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Research Article

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Formulation and evaluation of Tolnaftate loaded topical liposomal gel for effective skin drug delivery to treat fungal diseases

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ABSTRACT

A liposomal reservoir system bearing the anti-fungal, tolnaftate, was developed for better-permeable, controlled and localized delivery via topical route. Different charged liposomes were developed by dried thin film hydration technique and incorporated to carbopol gel. The developed gels were characterized for particle size, poly-dispersity index, drug content, spreadability, microbial assay and in vitro permeation. Antifungal efficacy of developed gels was assessed by the in vivo studies in albino wistar rats. The mean particle size of neutral, negative and positive liposomes was 119 nm, 143 nm and 284 nm respectively. Among the three different liposomes neutral charged has exhibited higher entrapment efficiency about 88.14%. The animals have shown complete sign of cure on the 13th day of the drug therapy. Small sized vesicle of developed liposomal gel formulation attributed higher permeation and faster cure rate than the available marketed formulation. So, this study ensures that liposomal carriers have a high potential in topical drug delivery and can overcome the permeability and efficacy problems.

Keywords: Tolnaftate, liposomal gel, permeability, Topical antifungal, *Candida albicans.*

INTRODUCTION

Topical drug delivery is a pleasing route for local and systemic treatment. The delivery of drug through the topical is most effective treatment for the skin diseases [1].Topical drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist. When the drug is applied to the topically, mainly hydrophobic drugs, only a minute quantity enters in to the systemic circulation remaining stays as such on the skin.

Tolnaftate is a synthetic thiocarbamate used as an anti-fungal agent. It inhibits the squalene epoxidase [2]. Squalene epoxidase is an important enzyme in the biosynthetic pathway of ergosterol (a key component of the fungal membrane). Tolnaftate was found to be only active by topical application and inactive by the oral and intra peritoneal routes of administration [3,4]. Tolnaftate is available in the market in different topical dosage forms like cream, powder, spray and liquid aerosol. But each have its own disadvantages like aerosols cause a mild temporary stinging, in case of creams and gels they are having poor penetration hence require long time of therapy for curing and decreases the patient compliance.

One of the approaches to increase penetrability of the drug thereby without compromising the efficacy is through colloidal drug delivery system. Colloidal/Sub-micron drug delivery systems are drawing attention in the

pharmaceutical research for the commercialization as they (a) enhancing the permeability (b) elevating the drug efficacy (c) slower release makes sustained and prolonged action (d) very low intrinsic toxicity (5)

Liposomes are the microscopic spheres made up of an aqueous core surrounded by the lipid shell [6,7,8]. Liposomes are (1) pharmaceutically acceptable & stable (2) superior carriers (3) ability to encapsulate hydrophilic and lipophilic drugs (4) has higher protection against the degradation (5) enhanced bio-availability of drug [9,10]. They can entrap hydrophilic and lipophilic drugs and exhibit higher accumulation at the site of action and deliver the drugs in sustained and controlled rates [8].An important aspect of liposomes is the protection that they afford as an encapsulating agent against potentially damaging conditions in external environments. Liposomes are also an important system in their own right in medical, cosmetic, and industrial applications [11]. They have affinity to pass keratin of horny layer of skin and can penetrate deeper into skin and hence give better absorption. Topical liposome formulations could be more effective and less toxic than conventional formulations [12]. Topical formulations are also smart route for local and systemic treatment [1]. Hence in this study an attempt has made to formulate tolnaftate liposomal gel for potent skin penetration thereby increasing its efficacy.

EXPERIMENTAL SECTION

Materials

Tolnaftate was obtained as gift sample from Mole craft life sciences, Hyderabad, India. Soya bean lecithin, cholesterol, stearyl amine, dicetyl phosphate were purchased from sigma Aldrich, Mumbai, India. Sodium hydroxide and PEG 400 was obtained from SD fine chemicals, Chennai, India. Chloroform and ethanol was obtained from Merck, Mumbai, India. carbopol 971 P was obtained from Lubrizol, Mumbai, India. The equipment used in this study is rotary vacuum evaporator (Rotavapor® R-215), UV-Visible spectrophotometer (Shimadzu 1700E), Equitron probe sonicator (42 KHz, 120 W).

Methods

Development of calibration curve for tolnaftate

A stock solution of 1mg/mL of standard drug was prepared, later dilutions were made with ethanol. From this stock solution 10, 20, 30, 40, 50 µg/mL dilutions were prepared using ethanol. The λ_{max} of the drug was determined by scanning the dilutions between 200 to 400 nm using a UV-Visible spectrophotometer [13].

Preparation of tolnaftate liposomes

The preparation of liposomes with soya lecithin containing tolnaftate was prepared by dried thin film hydration technique using rotary vacuum evaporator. Accurately weighed drug and other chemicals was dissolved in 10 mL of chloroform and stirred in mechanical stirrer to form a homogenous mixture. The mixture was dried in rotary evaporator with vacuum of about 25 mm Hg at 25 ^{0}C. The process was continued until all the chloroform gets evaporated to get a dried thin film on the surface of the vacuum flask. Add 10 mL of phosphate buffer saline (PBS) pH 7.4 and rotated at 25 ^{0}C without vacuum, to get a liposomal suspension of multi lamellar vesicles (MLVs). The composition and ratios of lecithin, cholesterol and stabilizers used for different types of liposomes were mentioned in the (Table 1). The liposomal dispersion obtained after hydration was sonicated for 30 min by bath sonicator to produce small and more uniform sized population of liposomes [14,15].

Preparation of liposomal gel

One gram of carbopol 971 P was dispersed into purified water under stirring. Then the specified quantity of benzyl alcohol was added to carbopol gel during stirring. The pH of carbopol was adjusted to 6.8-7.0 using 2N NaOH .The prepared liposomes was added to carbopol gel under mixing to get a 1% tolnaftate liposomal gel. Finally the pH was adjusted to 6.8-7.0 using 2N NaOH and make up the final volume [16].

Characterization of Liposomes

Particle size and poly dispersibility index determination

Mean droplet size and polydispersity of the liposomes were determined by dynamic light scattering (DLS) technique. The instrument used was Malvern Zetasizer Nano, Series ZEN1002 (Malvern, UK) in cuvette DTS0012 with a 532 nm green laser and a scattering angle of 173^0 [17].

Percentage of entrapment efficiency
Liposomes were centrifuged and the supernatant was diluted with aliquot amount of ethanol and the concentration was determined by UV-Visible spectrophotometer. The amount of drug loaded was determined using the formula.

$$\%\ Entrapment\ efficiency = \frac{Amount\ of\ drug\ loaded}{label\ claim} \times 100$$

Short term stability studies
The short-term stability was to monitor physical and chemical stabilities of the liquid form of tolnaftate liposomal formulations at 4 ^{0}C and room temperature for up to one month. The stability parameter, such as, percentage of entrapment efficiency and pH was determined as a function of the storage time [18].

Characterization of Liposomal Gel
Physical examination and homogenicity
The prepared liposomes and liposomal gel formulations were inspected visually for their colour intensity difference. All developed gels were tested for homogeneity by visual inspection after the gels have been set in the container. They were also tested for their appearance and presence of any aggregates [19].

Determination of viscosity
Brookfield DVE viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) was used for the determination of viscosity of the formulations. About 0.5 g of sample was taken for analysis without dilution the sample by using spindle no. 63 using different rpm at 25±0.5°C [20].

Determination of Spreadability
Spreadability was determined by apparatus which was suitably modified in the laboratory and used for the study. It consists of a wooden block provided by a pulley at one end. By this method, spreadability was measured on the basis of 'Slip' and 'Drag' characteristics of liposomal gels. A ground glass slide was fixed on the block. 2 gm of liposomal gel under study was placed on the ground slide. The liposomal gel was then sandwiched between ground slide and another glass slide having the dimension of fixed ground slide, provided with the hook. A 1 kg weight was placed on the top of the two slides for 5 min to expel air and to provide a uniform film of the liposomal gel between the slides. Excess of the liposomal gel was scrapped from the edges. The top plate was then subjected to weight of 100 gm with the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5 cm was noted. A shorter interval indicates better spreadability [21].

Spreading coefficient was determined by using the formula:

$$S = m\ X \frac{l}{t}$$

Where, S = Spreadability,
m = Weight tied to upper slide,
l = Length of glass slides
t = Time taken to separate the slides completely from each other.

Microbiological Assay
Tolnaftate is active against *Candida albicans* (*C. albicans*) which is the most common species for dermal fungal infections. Hence it was chosen as fungal inoculum model for both microbial and *in vivo* studies. Petri-dishes containing 20 mL medium (Sabouraud dextrose agar) were seeded with 100 µl of the fungal inoculums *C. albicans*. The plates were dried at room temperature for 15 min. Wells, each 2 cm in diameter, were cut out of the agar. 2 g of neutral, positive and negative gel formulations were placed into each well. marketed cream (1%) equivalent to 2 g of formulation was used as reference. The fungal plates of *C. albicans* were incubated at 25 °C for 2 days. The zone of inhibition was observed after 48 h. The results were recorded by measuring the zones of growth inhibition surrounding the wells.

***Ex Vivo* Permeation Studies**

Permeation studies of neutral, positive, negative liposomal gel formulations and marketed cream were performed using Franz diffusion cells with a diffusion area of 1.813cm^2. Ethanol:PBS (pH 7.4) in 20:80 ratio was used as diffusion media with constant stirring using a magnetic stirrer at 300 rpm. The water bath was maintained at a temperature of 32±0.5^{0}C. 200mg of each formulation was applied separately on the dorsal surface of pork skin. Five Franz diffusion cells were used simultaneously i.e. neutral, positive, negative, marketed cream and blank. One mL of sample was withdrawn from each in the time intervals of 0, 0.25, 0.5, 0.75, 1, 2, 4, 6 and 12 h respectively. The amount of tolnaftate in the collected sample was determined by UV-Spectrophotometer [22,23].

After the permeation study of 12 h, the percentage drug retained on the skin at end of 12 h was determined by washing the skin three to four times with the ethanol solution. The solution was filtered through 0.22 µm filter and was analysed for the entrapment efficiency using UV-Visible spectrophotometer. For estimating the drug retained in the skin, the skin was cut into small pieces and was homogenized, which was then sonicated in the ultrasonicator for 10 min followed by vortex mixing for 15 min. The sample was then centrifuged at 6000 rpm for 15 min. After centrifugation, supernatant was taken, filtered through 0.22 µm filter and was analyzed for entrapment efficiency [24].

Invivo Studies

Albino wistar rats (male) weighing 150±20 g were used for topical fungal cure rates studies. All animal experiments were approved by institutional animal ethical committee, J.S.S. College of Pharmacy, Ooty (Proposal no. JSSCP/IAEC/M.PHARM/PH CEUTICS/08/2012-2014). Animals were grouped in to 4 groups containing 3 animals in each group. Group I animals were treated neutral formulation, group II with positive formulation, group III with negative formulation and group IV with marketed cream. Negative control was maintained to confirm the cure is because of formulations but not a natural process.

The male wistar rats which are two months old were maintained by giving food and water at 21 ^{o}C. The infectious agent used for this *in vivo* study is *C. albicans*. The organism was obtained from J.S.S College of pharmacy, Ooty. The micromycetes were maintained on sabouraud dextrose Agar (SDA), containing 40 g of glucose, 10 g of agar and 10 g of peptone in pure distilled water. The cultures were stored at 4^{0}C and sub cultured once a month. At the dorsal surface of the each rat, the areas of 5 cm^2 were cleaned and depilated. The infectious inoculums were prepared from a 6 day old culture of *C. albicans.* The inoculum was applied on the animal's dorsal surface immediately after the depilation and left for 3 days. The treatment was started on the 5th day of post inoculation and continued until complete recovery from the infection was achieved. Animals were treated once a day and the infected areas were scored visually for inflammation and scaling, as well as the presence of the pathogens by cultivating skin scales from infected loci in SDA plates containing 100 units/mL of penicillin and streptomycin, each day [24,25].

RESULTS AND DISCUSSION

Preformulation Studies

Standard calibration curve of tolnaftate in UV spectrophotometer

The UV absorbance of tolnaftate standard solutions was in the range of 10-50µg/mL of drug in ethanol showed linearity at 257 nm. The linearity was plotted for absorbance (Abs) against concentration (µg/mL) with R^2 value of 0.996 and with the slope equation y =0.018x-0.003.

Preparation of tolnaftate liposomal gel

The liposomes were prepared by dried thin film hydration technique using rotary evaporator with drug and carrier (soya lecithin). The formulation containing tolnaftate were prepared with different stabilizers like stearylamine and dicetyl phosphate and all other parameters like temperature, vacuum and stirring speed were kept constant. The composition and ratios of compounds are shown in (Table 2). Liposomes containing tolnaftate of optimized batch were mixed into the carbopol 971 P gel with an electrical mixer and finally the pH was adjusted to 6.8-7.0 using 2N NaOH and made up the final volume.

Characterisation of Liposomes

The prepared liposomes were viewed under motic microscope (Figure 1). Mean particle size and polydispersity index of the liposomes were determined by Malvern Zetasizer. The mean particle diameter of neutral liposomes was found to be around 119 nm. The mean particle diameter of negatively charged vesicles was found to be 143 nm and

positively charged was found to be 284 nm. These results can be attributed to the inclusion of a charge inducer (Stearylamine, Dicetyl phosphate) in liposomes, which increased the spacing between the adjacent bilayers, resulting in the formation of liposomes larger in size compared with the neutral ones (Table 3).

Table 1: Composition and ratios of lecithin, cholesterol and stabilizers for different types of liposomes

Ratio of ingredients	Types of liposome		
	Neutral	Positive	Negative
Lecithin: cholesterol: Stearylamine: dicetylphosphate	5:5:0:0	4.5:4.5:1:0	4.5:4.5:0:1
	6:4:0:0	5:4:1:0	5:4:0:1
	7:3:0:0	6:3:1:0	6:3:0:1
	8:2:0:0	7:2:1:0	7:2:0:1
	9:1:0:0	8:1:1:0	8:1:0:1
	4:6:0:0	4:5:1:0	4:5:0:1
	3:7:0:0	3:6:1:0	3:6:0:1
	2:8:0:0	2:7:1:0	2:7:0:1
	1:9:0:0	1:8:1:0	1:8:0:1

Table 2: The composition of optimized liposomal formulation

Type of Liposome	Drug	Soya lecithin	Cholesterol	Stearylamine	Dicetyl phosphate
Neutral	2	8	2	-	-
Positive	2	7	2	1	-
Negative	2	7	2	-	1

Table 3: Particle size and Poly dispersity index (PDI) of tolnaftate liposomes

Type of liposome	Particle Size (nm)	PDI
Neutral	119	1.000
Positive	284	0.640
Negative	143	0.163

Table 4: Stability of liposomes at 4 ^{0}C and 25 ^{0}C

Type of Liposome	Entrapment Efficiency at 4^{0}C			Entrapment Efficiency at 25 ^{0}C		
	0 day	15 days	1 month	0 day	15 days	1 month
Neutral	88.14±0.28	87.12±0.89	88.01 ±1.21	88.14 ±0.72	87.66± 1.6	86.34± 2.34
Positive	82.8±0.47	83.92 ± 0.48	81.7 ±0.74	82.8± 0.96	81.51 ±1.12	80.15± 2.05
Negative	84.23±0.21	82.16±0.29	84.14 ±0.48	84.23± 0.75	83.92 ±0.97	82.69± 1.84

**Values are Mean±S.D (n=5)*

Table 5: Stability study of liposomal gel at 4 ^{o}C

Type of liposome	pH			Viscosity		
	0 day	15 day	1 month	0 day	15 day	1 month
Neutral	6.85	6.83	6.84	3724-3735	3732-3738	3725-3738
Positive	6.89	6.86	6.85	5316-5381	5378-5399	5385-5398
Negative	6.87	6.88	6.83	5030-5051	5030-5051	5027-5051

Table 6: Stability study of liposomal gel at room temperature (25 ^{0}C)

Type of liposome	pH			Viscosity		
	0 day	15 days	1 month	0 day	15 days	1 month
Neutral	6.85	6.85	6.84	3724-3735	3725-3738	3728-3739
Positive	6.89	6.88	6.85	5381-5399	5383-5395	5136-5191
Negative	6.87	6.86	6.84	5128-5316	5132-5319	5122-5198

Table 7: Comparative data of % drug permeated, retained in skin and on skin

% Drug	Neutral	Negative	Positive	Marketed cream
Permeated	58.2±0.6	51.19±0.81	44.8±0.9	19.78±1.12
Retained in skin	29.33±0.5	25.36±0.2	20.56±0.43	10.66±1.6
Retained on skin	5.71±1.6	27.46±0.3	32.33±0.94	56.32±0.3

**Values are Mean±S.D (n=3)*

Percentage of Entrapment efficiency
The percentages of entrapment efficiency in neutral and charged liposomes were found to be 88.14%, 82.8% and 84.23% respectively. The percentage drug loading was optimum in neutral liposomes when compared with charged liposomes.

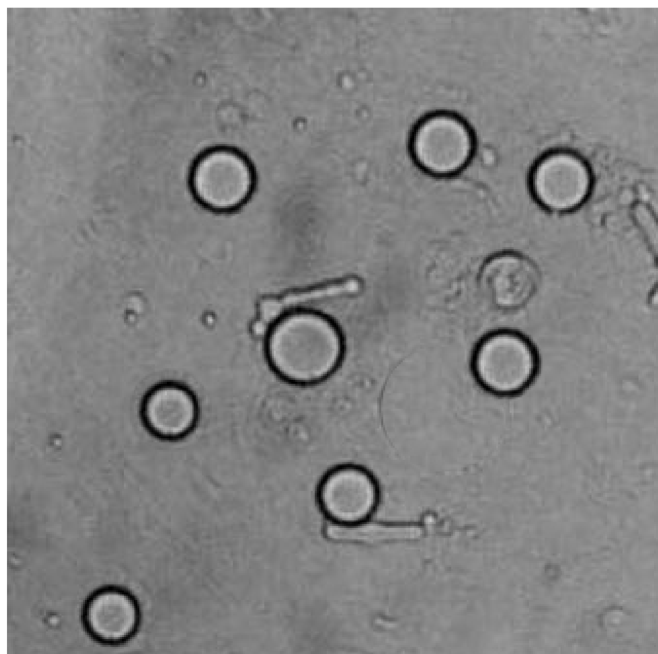

Figure 1: SEM image of formulated liposome

Characterization of Liposomal Gel
Physical examination and homogenicity
The prepared liposomal gel formulations were inspected visually for their colour, homogenicity, consistency and phase separation. All the liposomal gel formulations were found to be homogenous without any colour intensity differences and aggregates. The pH values of all the prepared formulations ranged from 6.8-7.0, which is considered to be acceptable for avoiding the risk of irritation upon application to the skin.

Spreading coefficient
The spreadability of neutral, positive, negative and marketed cream was found to be 39, 35, 38.30 and 20.57 respectively. Based on the values neutral and charged liposomal gel formulation having high spreadability coefficient than marketed cream because the gel formulations are having less viscous compare to creams. Acceptability and clinical efficacy of topical preparations require possessing optimal mechanical properties (ease of removal from the container, spreadability on the substrate), rheological properties (viscosity, elasticity, thixotropy and flowability) and other desired properties such as bio adhesion, desired drug release and absorption. The efficacy of topical therapy depends on the patient spreading the formulation in an even layer to deliver a standard dose. The optimum consistency of such formulation helps to ensure that a suitable dose is applied or delivered to the target site. The delivery of the correct dose of the drug depends highly on the spreadability of the formulation so spreadability is directly proportional to efficacy. Based on this it was concluded that formulated liposomal gel formulations were having more efficacy than marketed cream.

Microbial assay
The images for zone of inhibition are shown in the (Figure 2). The zone of inhibition of neutral, positive, negative and marketed cream were found to be 0.64±0.17 mm, 0.59±0.23 mm, 0.45±0.18 mm and 0.12±0.05 mm respectively. Based on these results, the neutral showed maximum inhibitory effect on fungal strain *C. albicans* followed by positive, negative formulations and marketed cream. The results indicated that neutral, positive, negative formulations were having higher release behavior and higher penetration capacity compare to marketed cream. It was also found that neutral, positive, negative have high zone of inhibition compared to marketed cream

this may be due to the drug in the vesicles of the 3 gels were highly lipophilic compared to drug as such present in marketed cream as a result high uptake of lipophilic compounds by fungal strains lead to higher zone of inhibition. This microbial assay has also exposed the fact that lower viscosity gel has higher release rates in addition to low vesicle sizes which has higher penetration and together leads to high zones of fungal inhibition. Hence, it was further concluded that as the vesicle size and viscosity of the formulation decreased, the zone of inhibition produced was also found to be increased.

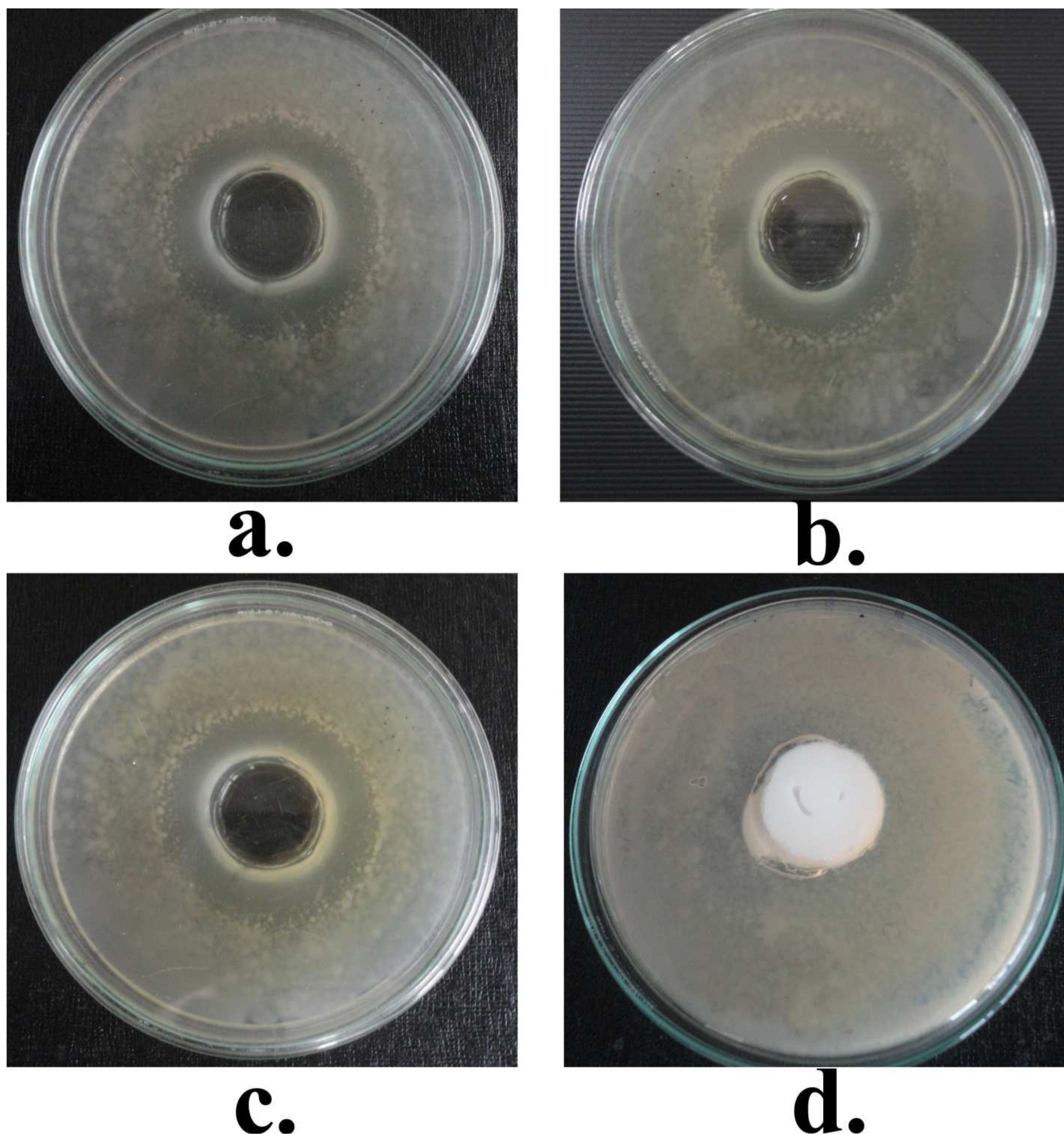

Figure 2: Images for zone of inhibition

Stability studies

Results of different parameters for liposomes (Entrapment efficiency) and liposomal gel (pH and viscosity) during stability period (Table 4, 5, 6). From results it can be concluded that at room temperature and freeze temperature

there was insignificantly variation in % entrapment efficiency. Similarly there was no significance of difference observed in pH and viscosity of liposomal gels.

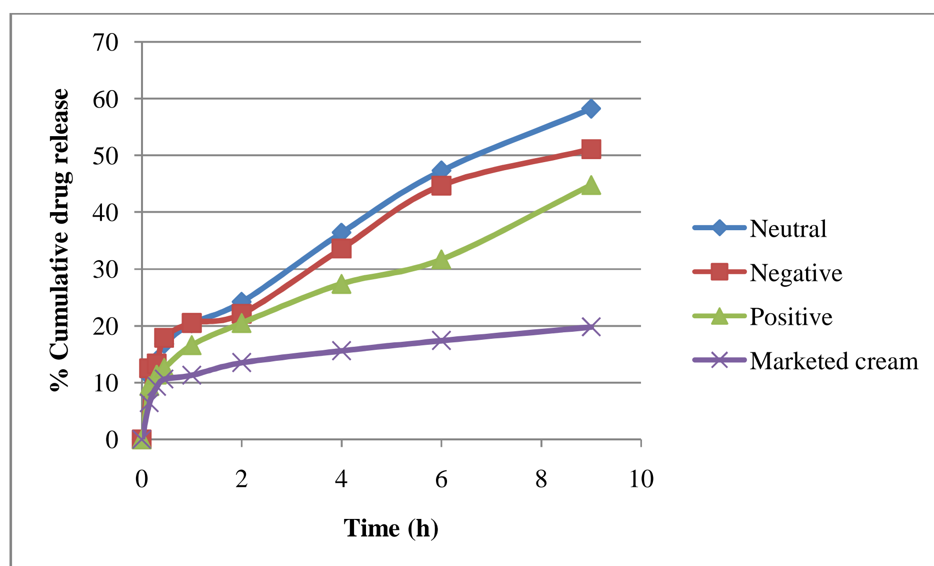

Figure 3: Comparative ex *vivo* diffusion profile at pH 7.4 study

Liposomal formulations

***Ex Vivo* Permeation Studies**

The permeation profile was obtained by placing neutral, positive and negative formulations and marketed cream (each containing 2 mg of Tolnaftate) on the pork dorsal ear skin in the Franz diffusion cell. The results for permeation studies i.e. % drug permeated (receptor chamber), % retained in the skin, % retained on the skin (donor chamber) were listed in the (Figure 3). It was observed that tolnaftate concentration steadily increased in the receptor medium with increase in time, where the permeation profile generally followed Fick's diffusion law. The % cumulative amount of tolnaftate permeated from neutral, negative, positive formulations and marketed cream at the end of 12th h after application was found to be 58.2±0.6, 51.19±0.81, 44.84±0.9 and 19.78±1.12. Smaller vesicle size of neutral, negative, positive provides larger area for permeation of drug in to skin and high drug concentration on the affected area results in a larger concentration gradient, which is a necessity for efficient dermal drug delivery. The permeation was observed maximum with formulated liposomal gels. The marketed cream which was used as a control had retained large amount of the drug on the skin (56.32±0.3).The retention of drug in the skin was observed maximum with neutral liposomal gel (29.33±0.5), followed by negative (25.36±0.2), positive (20.56±0.43) which is due to less viscosity and low vesicle size of the neutral liposomal gel followed by negative and positive liposomal gel (Table 7).

After permeation studies ANOVA by post tukey multiple comparison of all columns was used to calculate the significance ($P<0.05$) of permeation difference between each formulations. Based on the ANOVA data using post tukey multiple comparison of all columns at 12th h release of each formulation, it was conclude that the neutral, negative, positive, and marketed cream were extremely significant ($P<0.05$) in their permeation, possibly due to slower release and poor permeation of marked cream and lower vesicle size of neutral liposomal gel compare to negative and positive liposomal gel the killing of fungus is possible only if larger amount of drug retains into the skin layers and with higher permeability.

Invivo studies

First symptoms (small scaly redness) on the rats inoculated with *C. albicans* were observed on the 5th day of the experiment, while later (8th day), they were exhibiting in blood wounds, around 5 mm in diameter. The treatment was started on the 5th day of the experiment. The lesions and wounds started curing by the 8th day itself for neutral, positive, negative formulations. On the 13th day of the treatment with neutral, positive, negative formulations, the rats were completely cured, there were no visually observed symptoms, Animals treated with the commercial cream, were cured after 16 days of treatment (Figure 4).

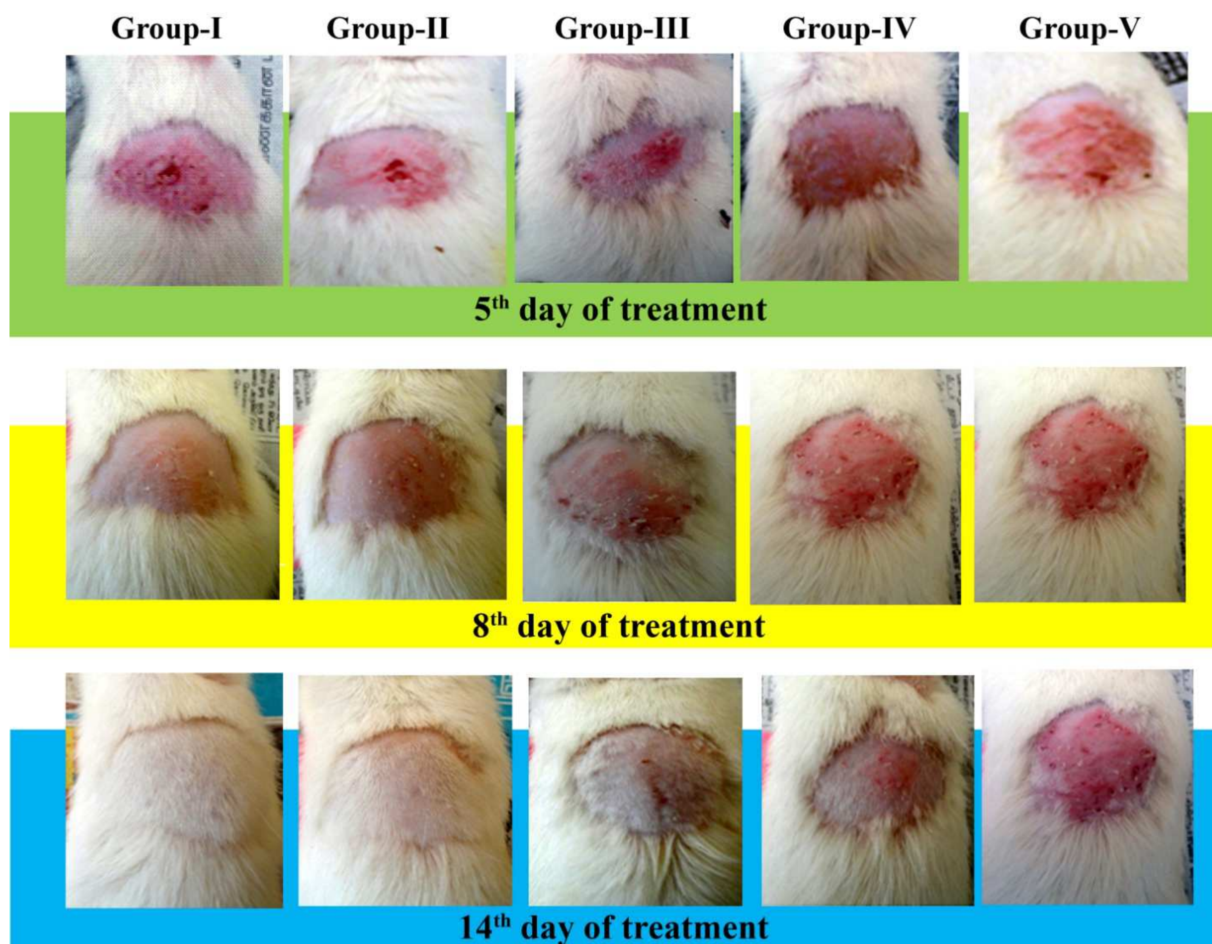

Figure 4: Images of animals during 5th, 8th and 14th day of treatment with neutral, positive, negative liposomal gel, Marketed cream and negative control

On the final day (Day 14) to make conformation of fungus was completely cured small quantity of skin was scrapped from infected area and cultured on SDA plates and then incubated. Up on 4 days of incubation the plates were scored for fungal colonies and statistical data produced using one way ANOVA followed by post-tukey multiple comparison test. Based on ANOVA data it was concluded that neutral, positive and negative formulations are extremely significant ($P < 0.05$) than marketed cream and negative control, since the number of colony forming units (CFU) were more for marketed cream which is near similar to that of negative control (Figure 5). This is due to the fact that neutral, positive and negative liposomes have less vesicle size and high lipophilicity which allow high concentrations of drug to penetrate to the skin and functionally create a drug depot in the stratum corneum and epidermis. Hence neutral, positive and negative formulations have a good therapeutic and antifungal effect and could represent possible alternative for the treatment of patient infected by dermartomycoses compared to marketed cream.

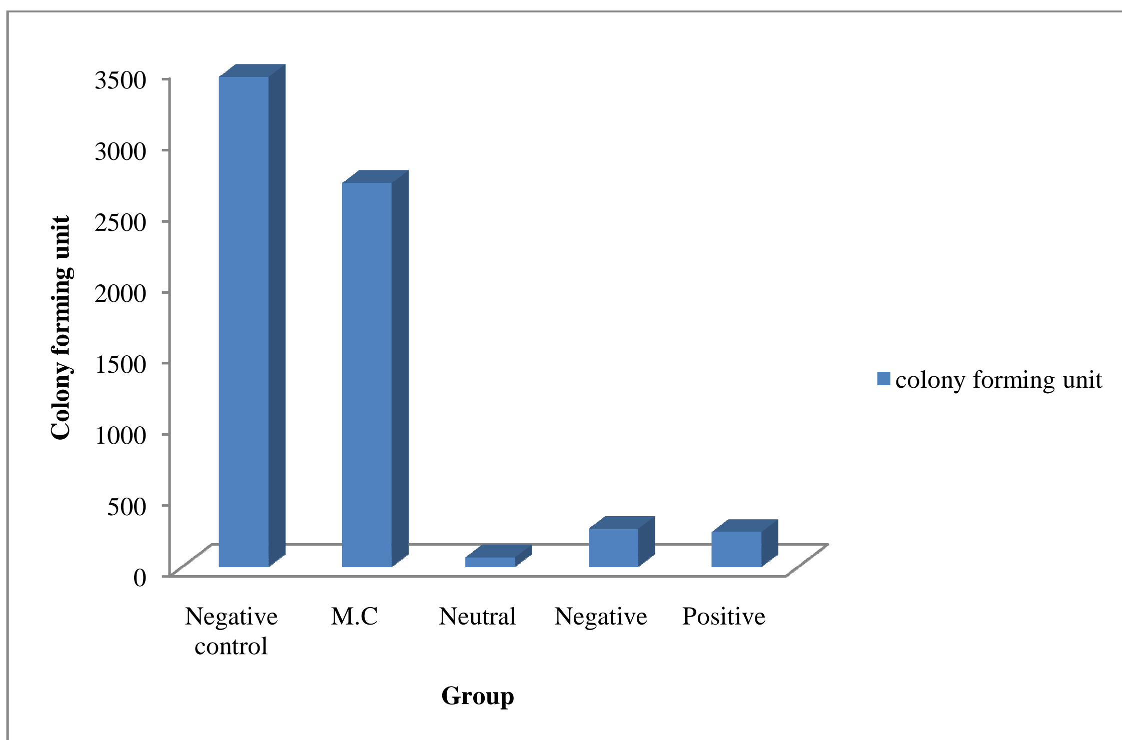

Figure 5: Comparative representation of number of CFU after post-antifungal animal study

CONCLUSION

The formulated liposomal gels shown a significant permeation and topical fungal cure rates compared to marketed cream. A higher permeation and cure rate is due to less size of particles. This study further confirms that liposomal gels provided greater permeation followed by cure rates of poorly soluble drugs which are intended for topical use to overcome the permeability and efficacy problems.

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