DEVELOPMENT & OPTIMIZATION OF ALLYL AMINE ANTIFUNGAL NANOEMULGEL USING 2^3 FACTORIAL DESIGN: FOR THE TREATMENT OF *TINEA PEDIS*

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Abstract

Drug permeation through skin from topical nanoemulsion (NE) depends on the concentration of the oil phase, aqueous phase, surfactant and co surfactant. Hence it is very important in optimizing the composition of NE. The conventional method of experimentation is costlier and is restricted to changing one factor at a time, the other factors being kept constant. Hence, there is a need of design of experiment (DoE), a statistical tool for finding optimum combination, using relationship between factors affecting a process and output of that process. The levels of the critical factors influencing the NE formation, were obtained from pseudo ternary phase diagram. From the design matrix of DoE, formulation matrix was generated which were analyzed for globule size as response. The model was found to be highly significant from the F ratio (71.49) and R^2 value of 0.9940. The optimized formulation was found to pass the stability and viscosity test (approx.120 cP). *Ex-vivo* skin permeation test of nanoemulgel (NG) formulation showed skin permeability of approximately 20% of the drug, while the marketed cream (MC) showed a permeability of 18% only. The amount of drug retained in the skin by the NG formulation was found to be approximately 31% while for the MC it was found to be only 20%. The NG formulation was found to treat the infected rat skin within 12 days of treatment while the MC took about 16 days for complete removal of the infection. Thus the NG formulation effectively permeates to the layers of the skin and treats the fungal infection more effiently than the MC.

Keywords: Nanoemulsion, design of experiment, *ex-vivo* skin permeation tests, *in-vivo* treatment of infected skin

Introduction

Topical preparations are those formulations meant for application to the surface of the skin for localized effect to treat various skin infections, as pain releaving medications etc.,.(1). Dermatophytes, in the evolutionary process have acquired the ability to metabolize and subsist upon keratin, a protein resistant to most other organisms (2). The fungi attack skin, nails, and hair, where keratin is the major structural protein, leading to a wide variety of disease states. Three types of dermatophytes account for the majority of infections: *Epidermophyton*, *Trichophyton*, and *Microsporum*. Dermatophytoses have varied presentations, are named by location, and have similar treatments. The major types of dermatophyte infections include: involvement of the scalp (tinea capitis), feet (tinea pedis), groin (tinea cruris), and other body surfaces (tinea corporis). These are typically superficial, involving the epidermis (2,3). The conventional topical agents like creams, ointments, lotions pose many disadvantages . They are sticky causing uneasiness when applied, have less spreading coefficient, exhibit stability problems also. Nano emulsions are heterogenous,

isotropic, thermodynamically stable, transparent systems, where the dispersions of oil and water are stabilized by use of surfactant molecules (4).

Drug permeation through skin from topical nanoemulsion formulations depends on their composition of the oil phase, aqueous phase, surfactant and co surfactant. Hence it is very important in optimizing the composition of these 4 factors in the formulation of nanoemulsion (5). The conventional method of experimentation is costlier and is restricted to changing one factor at a time, the other factors being kept constant. This fails to show interaction effect that may exist between some of the factors consequent on which optimum combinations are difficult to be determined. Hence, there is a need of design of experiment (DoE), a statistical tool for finding optimum combination, using relationship between factors affecting a process and output of that process (6).

Butenafine is a synthetic benzylamine derivative used for the topical treatment of *tinea pedis*. It works by inhibiting the synthesis of ergosterol by inhibiting squalene epoxidase, an enzyme responsible for the creation of sterols needed in fungal cell membranes (7). Lacking ergosterol, the cell membranes increase in permeability, allowing their contents to leak out. Though the marketed formulations effectively treat the fungal infection they require prolonged used of the drug. The present work is aimed at formulating nanoemulgel of butenafine hcl, with increased permeability and thus reduce the duration of treatment.

Materials

Butenafine Hcl was obtained as a gift sample from Vasista laboratories (Hyderabad, India). Capryol 90, Capmul MCM, Labrafac (LF), Isopropyl myristate (IPM), Lauroglycol (LG), Labrafac Lipophile (LL) and Soya oil (SO) were received as a gift sample from Gattefosse Pvt. Ltd. (Mumbai, India). Cremophore RH 40 was obtained as gift samples from Gattefosse (Mumbai, India). Ethanol was purchase from Rankem Pvt. Ltd. Carbopol 934P was purchased from Corel Pharma (Ahmedabad, India). T. rubrum (MTCC No. 296) and C. albicans (MTCC No.3018) were procured from Institute of Microbial Technology (Chandigarh, India).Double distilled water was used throughout the study. All other chemical reagents and solvents used were of analytical grade.

Method

Nanoemulsion was formulated by spontaneous emulsification technique using slow aqueous titration method and is dispersed into the carbapol gel (8).

Solubility studies (9)

Solubility of Terbinafine was determined in various oils such as Capryol 90, Capmul MCM, LF, IPM, LG, LL, and SO by shake flask method. An excess amount of drug was taken in 2 ml of the oil in vials, and mixed using vortex mixer. The vials were then kept at 25 ± 1^0C in an isothermal shaker for 72hrs to reach equilibrium. The equilibrated samples were then centrifuged at 3,500rpm for 15min. The supernatant was filtered through a 0.45µm membrane filter. The amount of drug dissolved in the oil was determined using UV spectrophotometer at their respective wavelength (table 1).

Construction of pseudoternary phase diagram (10)

Pseudo ternary phase diagrams were constructed to examine the formation of oil in water nanoemulsion with 4 components oil, surfactant, cosurfactant, and aqueous phase. The 4-component system consists of (i) Labrafac Lipophile (Selected from solubility studies) (ii) surfactant Cremophore RH40 (iii) a Cosurfactant (Ethanol) and (iv) distilled water (aqueous phase). Surfactant and cosurfactant in each group were mixed in different weight ratios (1:1 to 1:9 and 1:1 to 9:1**)**. These ratios were choosen in increasing concentration of surfactant

with respect to co surfactant and viceversa. For each phase diagram, oil and specific surfactant and co surfactant mixture (ScoS) was mixed in different weight ratios freom 1:9 to 9:1 in different glass vails. Seventeen combinations of oil and SCoS, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1 , 2:1 were made so that maximum ratios were covered for the study to depict the boundaries of phases precisely formed in the phase diagrams Slow titration with aqueous phase was done to each weight ratio of oil and SCoS and visual observation was carried out for transparency and flowability of the nanoemulsions. Only the nanoemulsion regions were plotted in the pseudoternary phase diagram. The pseudo-ternary phase diagrams were built using CHEMIX School software (fig 1 to 6).

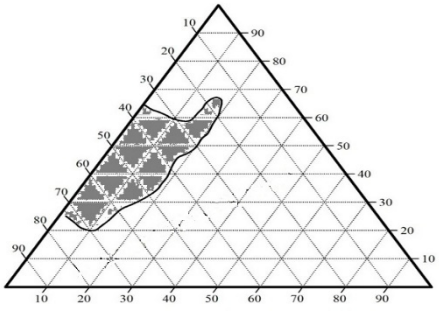


Fig 1: ScoS 2:1

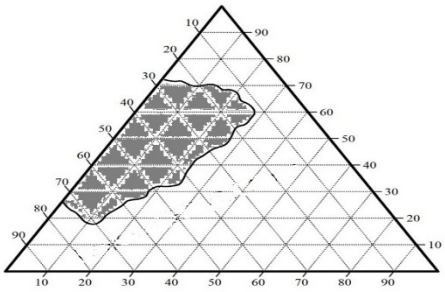


Fig 2: ScoS 2:3

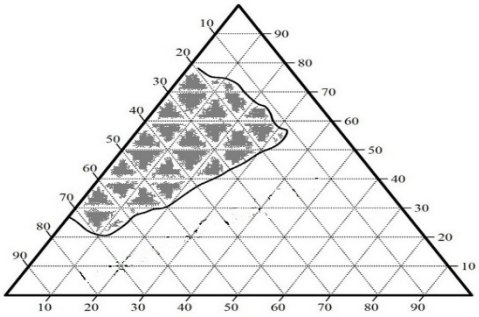


Fig 3: ScoS 3:2

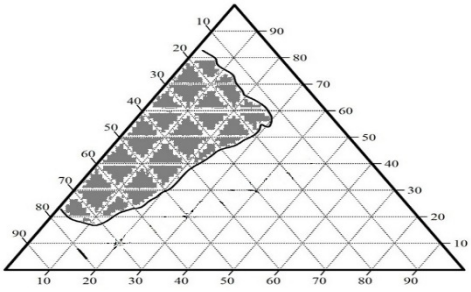


Fig ScoS 4:1

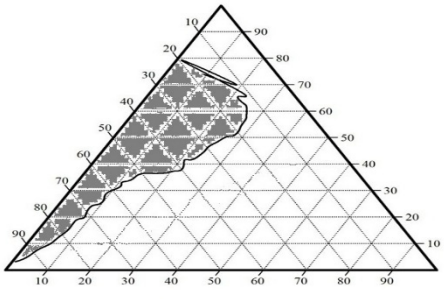


Fig 5: ScoS 5:2

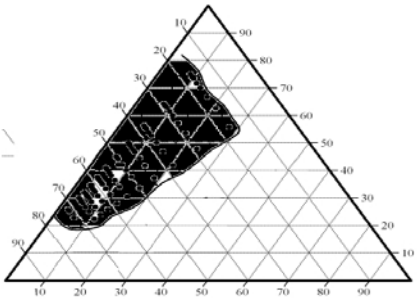


Fig 6 : ScoS 6:3

4.3 2^3 Factorial design (11)

Full factorial design allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable. The minimum and maximum levels of the independent variables were obtained from the pseudo ternary phase diagrams and are incorporated in the DoE design matrix (table 2). The optimized formulation of the nanoemulsion was achieved by analysis of the results of the design matrix using UNSCRAMBLER 10.3X software, with three critical factors which influence globule size of the nanoemulsion (table 3).

The batches were formulated and evaluated for globule size of the emulsion and the design matrix was analysed (table 4). Three batches of the optimized formulation (A, B, C) as given by the results of DoE were prepared and stability studies were performed.

Thermodynamic Stability studies (9)

The batches A, B and C were formulated with the optimized composition of SCoS 2:1 and Oil:SCoS 1:1 and were subjected to different thermodynamic stability studies such as centrifugation, heating cooling cycle and freeze thaw cycle, to avoid the selection of metastable formulations (table 5).

Incorporation of drug

Stable and optimized nanoemulsion formulations from stability studies were selected and formulated by incorporating the drug in the oil phase such that it accounts to 1% of the total nanoemulgel.

Preparation of gel

4% Carbapol gel was prepared using 10% Triethanolamine and the pH was adjusted to 7.2.

Incorporation of nanoemulsion into the gel

The nanoemulsion was incorporated into the gel by simple dispersion.

Evaluation of nanoemulgel

Thus the formulated nanoemulgel is evaluated for viscosity **(12)** (table 6) using Brookfield DV III ultra V6.0 RV cone and plate rheometer using spindle # CPE40 at 25 ± 0.5^0C, homogeneity by visual characterization, *ex-vivo* drug permeation through skin using Franz diffusion cell, and *in vivo* treatment of infected rat skin.

***Ex-vivo* drug permeation through rat skin using Franz diffusion cell (13):**

Ex-vivo skin permeation studies for formulation A, B and C, marketed formulation and a blank were performed in a Franz diffusion cell with a diffusion area of 2.26cm^2 and receptor volume of 22.5mL using abdominal rat skin. Abdominal rat skin was excised and washed with isotonic NaCl. The excised skin was then mounted between the donor and the receptor chambers of the Franz diffusion cell with the dermal side in contact with the receptor medium and the stratum corneum side facing upwards into the donor compartment. Then, optimized NE formulation equivalent to 250 mg of Butenafine Hcl was evenly applied on the surface of the rat skin in the donor compartment. The receptor compartment was filled with physiological saline solution (pH 5.5 acetate buffer/methanol (9:1)). The temperature in the receptor compartment was maintained at 37±0.5 °C to simulate the skin temperature and was magnetically stirred at 50 rpm. Samples were withdrawn at predetermined time intervals of 0.15, 0.30, 0.45, 1, 2, 4, 6, and 9hrs, filtered through 0.45µm cellulose membrane filter and analyzed for butenafine content by UV spectroscopy (fig 7). Fresh buffer solution was immediately replaced into the receptor chamber after each sampling.

After 9hrs the % drug retained on the skin at the end of 9th hr was determined. The rat skin is minced, equilibrated with physiological buffer solution to extract the drug from the skin layers. The equilibrated solution is filtered through 0.22µm filter and was analyzed for drug content spectrophotometrically.

Infecting the rat skin (14)

12 male wistar rats, each weighing 180 to 200gms were selected with 3 in each group. One group for formulation A, the other for formulatio B , the third for marketed formulation

and the fourth group is maintained as control. On the back of each animal, the areas of 4 cm were cleaned and depilated. The infectious inoculum was prepared from a 7 day old culture of *Trychophyton mentagrophytes*. The inoculum was applied on the animal back immediately after the depilation and left for 7 days. The establishment of active infection was confirmed by visual examination of the erythematous lesions.

***In vivo* treatment of infected rat skin**

To the infected area the formulated gel was applied and the animals were kept undisturbed for 24hrs. They were examined for the disappearance of the lesions and this procedure was repeated until complete disappearance of the lesions.

Results and discussion

Solubility studies

Solubility of the drug in the oil phase plays a crucial role in the formulation of nanoemulsion. So the oil in which the drug shows maximum solubility is selected for further studies. From table 1 it shows that Labrafac lipophile shows maximum drug solubility of 127mg/mL. Hence LL is selected as the oil phase for nanoemulsion formulation.

Table 1: Solubility studies

OIL	SOLUBILITY(mg/mL)
CAPRYOL 90	48
CAPMUL MCM	27
LF	62
IPM	21
LG	34
LL	127
SO	40

Pseudo ternary phase diagram study

Phase behavioral studies were performed by constructing phase diagrams that depict the boundaries of different phases, as a function of composition and temperature, to investigate the structural organization of the emulsions formed. The concentration of surfactant and co-surfactant is responsible for the barrier formation at the interface required to prevent the coalescence of the formed nanoemulsion. SCoS gets adsorbed at the interface, reducing the energy required for nanoemulsion formation thus improving the thermodynamic stability of the nanoemulsion formulation. From the pseudoternary phase diagram study, it is evident that the oil concentration of 1 to 5mL and surafcatant concentrationof 2 to 6 mL and cosurfactant concentration of 1 to 3 promotes the nanoemulsion region with required consistancy. Hence these limits were inserted in the design matrix and analysed for optimized formulation.

Table **2**: Variables for optimization and their limits

INDEPENDENT VARIABLES			LEVELS	
			LOW	HIGH
SL.NO	VARIABLE	UNIT	-1	+1
1	OIL	mL	1	5
2	CREMOPHORE RH 40	mL	2	6
3	ETHANOL	mL	1	3

Table 3: Variable optimization using DoE: Matrix generated by UNSCRAMBLER 10.3 X software and the response values

STD	RUN	BATCH	FACTOR 1, OIL (mL)	FACTOR 2, CREMOPHORE RH 40 (mL)	FACTOR 3, ETHANOL (mL)	GLOBULE SIZE (nm)
1	9	F1	1	2	1	40
2	2	F2	5	2	1	25
3	3	F3	1	6	1	18
4	4	F4	5	6	1	12
5	10	F5	1	2	3	56
6	1	F6	5	2	3	60
7	6	F7	1	6	3	20
8	11	F8	5	6	3	60
9	5	F9	3	4	2	15
10	7	F10	3	4	2	32
11	8	F11	3	4	2	28

Table 4: ANOVA results of DoE

ANOVA	SS	DF	MS	F-RATIO	p-VALUE
SUMMARY					
MODEL	1113.5	7.0	159.0714	71.4198	0.0025
ERROR	6.6818	3.0	2.2273		
CORRECTED TOTAL	1120.1820	10.0			
VARIABLES					
OIL(A)	578.0	1.0	578.0	259.5102	0.0005
CREMOPHORE RH 40 (B)	480.0	1.0	480.5	215.7347	0.0007
ETHANOL (C)	50.0	1.0	50.0	22.4490	0.0178
OIL*CREMOPHORE RH 40	2.0	1.0	2.0	0.8980	0.4132
OIL* ETHANOL	0.5	1.0	0.5	0.2245	0.6680
CREMOPHORE RH 40 * ETHANOL	2.0	1.0	2.0	0.8980	0.4132
OIL*CREMOPHORE RH 40 * ETHANOL	0.5	1.0	0.5	0.2245	0.6680
MODEL CHECK					
LINEAR	1108.5	3.0	369.5	165.8980	0.0008
INTERACTION 2	4.5	3.0	1.5	0.6735	0.6234
INTERACTION 3	0.5	1.0	0.5	0.2245	0.6680
LACK OF FIT					
LACK OF FIT	6.6818	1.0	6.6818		
PURE ERROR	0	2.0	0		
ERROR	6.6818	3.0	2.2273		
QUALITY					
METHOD USED	FULL FACTORIAL DESIGN				
R^2	0.9940				
ADJUSTED R^2	0.9801				
PREDICTED R^2	-0.4050				
S	1.4924				
MEAN	22.7273				
C.V IN %	6.5666				
PREDICTED SS	573.88				

SS= SUM OF SQUARES; DF= DEGREE OF FREEDOM; MS= MEAN SQUARED;

The actual and predicted plots fall close to the line. ANOVA (partial sum squares-type-III) reveals the model with F ratio of 71.4198, and R^2 value of 0.9940 which implies the model to be significant. The optimized batch as given by the analysis of the results of DoE is ScoS ratio of 2:1 and Oil: ScoS ratio of 1:1.

Thermodynamic stability studies

Table no 5: Thermodyanamic Stability studies

FORMULATION	THERMODYNAMIC STABILITY STUDIES		
	CENTRIFUGATION	HEATING & COOLING CYCLE	FREEZE THAW CYCLE
A	Pass	Pass	Pass
B	Pass	Pass	Pass
C	Pass	Pass	Pass

Thermodynamic stability studies differentiate those nanoemulsion formulations from those of kinetically stable formulations which undergo phase separation. The formulations which pass the thermodynamic stability studies are those formulations which contain adequate amounts of ScoS concentration required for nanoemulsion formulation, and which decreases the energy required for nanoemulsion formation. This decreased energy contributes to the stability of nanoemulsion. From table 5 it is evident that the formulation A, B, C passed the stability studies. Hence formulations A, B, C were kinetically stable with adequate amounts of SCoS concentration.

Evaluation of nanoemulgel

Viscosity and homogeneity studies

Table 6: Viscosity and homogeneity studies

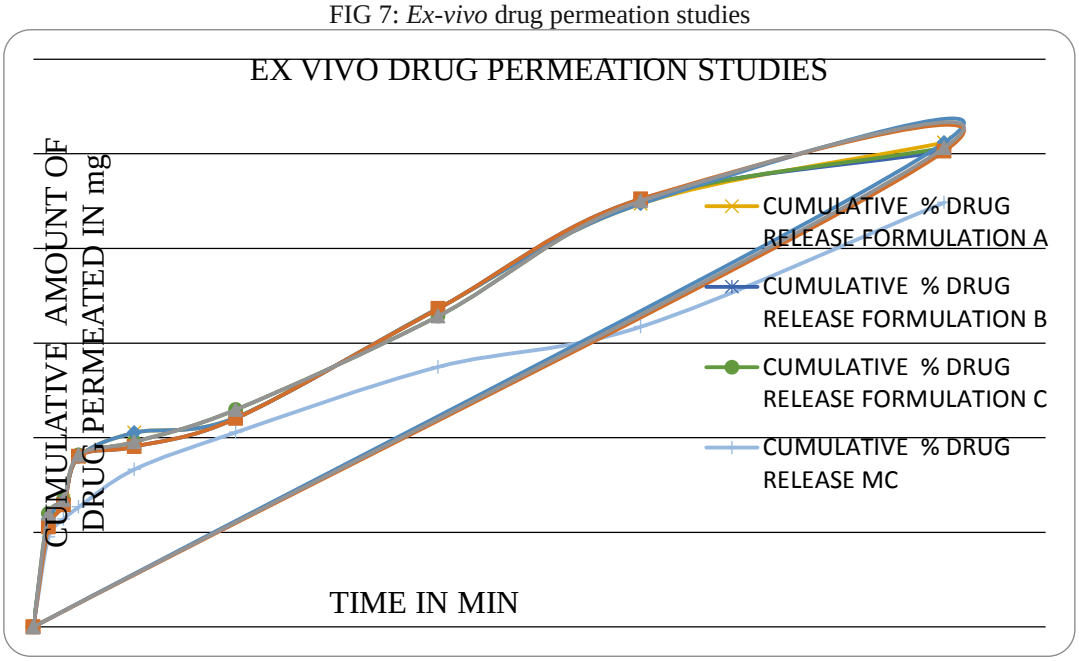
FORMULATION	MEAN VISCOSITY (CP)	HOMOGENEITY
A	119.86	Clear & translucent
B	119.28	Clear & translucent
C	120.05	Clear & translucent

The viscosities of the formulations A, B, and C were not higher, which indicates that the rate of drug permeation from the NG formulation is not hindered by the gel structure of the gelling agent and they also have good spreadability on the surface of the skin.. Moreover the concentration of SCoS and the oil did not oppose for the flow and hence the viscosity. This adds and additional advantage to the NE formulation. The formulations were found to be clear and translucent.

***Ex-vivo* drug permeation through rat skin using Franz diffusion cell**

From the drug permeation studies it was observed that butenafine concentration steadily increased in the receptor chamber with increase in time, where the permeation profile generally followed Fick's law of diffusion. The amount of butenafine that permeated through the skin by the end of 9hrs after application was found to be 51.19 mg, 50.28 mg, 50.64 mg and 44.84 mg from formulation A, B, C and M.C respectively. This may be attributed to the smaller globule size of the NE formulation which 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 necessary for efficient dermal drug delivery. The increase in drug concentration in the receptor compartment may also be due to the decreased interfacial tension by SCoS, which is responsible for the decreased globule size. The presence of triglyceride in the formulation also might have accounted for the permeation. Since the basic nature of stratum corneum is lipid and the lipophilic nature of the NE droplets are responsible for loosening of the structure of stratum corneum instead of rupturing it, which is the case with other permeation enhancers.

FIG 7: *Ex-vivo* drug permeation studies



The amount of drug that retained in the skin was found to be 30.33, 31.52, 30.86 and 19.78 for formulation A, B, C, and marketed cream respectively. This indicated that the nanoemulgel formulation is more permeable through skin and is retained in the skin more effectively due to less globule size and viscosity when compared to the marketed cream formulation.

***In vivo* treatment of infected rat skin:**

Symptoms of erythematous lesions around 20mm diameter were observed on the 5th day of infection. Treatment of these bloody lesions was initiated on the 8th day of infection which continued for 14 days. The lesions and the wounds started to cure from the 3rd day of treatment. By the end of 11th day of treatment there were no visually observed symptoms. Animals treated with marketed cream, were cured after 16 days of treatment. This may be attributed to the fact that the nanoemulgel formulations has less globule size, decreased interfacial tension and high lipophilicity which allows high concentrations of drug to penetrate the skin and is effectively retained in the layers of the skin. Due to sufficient quantity of drug in the aqueous bed of the skin which the MC formulation could not reach, while the NG could because of its decreased globule size and lipophilic nature, the drug effectively inhibits the synthesis of ergosterol by inhibiting squalene epoxidase, an enzyme responsible for the creation of sterols needed in fungal cell membranes. Lacking ergosterol, the cell membranes increase in permeability, allowing their contents to leak out.

Conclusion

NG formulation of Butenafine Hcl was formulated by the use of DoE with optimized concentrations of oil phase, and SCoS. Pseudo ternary phase diagrams were constructed from which the levels of oil, surfactant and co surfactant at which NE is formed was obtained. The optimized concentration of NE formulation was derived by the use of DoE, and analyzing the results of DoE. The actual and predicted plots fall close to one another. ANOVA (partial sum squares-type-III) reveals the model to be significant with F-ratio 71.4198 and R^2 value 0.9940. The optimized formulations were thermodynamically stable, with good spreadability and were homogeneous. From the *ex-vivo* skin permeation studies it can be concluded that the NG formulation was more permeable through skin and was retained in the skin more effectively than the MC formulation due to less decreased interfacial tension, hence

decreased globule size and viscosity when compared to the MC formulation. From *In-vivo* treatment of infected skin studies it can be concluded that the NG formulation effectively treats the fungal infection when compared to the MC formulation.

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