wide range of fungal infections. Work aimed to formulate the amphotericin B (AmB) ethosomal gel (EF), evaluate its antifungal activity against fungal isolates of human origin and compare its antifungal activity with marketed liposomal gel (MLG). AmB ethosomal gel (EF) was formulated and characterized for their physical appearance, pH, spreadability, viscosity, drug content, zeta potential, *in-vitro* diffusion study and *in-vitro* and *in-vivo* antifungal study. The results showed that pH value was within acceptable limits (6.2 ± 0.021). EF showed better spreadability than MLG. EF showed highest drug content (97.3 ± 0.43 %) than that of MLG (76 ± 0.32%), better spreadability and more negative zeta potential to confirm the stability of the formulation. AmB EF showed the highest zone of inhibition (28 ± 0.20 mm), in contrast to MLG (24 ± 0.13 mm) against *Candida species*. The *in-vitro* and *in-vivo* studies revealed effective therapeutic potential against *Candida albicans* induced dermal mycosis. Result of this study suggested that ethosomal gel be the most proficient carrier system for dermal and transdermal delivery of Amphotericin B for the treatment of dermatomycoses.

INTRODUCTION: Fungal infections are most commonly caused by *Candida spp*., particularly *C. albicans*, *Candida tropicalis*, and *Candida parapsilosis* [1, 2]. Among *Candida species*, *C. albicans* is the most common cause of invasive infections. It is the leading cause of disseminated fungal infection in neonates, immunocompromised hosts, diabetics, and postoperative patients [3]. Several treatment options are available for cutaneous fungal infection [4]. Some drugs such as miconazole, clotrimazole, and ketoconazole have been used for the treatment of fungal and yeast skin infections. Amphotericin B has been the agent of choice for the treatment of serious fungal infections for more than 35 years. However, administration of the most common preparation of amphotericin B (a sodium deoxycholate colloidal suspension) is associated with severe, dose-limiting acute and chronic toxicities, particularly nephrotoxicity [5].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(1).413-19

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(1).413-19

Also, topical application is limited due to its low absorption through mucosa or skin. AmB molecule is highly lipophilic and cannot be dissolved in an aqueous medium. Several compounded topical formulations of AmB in the form of cream, lotion, and ointment are available but are not approved by regulatory agencies of most countries.

Application of these topical formulations of AmB have shown very limited efficacy even at high doses of drug and results in several adverse reactions such as severe blistering, itching, redness, peeling, dryness or irritation of the skin and failed to achieve cure against fungal infections [6]. Considering the benefits of topical drug delivery, we have developed a gel formulation of AmB and evaluated the antifungal activity of AmB ethosomal gel formulation.

MATERIALS AND METHODS:

Materials: Carbopol 934, methyl and propyl parabens, light liquid paraffin, propylene glycol, and ethyl alcohol were purchased from Central Drug House, India. *Candida albicans* strain (Microbial Type Culture Collection) was obtained from Institute of Microbial Technology, Chandigarh.

Formulation of AmB Ethosomal Gel:

Preparation of Ethosomes: Ethosomes were prepared by the cold method using probe sonicator. Phospholipid was dissolved in ethanol at 30 °C on a magnetic stirrer. The drug was dissolved in propylene glycol, and the solution was vortexed so that the drug completely gets dissolved in propylene glycol later the mixture was added to the phospholipid and ethanol mixture at 30 °C on a magnetic stirrer. Further warm water heated up to 30 °C was added dropwise drop using syringe kept on sonicator and was sonicated for 3 cycles, each cycle of 5 min to get nanosized ethosomes [7].

Preparation of Carbopol Gel Base: 2.0 gm Carbopol 934 was weighed and dispersed in water with mild stirring and allowed to swell for 24 h to obtain 2.0% gel. Later 2 ml of glycerin was added for gel consistency. Further, 0.02% of methylparaben was added.

Preparation of Ethosomal Gel: 1 gm of ethosome formulation was dissolved in 10 ml of ethanol and centrifuged at 6000 rpm for 20 min to remove the unentrapped drug. The supernatant was decanted, and sediment was incorporated into the gel vehicle. The incorporation of the ethosomes into gels was achieved by slow mechanical mixing at 25 rpm for 10 min [8, 9].

Characterization of AmB Ethosomal Gel:

Physical Appearance: The formulated ethosomal gel (EF) and marketed liposomal gel (MLG) were tested for their physical appearances such as color, consistency, and texture.

Determination of pH: Weighed 5 gm of each gel formulation were transferred in 10 ml of the beaker, and the pH was measured by using the digital pH meter. The pH of the topical gel formulation should be in range 3-9 to treat the skin infections [10].

Spreadability: A modified apparatus was used for determining spreadability. The spreadability was measured based on slip and drag characteristics of the gels. The modified apparatus was fabricated and consisted of two glass slides, the lower one was fixed to a wooden plate, and the upper one was attached by a hook to a balance. The spreadability was determined by using the formula:

$$S = ML/T$$

Where S= is spreadability, M= is weight in the pan tied to upper slide, T= is the time taken to travel a specific distance, and L= is the distance traveled.

For the practical purpose of the mass, the length was kept constant, and 't' was determined. The measurement of spreadability of each formulation was in triplicate (n=3), and the average values are presented [11].

Measurement of Viscosity: The viscosity of gels was determined by using a Brookfield viscometer LV (DV-E) model. A T-Bar spindle, in combination with a helipath stand, was used to measure the viscosity and have accurate readings. The spindle (64) was lowered perpendicularly in the center keeping in mind that the spindle does not touch the bottom of the jar. The spindle (64) was used for determining the viscosity of the gels. Various factors like temperature, pressure and sample size, *etc.* which affect the viscosity were maintained during the process. The spindle was moved up and down giving viscosities at a number

of points along the path. The torque reading was always greater than 10%. Five readings taken over 60 sec were averaged to obtain the viscosity.

Drug Content: 1 gm of the EF and MLG were mixed with 100ml of phosphate buffer of pH 7.4 separately. Aliquots of different concentrations were prepared by suitable dilutions after filtering the stock solution and the absorbance was measured at 386 nm. Reading were taken in triplicates (n=3). Drug content was calculated by linear regression analysis of the calibration curve.

Determination of Zeta Potential: The zeta potential of both EF and MLG was determined by Zeta meter.

***In-vitro* Diffusion Study:** An *in-vitro* drug release study was performed using modified Franz diffusion cell. Egg membrane was placed between receptor and donor compartments. EF was placed in the donor compartment, and the receptor compartment was filled with 1% tween 80 solutions consisting of 1 ml of tween 80 in 100 ml of phosphate buffer of pH 7.4(50 ml). The diffusion cells were maintained at 37 ± 0.5 ℃ with stirring at 50rpm throughout the experiment. At different time interval, 3 ml of aliquots were withdrawn from the receiver compartment through the side tube, and the fresh buffer (3 ml) was added to the receiver compartment. The withdrawn sample was analyzed for drug content by UV Visible spectrophotometer. The readings were taken in triplicates (n=3). The same procedure was repeated, and the sampling was done at 2h, 4h, 6h upto 24 h. The same procedure has been followed for MLG, the readings were taken in triplicates (n=3) [12].

***In-vitro* Antifungal Activity:** *C. albicans* was used as an indicator strain. The nutrient agar media was prepared for this purpose and the final pH of the medium was kept at -5.6 ± 0.2 to retard the growth of unlike organisms. The medium was sterilized using an autoclave for sterilization at 15 lbs /inch^2 (121 ℃) for 15 min.

The microbial cultures used in the study were obtained in lyophilized form. This lyophilized culture was inoculated in sterile nutrient broth and potato dextrose broth for fungus growth than incubated for 24 h at 37 ± 0.5 ℃. After incubation, the growth was observed in the form of turbidity. These broth cultures were further inoculated on to the nutrient agar and potato dextrose agar plates with loop full of bacteria and further incubated for next 24 h at 37 ± 0.5 ℃ to obtain the pure culture and stored as stocks that are to be used in further research work.

The paper disc diffusion method was used to determine the antifungal activity of gel formulation using standard procedure [13]. For this experiment, 6 mm diameter Whatman filter paper discs were impregnated with a stock of 100 mg/ml then dried in aseptic conditions. A nutrient potato dextrose agar plate with particular fungus is seeded with the help of spread plate technique prior and left for 5 min. Now the drug impregnated filter paper discs were placed in the center of pre-inoculated culture plates then incubated for 24 h at 37 ± 0.5 ℃. After incubation, plates were observed to see the sensitivity of EF and MLG towards test fungus at different concentration (25, 50, and 75 mg/ml) in the forming zone of inhibition [13].

***In-vivo* Performance Studies:** To determine the *in-vivo* efficacy of the formulation, *C. albicans* induced mycosis model [14] was used for the studies. All the experiments were conducted with the prior permission of the Institutional Animal Ethics Committee ((Req.no: 891/CPCSEA).

Briefly, rat's hair (Wistar albino rats, 100-150 g) were removed using the depilatory cream and an area of 3 cm^2 × 3 cm^2 was marked. On next day, the skin of the animal was slightly abraded using sandpaper. Previously prepared inoculum of *C. albicans* was applied using a glass rod. Animals were divided into 4 groups containing 3 animals each. The first group served as the control, which received no treatment. Second group received an ethanolic solution of AmB. AmB liposomal formulation (MLG) was applied to animals of the third group, and AmB ethosomal gel (EF) was applied to the fourth group. Animals received the treatment for 6 consecutive days post-infection except control group. The animals were observed for any gross morphological changes. After 6 days, in order to determine the efficacy of treatment, animal skin was wiped with a cotton swab (ethanol 70%). The skin was excised from the treated site, homogenized in 5 ml saline in a tissue homogenizer. The so formed homogenate was

streaked on solid yeast extract-peptone-dextrose medium and incubated at 25 °C for 4 days.

Numbers of colony forming units (CFUs) on agar plate were counted, and the logarithm of the number of CFUs per infected site was calculated. An animal with more than one fungal colony was considered as positive.

Statistical Analysis: Data were expressed as Mean ± standard error of the mean from 3 independent experiments in each group, respectively. Results were analyzed statistically using one-way analysis of variance test followed by post-hoc Dunnet test for a level of significance at *P<0.05; **P<0.01; ***P<0.001.

RESULTS AND DISCUSSION:

Physical Appearance: The EF and MLG were pale yellow, with gel-based consistency and uniform texture.

Determination of pH: In transdermal drug delivery system pH plays an important role, the result of EF shows that the formulation is suitable for skin delivery. The pH value of the EF was within acceptable limits, and it was estimated to be 6.2 ± 0.021 for and was 5.9 ± 0.043 for MLG.

TABLE 1: RESULTS OF PH, SPREADABILITY, VISCOSITY, ZETA POTENTIAL AND DRUG CONTENT STUDY OF EF AND MLG FORMULATIONS

Formulation Code	pH	Spreadability (gm.cm/sec.)	Viscosity (cps) at 2rpm	Zeta Potential (mV)	Drug content (%)
EF*	6.2 ± 0.021	11.1 ± 0.075	9400 ±1.70	-44.5	97.3 ± 0.43
MLG**	5.9 ± 0.043	13.3 ± 0.061	9950 ± 1.45	-37.5	76 ± 0.32%

*EF – Ethosomal Formulation; **MLG – Marketed Liposomal Gel

Thus, the pH values of all the prepared formulations ranged from 6.0 to 6.5, which is considered acceptable to avoid the risk of irritation upon application to the skin by Ganeshpurkar *et al.*, 2014. The result of pH determination is given in **Table 1**.

Spreadability: The result of spreadability study is given in **Table 1**. The spreadability of EF was in the range 11.1 ± 0.075 gms.cm./sec and that of MLG is 13.3 ± 0.061. The concentration of polymer plays a vital role here. A good spreadability of the gel depends on the accurate concentration of polymer used. The gels having a very high and very low spreadability value indicate that the application of the gel to the site is difficult. Thus, this study concluded that EF has better Spreadability than MLG.

Viscosity Measurements: The results of viscosity study **Table 1** showed that the viscosity of the EF and MLG were almost the same. There was not much difference between the viscosities of both the gel. The viscosity of the EF was calculated to be 9400 ± 1.70cps at 2rpm, and that of MLG it was 9950 ± 1.45cps.

Drug Content: Drug content is the most important evaluation in ethosomal formulation and the data found are satisfactory. From the study **Table 1,** it was found that the drug content of EF was calculated to be 97.3 ± 0.43 % and that of MLG was calculated to be 76 ± 0.32%. Thus, ethosomal gel shows the greater capacity to hold the drug than that of liposomal gel.

Zeta Potential: The result of zeta potential study is given in **Fig. 1** and **2** for MLG and EF formulations, respectively. From the study zeta potential of MLG and EF was found to be -37.5mVand -44.5mv respectively **Table 1,** which shows that ethosomal formulation tends to be more negative than the other, thus being more stability. Thus, it is clear that EF formulation shows good stability.

***In-vitro* Drug Release Study:** The cumulative percentage drug release from EF and MLG formulations is shown in **Fig. 3**. EF Formulation showed higher cumulative drug release of 98.6420 ± 0.053 in 24 h than the MLG formulations (74.6521 ± 0.056). Thus, after comparing the release study of both the gels, it was concluded that the EF gave better release than MLG, which clearly states the role of the presence of ethanol.

***In-vitro* Antifungal Activity:** The lawn cultures were prepared with the pathogenic microorganism used under present study and sensitivity of microorganism towards the antifungal gel

formulation was studied at the concentration of 100 mg/ml using disc diffusion method. *Candida albicans* were inhibited by the standard antifungal used in present work *i.e.* AmB, at all the concentration (25, 50 and 100 mg/ml) used in the study for comparison. The results of the anti-fungal activity are shown in **Table 2**. In present work, EF and MLG showed antifungal activity against *Candida albicans* with the zone of inhibition lying in the range of 17 to 28 mm.

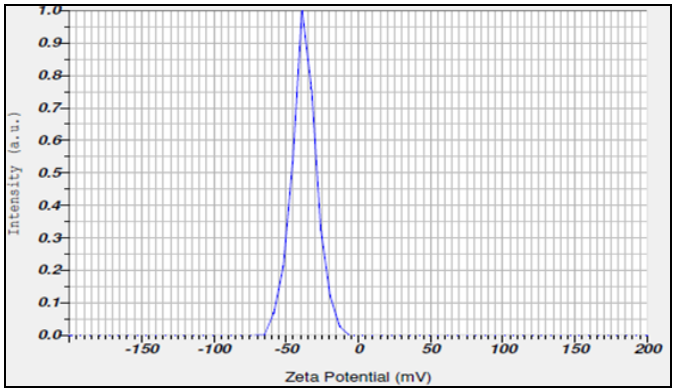

FIG. 1: ZETA POTENTIAL OF MLG

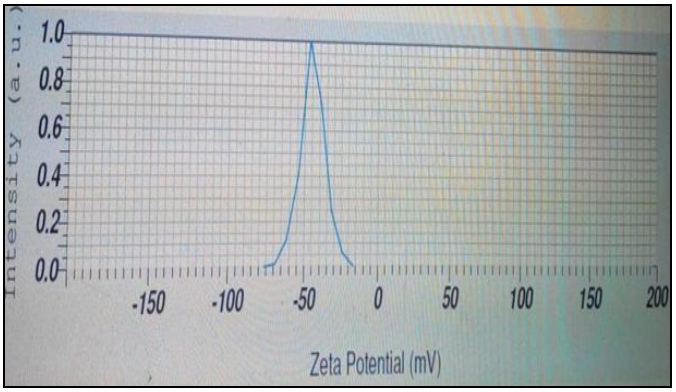

FIG. 2: ZETA POTENTIAL OF EF

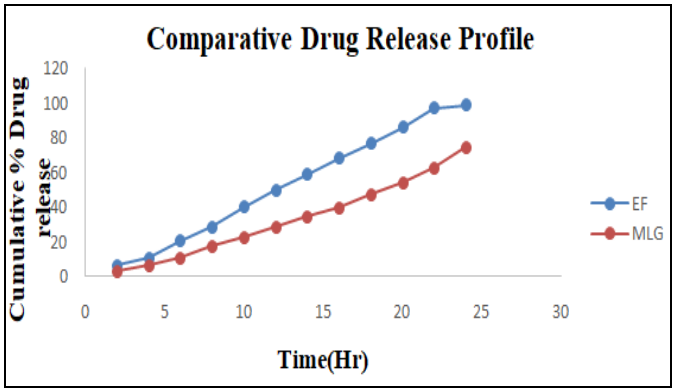

FIG. 3: COMPARATIVE DRUG RELEASE PROFILE OF EF AND MLG FORMULATION

TABLE 2: ANTIFUNGAL ACTIVITY OF DIFFERENT GEL FORMULATIONS AGAINST *CANDIDA ALBICANS*

Sample	Zone of inhibition (mm)		
	25 mg/ml	50 mg/ml	100 mg/ml
Marketed liposomal gel (MLG)	15±0.20	20±0.15	24±0.13
Ethosomal gel (EF)	17±0.11	24±0.21	28±0.20
Standard drug (SD)	20±0.12	25±0.13	29±0.27

Data are expressed as mean ± standard deviation (n = 3)

FIG. 4: PHOTOGRAPH SHOWING ANTIFUNGAL ACTIVITY (A) MLG; (B) EF; (C) SD

EF showed a greater percentage of inhibition of fungal infection against *Candida albicans.* On comparison of EF with MLG of AmB showed a greater percentage of inhibition of fungal infection

against *Candida albicans*, which is basically due to greater permeation of ethosomes due to the presence of ethanol.

***In-vivo* Performance Studies:** *C. albicans* induced rat mycosis model was used to determine *in vivo* efficacy of the ethosomal gel formulation, and results are shown in **Table 3**. **Table 3** demonstrates the significant efficacy ($P<0.05$) of ethosomalgel (EF) formulation in treating *Candida* infections when compared with AmB-ethanol solution (SD) treated group and AmB liposomal gel (MLG). Isolates of *Candida* were removed from the skin and tested for their viability.

In EF treated group, only one out of three animals exhibited positive culture test. Rapid recovery of infection was observed with AmB loaded ethosomal gel.

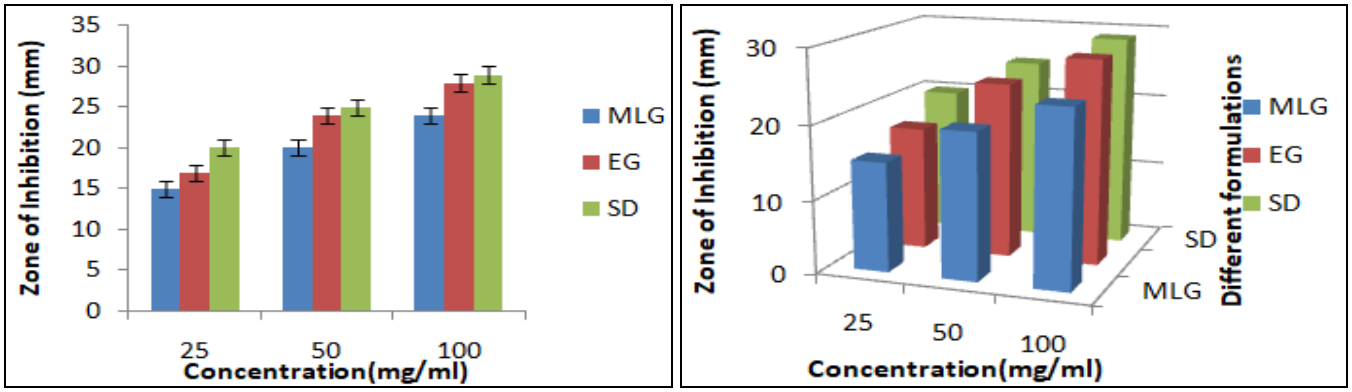

FIG. 5: COMPARATIVE ZONE OF INHIBITION OF DIFFERENT FORMULATION. Each data point is the average value of 3 determinations with error bars indicating ± standard deviation (SD); MLG= Marketed Liposomal Gel: EG= Ethosomal Gel: SD= Standard Drug.

TABLE 3: COLONY FORMING UNIT OF *CANDIDA ALBICANS* IN SKIN OF RATS AFTER TREATMENT WITH AMB ETHOSOMAL GEL

Treatment	No. of animals with positive culture/ total no. of animals	Log CFU/ infected sites
Control	3/3	4.16±0.83
AmB ethanol solution	2/3	3.02±0.53***
AmB liposomal gel (marketed)	2/3	3.02±0.53***
AmB ethosomalgel	1/3	1.16±0.70**

Data were expressed as mean±SEM from three in each group, respectively. Results were analyzed statistically using one-way analysis of variance (ANOVA) test. Level of significance at *P<0.05, **P<0.01, ***P<0.001. AmB: Amphotericin-B; CFU: Colony forming unit.

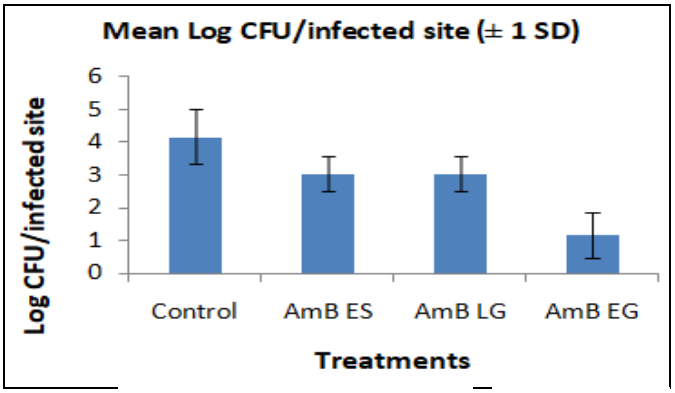

FIG. 6: COLONY FORMING UNIT OF *CANDIDA ALBICANS* IN SKIN OF RATS AFTER TREATMENT WITH EF

Recently, much focus has turned to these opportunistic fungi responsible for causing infections in vulnerable patients, such as patients treated with immune suppressors, or other acquired immunodeficiency conditions and chemotherapy patients [15]. Due to the rapid development of bacterial and fungal resistance, many antimicrobial medications can turn out to be ineffective.

Hence, researchers continue to search for new antimicrobials from natural products. Many studies are conducted towards reducing the AmB dose by combining it with new products that have antifungal action in an attempt to produce an effective synergetic action as well as reduce the adverse effects of AmB [16, 17]. The ethosomal gel is a versatile transdermal drug delivery approach, which has merits of both emulsion and gels, making it suitable for delivery of water-soluble and insoluble drugs.

The study results of antifungal activity of AmB suggest the treatment of infections caused by *C. albicans* and may reduce the efficacious dose of AmB, thus minimizing its side effects. Also, *in-vivo* experiment results confirmed the potential for therapeutic applications for the AmB ethosomal gel.

CONCLUSION: The fungal strains used in this study are mostly responsible for superficial mycoses. However, treatments of these infections are sometimes ineffective. This study has shown that AmB ethosomal gel exerted antifungal activity against dermatophytes, some of which are the most frequent species simplicated in dermatomycoses. Further research is needed to determine the toxicological profile of this ethosomal gel formulation and as well as develop it further for subsequent clinical evaluation.

ACKNOWLEDGEMENT: I am extremely thankful to Mr. Prabhat Jain, Scan Laboratory, Bhopal for providing the necessary support for the work.

CONFLICTS OF INTEREST: Nil

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How to cite this article:
Kaur C and Maurya P: Antifungal activity of amphotericin-B ethosomal gel against *Candida albicans*: a comparative study. Int J Pharm Sci & Res 2019; 11(1): 413-19. doi: 10.13040/IJPSR.0975-8232.11(1).413-19.