Design and Development of Ciclopirox Topical Nanoemulsion Gel for the Treatment of Subungual Onychomycosis

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ABSTRACT

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Onychomycosis is the most common infection of nails caused majorly by Trichophyton rubrum and Trichophyton mentagrophytes and minorly by yeasts. Topical delivery of available ciclopirox formulations as nail lacquer, cream, lotion, gel is hindered by the low permeability of human nail plates, and so there is need of repeated dosing for a longer period of time for effective treatment. In the present research work an attempt is made for effective delivery of ciclopirox olamine (CIC) across human nail plates by enahancing the penetration of CIC and retention time in skin layers. For this purpose nanoemulsion gel, composed of oil, surfactant, cosurfactant, and cabopol was developed by aqueous phase titration method and was evaluated for various in-vitro attributes. Oleic acid, tween 80, and PEG 400 were selected as oil, surfactant and co-surfactant respectively. Pseudoternary phase diagrams were plotted to get the range of nanoemulsion area. For the optimization of the formulation Box Benkhem model (RSM) was applied by taking size and zeta potential as dependant variables and formulation components were taken as independent variables. Total 17 formulations were suggested by the model, which were formulated and subjected to thermodynamic stability study and permeation study. Among thermodynamically stable formulation, SF4 had shown lowest permeation across skin (56.672 µg/cm^2/h). SF4 formulation was further evaluated for skin retention study by fluorescent microscopy. Fluorescence microscopy studies were done over a period of 6 hrs clearly giving an indication of longer retention capability of the nano-gel formulation, for the desired topical action.

Keywords: Onychomycosis, Nanoemulsion, permeation, fluorescent microscopy. thermodynamic stability

INTRODUCTION

Onychomycosis is a chronic persistent fungal infection of the nail bed caused by *T. rubrum* and *T. mentagrophytes* [1,2,3]. Fungi invade the distal and lateral under surfaces of the nail, resulting in thickening and discoloration of the nail, which sometimes can be accompanied by serious pain and disability. The disease seriously affects quality of life and the condition becomes much more complicated in patients suffering from diabetes [4]. The compromised immunological condition and slow wound healing in diabetes facilitates disease progression and aggravation[5].

The treatment modalities of onychomycosis include topical, mechanical, chemical and systemic treatments or a combination therapy [6]. Most treatments require long term use from as much as 3-9 months to be most effective .The long term use of drug increases the chance of long term side effects. Therefore, it is better to concentrate on combination of topical and systemic drug to reduce the duration of therapy. An ideal topical therapy in such conditions should have low systemic absorption and longer retention in skin layers. However, most of the topical formulations lack this essential characteristic and can leads to systemic side effects as well as sub-optimal levels of drug at the site of action.

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Ciclopirox olamine belongs to the antimycotic drugs used for the treatment of superficial mycoses. Though the mechanism of action of ciclopirox is not preciously known, involvement of loss of function of certain catalase and peroxidase enzymes has been suspected[7]. It acts by inhibiting the membrane transfer system by interfering with the Na+ K+ ATPase.

Nanoemulsion drug delivery system consists of an efficient solvent-free topical vehicle, based on drug entrapment in stable, submicron particles of oil-in-water emulsions with a mean droplet size between 100 and 200 nm that are uniformly dispersed in an aqueous phase. The size of nanoemulsion should be optimized in such a way that it penetrates through the skin and is retained there for longer duration and should not go to the systemic circulation. One of the unique characteristics of the nanoemulsion technology is the relatively high percentage of total particle volume occupied by the internal hydrophobic oil core of the droplets. This provides high solubilization capacity for lipophilic compounds compared to other lipoidal vehicles such as liposome. Viscosity-imparting agents are used for nanoemulsion thickening to result in creams/gels with the desired semisolid consistency for application to the skin. Another unique characteristic of nanoemulsion technology is that it does not employ the chemical penetration enhancers commonly used in other topical drug delivery vehicles, which may cause skin irritation and sensitization. Hence a nano-emulsion gel acts as a reservoir, which maintains a constant concentration gradient over the skin for a long period[8].

MATERIALS AND METHODS

2.1. Materials:

Ciclopirox base was obtained a gift sample from Encube Ethicals Pvt. Ltd. (Goa, India). Medium chain triglyceride (Labrafac), Caprylo caproyl macrogol-8-glyceride (Labrasol), were obtained as gift samples from Gattefosse´ (Saint Priest, Cedex France), Propylene glycol mono caprylic ester (Safsol 218) was obtained as gift sample from Nikko Chemicals (Tokyo, Japan). Isopropyl myristate (IPM), Glycerol triacetate (Triacetin), Castor oil, Tween 20, Diethylene glycol, from Merck (Schuchardh, Hokenbrunn, Germany). All other chemicals were of analytical grade.

2.2. Screening of components: The solubility of Ciclopirox olamine was determined in different oils viz. oleic acid, isopropyl myristate (IPM), olive oil, triacetin, clove oil, castor oil, safsol, labrafac, sesame oil and soyabean oil by adding excess of drug to 2 ml of the different components. The vials were tightly stoppered and were continuously stirred at 37 ± 0.5ºC using isothermal shaker (Nirmal International, New Delhi, India) for 72 hours to achieve equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 10,000 rpm for 15 minutes. The supernatant was taken and filtered through a 0.45-µm membrane filter. The concentration of Ciclopirox was determined in each oil, surfactant, cosurfactant, and combination of oils by HPLC.

2.3. Pseudo-ternary phase diagram study: On the basis of the solubility studies of drug, oleic acid was selected as the oil phase. Tween-80 and PEG 400 were selected as surfactant and cosurfactant respectively as per their emulsification capability for the system. Distilled water was used as an aqueous phase for the construction of phase diagrams. For the determination of existence zone of nanoemulsion, pseudo-ternary phase diagrams were constructed using aqueous titration method. To construct pseudo-ternary phase diagrams, the oil phase was mixed with surfactant: co-surfactant phase (Tween 80 and PEG 400 respectively) and the ratios of Smix (surfactant and co-surfactant mixture) used for titration are 1:0, 1:1, 1:2, 1:3, 1:4, 2:1, 3:1 and 4:1. The mixture was titrated with distilled water until it turned turbid. The volume of water used was then recorded. Water titration was continued until a clear, isotropic and thermodynamically stable dispersion with low viscosity was obtained. The pseudoternary phase diagrams were constructed by plotting water phase, oil phase and surfactant: co-surfactant phase used in the experiment.

2.4. Optimization of formulation: A Box-Behnken statistical design with 3 factors and 17 runs was selected for the optimization study[9]. The experimental design consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube. The range of Nanoemulsion area was taken by the aqueous titration method. On the basis of aqueous titration method the range of oil, Smix and water was obtained in the given range. Seventeen experiments were required for the response surface methodology based on the Box- Behnken design. Particle size and zeta potential were taken as dependent variables. The range of independent variable are given in Table 1.

2.5. Thermodynamic stability studies: Thermodynamic stability of the nano-emulsion systems were determined by performing following tests[11]:

1. Freeze thaw cycle: The formulations were exposed to six cycles between refrigerator temperature of 4 °C and 45 °C with storage at each temperature of not less than 48 h. Those formulations, which were stable at these temperatures, were subjected to centrifugation test.

2. Centrifugation: Formulations which remained stable after freeze thaw cycling were centrifuged at 3500 rpm for 30 min. Such of those formulations which did not show any phase separation were taken for the freeze-thaw stress test.

3. Freeze thaw stress cycle: Three freeze thaw cycles between - 21 °C and +25 °C with storage at each temperature for not less than 48 h was done for the formulations. Those formulations, which passed these thermodynamic stress tests, were further taken for the dispersibility test for assessing the efficiency of self-emulsification.

2.6. Surface morphology: Morphology of the nanoemulsion was studied using TEM (Morgagni 268D SEI, USA) operating at 200 KV and of a 0.18 nm capable of point-to-point resolution. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of the nanoemulsion. In order to perform the TEM observations, the diluted nanoemulsion was deposited on the holey film grid and observed after drying.

2.7. Viscosity: The viscosity of the formulations was determined using Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) using spindle # CPE40 at 25 ± 0.5 °C. The software used for the calculations was Rheocalc V2.6.

2.8. Ex-Vivo skin permeation and skin deposition study: Ex-vivo permeation studies through properly treated rat's skin were performed using an automated diffusion cell sampling system (SFDC, LOGAN Inst, Nj USA). Epidermal membrane samples were mounted into the diffusion cells (area 0.653cm^2) between donor and receptor compartment, equilibrated at 37±0.5ºC for 8-10hrs. The receptor cell was filled with the media and 6 mg of ciclopirox olamine in

nanoemulsion was applied on the skin surface in the donor compartment. To study the permeation from neat components (neat surfactant, oil), same amount of drug was dissolved in surfactant mixture, and oil. The receptor media was maintained at 35 ± 0.5º C and magnetically stirred at 600 rpm. After application of the test formulation on the donor side, 0.5 ml of aliquot was collected from the receiver cell at designated time intervals (viz. 0, 1, 2, 3,4, 5 , 6, 8, 10, 12 and 24 hr) for 24 hr period and replaced immediately with the same volume of fresh media maintained at 35 ± 0.5º C. After appropriate dilutions, the amount of drug in the receptor media was analyzed using UV spectrophotometer at 304 nm.

2.9. FORMULATION OF NANOEMULSION GEL AND ITS EVALUATION:

2.9.1. Formulation of optimized nanoemulsion into gel: On the basis of the permeation studies, skin deposition studies and *in vitro* characterization studies, the optimized nanoemulsion Sf4 was selected to be formulated into gel by the use of chitosan and carbopol 934. Carbopol 934 possesses an ability to form gels at acidic pH values, because it is hydrophilic and can retain water in its structure. Gels containing 1%, 1.5% and 2% of chitosan and carbopol were prepared and 1.5% of chitosan and 1.2 % of carbopol gel was found to be suitable for gelling the nanoemulsion because of its desirable transparency, consistency, feel and ease of spreadability. The carbopol gel was found to be more transparent than chitosan. Further evaluation was done with the carbopol 934. The nanoemulsion gel of carbopol 934 was prepared by using 1% ethanolamine. Chitosan was mixed with 1% aqueous solution of acetic acid manually in a mortar. After that it was hydrated for 1 hr at room temperature to obtain a gel of desirable viscosity and compatibility with the nanoemulsion[11]. The carbopol was hydrated for 10 minutes and then 1% triethanolamine is added. After 30 minutes, it gives transparent viscous gel. The optimized nanoemulsion was than mixed with the gel in 1:1 ratio with gentle stirring. The nanoemulsion gel (NEG) was further evaluated for its skin permeation by adopting the same protocol that was used for the *In vitro* skin permeation study of nanoemulsion formulations.

2.9.2. Spreadability[10] 0.5 g test formulation was placed with in a circle of 1 cm diameter pre marked on a glass plate over which a second glass plate was placed. A weight of 5 g was allowed to rest on the upper glass plate for 5 min. the increase in the diameter due to spraedability of the formulation was noted.

2.9.3. Drug content: 1 g of the gel was weighed in to a 100 ml beaker and dissolved in methanol; it was diluted appropriately and analysed at 304.5 nm by UV spectrometry.

2.9.4. The Skin retention study by fluorescence microscopy: Fluorescence microscopic evaluations were carried out to determine the skin retention and permeation of the nano emulsion gel (NEG), and marketed formulation. Flourescine was used to tag the formulations. For NEG the first skin sample was fixed for visualisation after 1 hr., the second skin sample was fixed after 2hr., whereas, the third sample was fixed after 6 hr. The blank sample consisting of oleic acid solution with flourescine was similarly applied on a separate abdominal skin sample of rat skin. Marketed formulation was triturated with fluorescent dye and applied on the skin. All the skin sample slides were prepared and fixed in 10% formic acid to washout the applied extra fluorescent dye from the skin surface before its microscopic evaluation. Two fluorescence filters namely rhodamine B and flourescine were used to make observations. The former showed orange colour while the later depicted green colour.

RESULT AND DISCUSSIONS

3.1. Component selection: After performing solubility study in different oils (Fig. 1), it was found that Ciclopirox olamine exhibited maximum solubility in the Clove oil (190 mg/ml) but during the solubility study it was observed that in oil, colour of drug changed because of physical incompatibility. It was inferred from these studies that ciclopirox olamine was incompatible with clove oil and it was dropped out and therefore oleic acid was chosen as the oil phase. The drug solubility is good in oleic acid (160 mg/ml).The other advantage with the use of oleic acid is that it has been reported to be a powerful enhancer for transdermal delivery as it increases the fluidity of the intercellular lipid barriers in the stratum corneum by forming separate domains which interfere with the continuity of the multilamellar stratum corneum and induce highly permeable pathways in the stratum corneum[12].

3.2. Pseudo-ternary phase diagram study: This work has been carried out with several surfactant mixtures. However oleic acid solublization in oil in water nano-emulsion for binary mixture of tween-80 and PEG 400 was found to be maximum. The surfactant mixture that provided higher oil solubilization was Tween 80: PEG 400 - 5:1 (Fig. 2).

3.3. Optimization by Box Benkhem Model: Box-Benkhem model was applied to evaluate the effect of concentrations of

Table 1: Variables and levels of optimization design

Independent Variables	Dependent variables	Level	Low Level	High Level
Oil	Size	9.50	7.00	12.00
Smix		52.50	45.00	60.00
Water		22.50	15.00	30.00

Table 2: The Optimization of Formulation by Box Benkhem Method

S. no.	Formulation Code	Run	Factor 1 % Oil	Factor 2 % Smix	Factor 1 % Water	Response 1 Particle size	Response 2 Zeta potential
1	Sf 1	6	7.00	45.00(5:1)	22.50	113.1	-32.5
2	Sf 2	16	12.00	45.00(4.5:1	)22.50	123.5	-25.5
3	Sf 3	12	7.00	60.00(4:1)	22.50	176.4	-40.7
4	Sf 4	13	12.00	60.00(4:1)	22.50	186.3	-40.4
5	Sf 5	4	7.00	52.50(4:1)	15.00	444.4	-39.0
6	Sf 6	7	12.00	52.50(4.5:1)	15.00	411.3	-32.2
7	Sf 7	3	7.00	52.50(5:1)	30.00	310.4	-36.8
8	Sf 8	21	2.00	52.50(4.5:1)	30.00	457.6	-32.9
9	Sf 9	10	9.50	45.00(5:1)	15.00	256.9	-36.4
10	Sf 10	1	9.50	60.00(4.5:1)	15.00	66.2	-33.4
11	Sf 11	15	9.50	45.00(4:1)	30.00	36.5	-39.6
12	Sf 12	8	9.50	60.00(4:1)	30.00	314.7	-37.3
13	Sf 13	14	9.50	52.50(5:1)	22.50	186.3	-45.2
14	Sf 14	9	9.50	52.50(5:1)	22.50	176.4	-46.8
15	Sf 15	17	9.50	52.50(5:1)	22.50	154.5	-72.4
16	Sf 16	5	9.50	52.50(4:1)	22.50	82.5	-54.5
17	Sf 17	11	9.50	52.50(4:1)	22.50	76.8	-71.4

Fig. 1: Solubility of drug in different nano-emulsion components

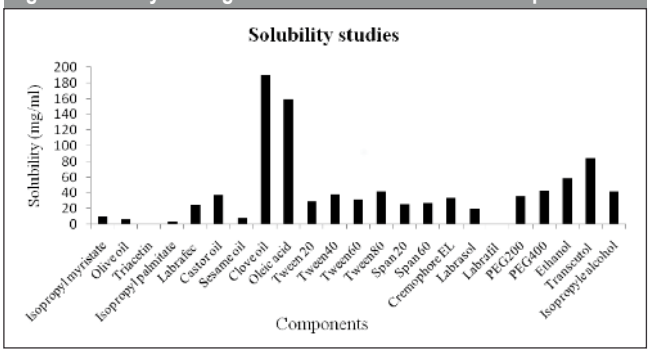
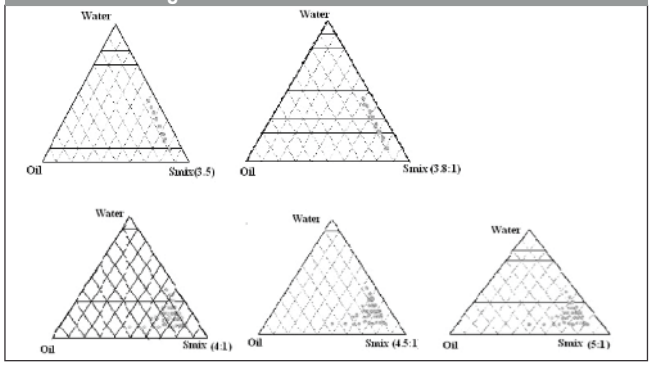


Fig. 2: Pseudo-ternary phase diagrams indicating o/w nanoemulsion region at different Smix ratios.



oil, water and Smix on size and zeta potential of the nano-emulsion. An extensive data was generated for all seventeen formulations suggested by the model (Table 2).

The polynomial equation generated by this experimental design (using Statistica Release 6, Statsoft Inc) was found to be useful for further calculations –

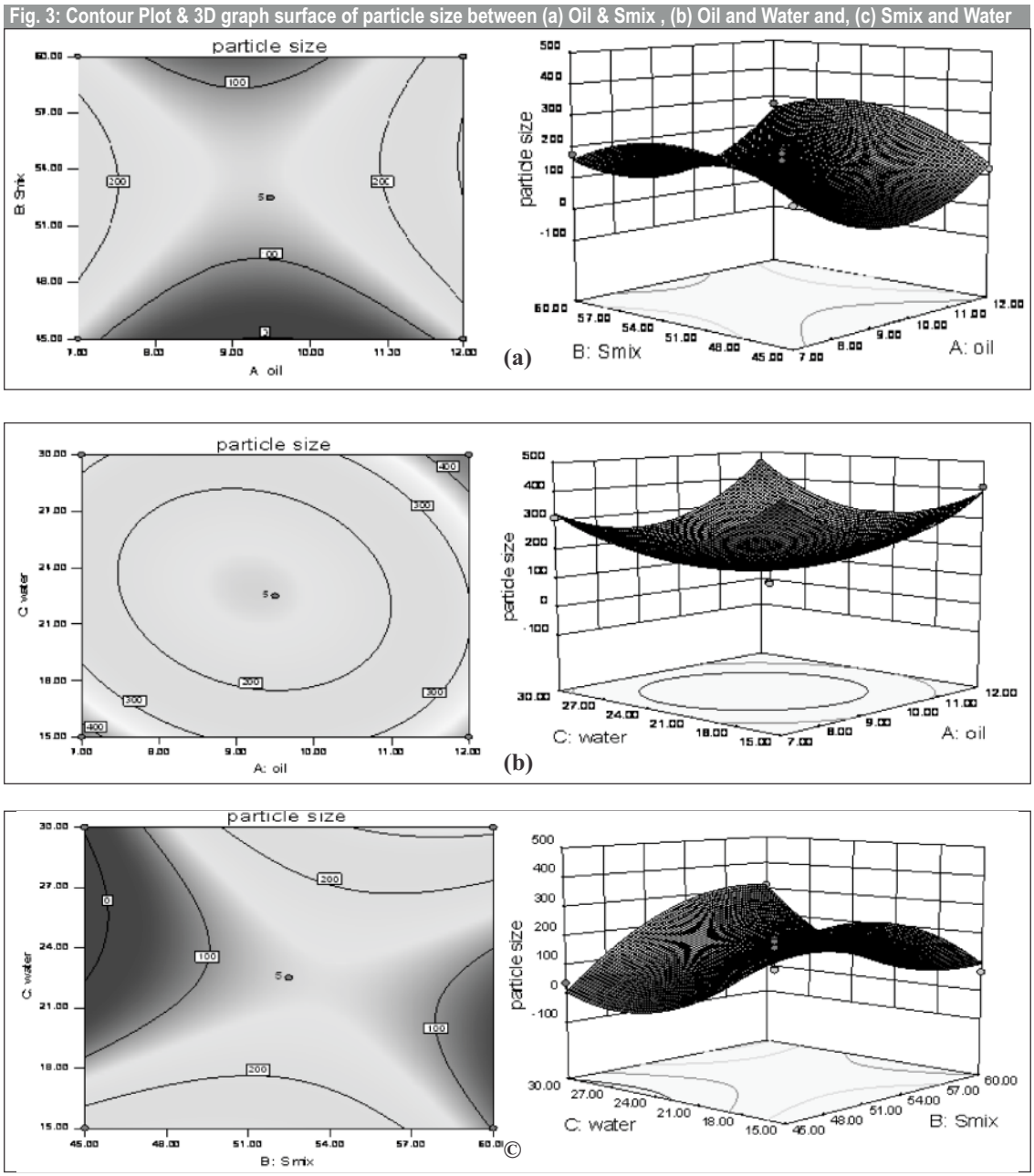
Particle Size = +135.30 +27.79 × A + 37.69 ×B - 7.45 × C + 21.85 ×A × B + 45.08 × A × C+ 117.23 × B × C + 136.92 × A^2 - 100.42 × B^2 + 133.70 × C^2

Final Equation in Terms of Actual Factors:
Particle Size = +1604.70700 - 520.40700 × oil + 134.5243 × Smix - 240.20133 × water + 1.16533 × oil × Smix + 2.40400 × oil × water + 2.0840 × Smix × water + 21.90800 × oil^2 - 1.78533 × $Smix^2$ + 2.37689 × $water^2$

The contour plot is a two-dimensional representation of the response across the selected factors. The full range of two factors at a time can be displayed. If there are more than two factors the 2D surface can be thought of a slice through the factor space. The independent variables are usually restricted to a regular grid. The actual techniques for determining the correct iso-response values are rather complex and are almost always computer generated (Fig. 3 and 4).

The more the number of responses is examined, the more busier the overlaid contour plot becomes. For this reason, only a maximum and a minimum contour are typically drawn

Fig. 3: Contour Plot & 3D graph surface of particle size between (a) Oil & Smix , (b) Oil and Water and, (c) Smix and Water



for each response. This minimizes the clutter and makes the overlaid contour plot easier to interpret. Contour plots show the interrelationship between the three factors.

Optimization of one response or the simultaneous optimization of multiple responses can be performed graphically or numerically. Simultaneously it can also evaluate all the response models for any value of the independent variables using the point prediction node. The relationship between the dependent and independent variables was further elucidated using contour and response surface plots. The contour profiler will create an overlaid contour plot. This plot will use one contour line for each response. Small dots are placed on one side of the contour line to indicate the direction of a higher response. The contour profiler makes it easier to change settings of any independent variables that cannot be shown on the plot as well as shading out regions of the contour plot that are not desired. The effect of factor 1, 2 and 3 on response 1 particle size was elucidated in Fig: 3-4. The factor 1 is kept constant and other factors varied in formulation 10-12 and on increasing the factor 2 (Smix) the response 2 (zeta potential) decreases.

The plot of predicted vs. actual graph shows the variation between the actual and predicted values of particle size (A) and zeta potential (B). A best fit line is drawn between predicted and actual values (Fig: 5). The actual values are very close to the predicted values.

Fig. 4: Contour Plot & 3D graph surface of Zeta potential between (a) Oil & Smix , (b) Oil and Water and, (c) Smix and Water.

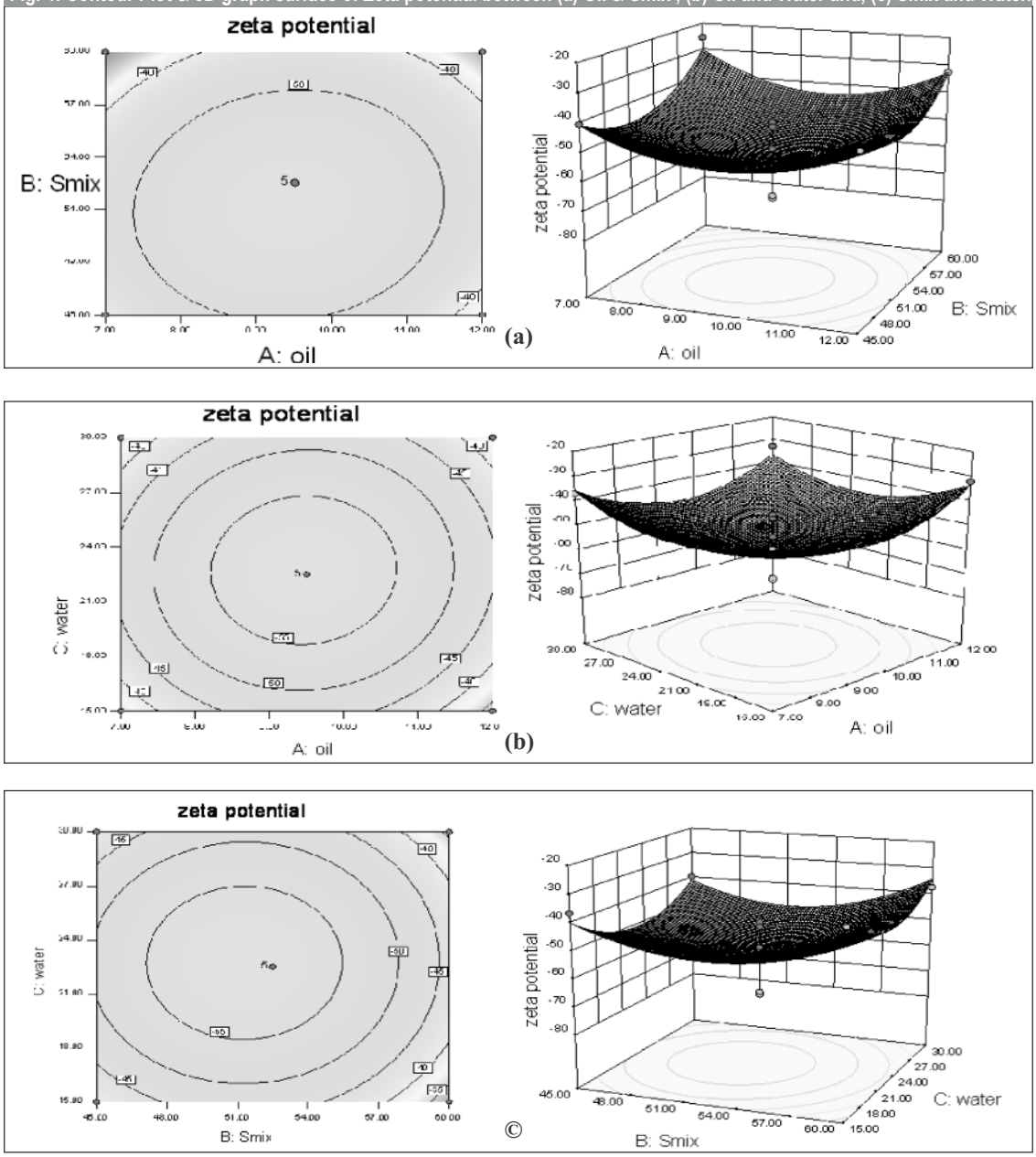


Table 3: The Optimized Formulation by the point prediction methods

Factor	Name	Level	Particle size		Zeta potential	
A	Oil	9.36	Predicted	Actual	Predicted	Actual
B	Smix	45.81	24.75	25.51	-52.61	-54.60
C	Water	22.50				

The cube plot shows the effect of 3 factors on the formulation. On the basis of point prediction method the design expert software predicts the following combinations (Table 3).

3.4. Thermodynamic stability studies: The thermodynamic stability testing was done to ascertain that the prepared nanoemulsions were stable when subjected to freeze thaw cycle (to check stability at low temperature) and centrifugation studies (to check stability at high shear). The stable formulations were selected and unstable ones (phase separation, turbidity, change in color or drug precipitation) were rejected. The formulations Sf 2, Sf 5, Sf 9, Sf 11, Sf 12 failed the thermodynamic protocol (Table 4). The remaining formulations were characterized for various *in vitro* attributes.

3.5. Surface morphology: The nano-emulsion droplets were visualized as bright spots surrounded in dark background (Fig. 6). Droplet size was found to be quite uniform and in agreement with the size values suggested by zetasizer.

Fig. 5: Plot between Predicted vs. Actual (A) Particle size and (B) Zeta Potential.

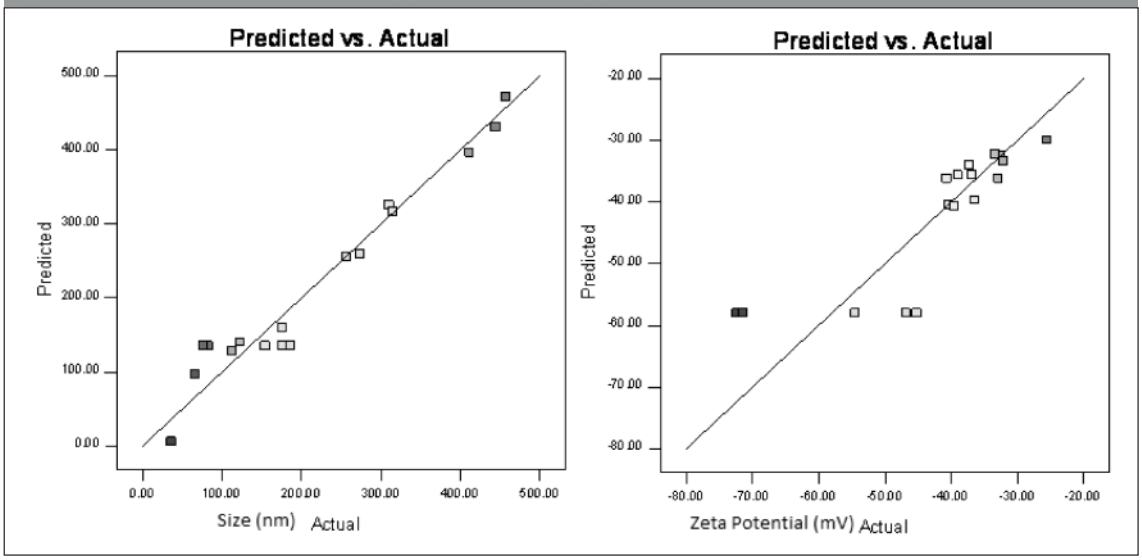


Fig. 6: Transmission electron microscope photograph of optimized nano-emulsion.

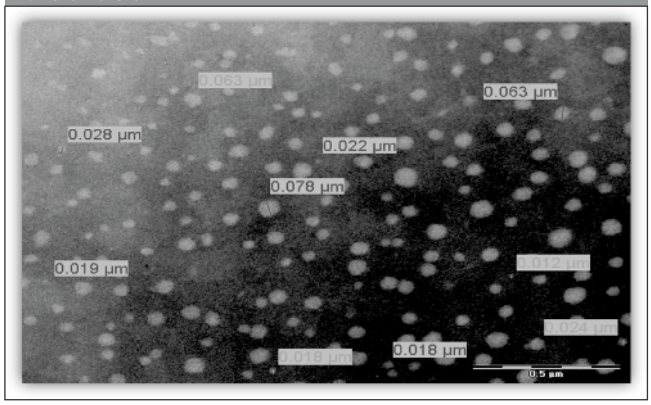
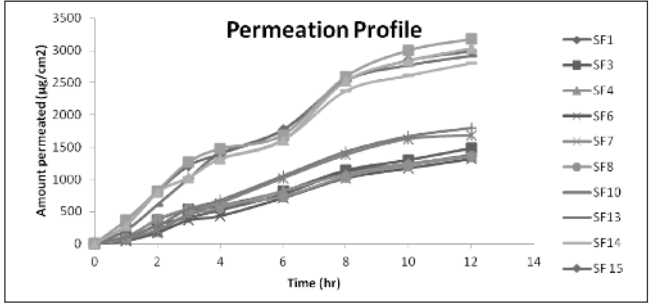


Fig. 7: Ex-vivo permeation profile of different nano-emulsion formulations



3.6. Viscosity: Viscosity of the nano-emulsion formulation (SF-4) was found to be very low as expected for o/w emulsion (0.8872 cP)[13]. The low viscosity may be due to presence of low amount of tween 80 (a fatty acid polyhydric alcohol ester having high intrinsic viscosity) as compared to carbitol (a short chain alcohol having low intrinsic viscosity) and also the low concentration of oil.

Table 4: The Thermodynamic Stability Study of Different Formulations

S. no.	Formulation code	Freeze Thaw cycle	Centrifugation cycle	Heating -cooling cycle	Inference
1.	Sf 1	✔	✔	✔	Passed
2.	Sf 2	x	✔	✔	Failed
3.	Sf 3	✔	✔	✔	Passed
4.	Sf 4	✔	✔	✔	Passed
5.	Sf 5	✔	✔	x	Failed
6.	Sf 6	✔	✔	✔	Passed
7.	Sf 7	✔	✔	✔	Passed
8.	Sf 8	✔	✔	✔	Passed
9.	Sf 9	✔	✔	x	Failed
10.	Sf 10	✔	✔	✔	Passed
11.	Sf 11	x	✔	✔	Failed
12.	Sf 12	✔	✔	x	Failed
13.	Sf 13	✔	✔	✔	Passed
14.	Sf 14	✔	✔	✔	Passed
15.	Sf 15	✔	✔	✔	Passed
16.	Sf 16	✔	✔	✔	Passed
17.	Sf 17	✔	✔	✔	Passed

3.7 Ex-Vivo skin permeation and skin deposition study: The permeation ability of the selected nanoemulsion formulations was evaluated conducting the ex-vivo permeation experiments. The permeation parameters of the tested nanoemulsion formulations are presented in Table 5 and Fig 7. On the basis of permeation study the nanoemulsion formulation Sf4 revealed minimum permeation. The Sf4 formulation was selected for further fluorescence microscopy and for the nanoemulsion gel formulation.

Fig. 8: Fluorescent images showing permeation through the skin at 1 hour (a) Control (oil only) (b) Marked formulation.

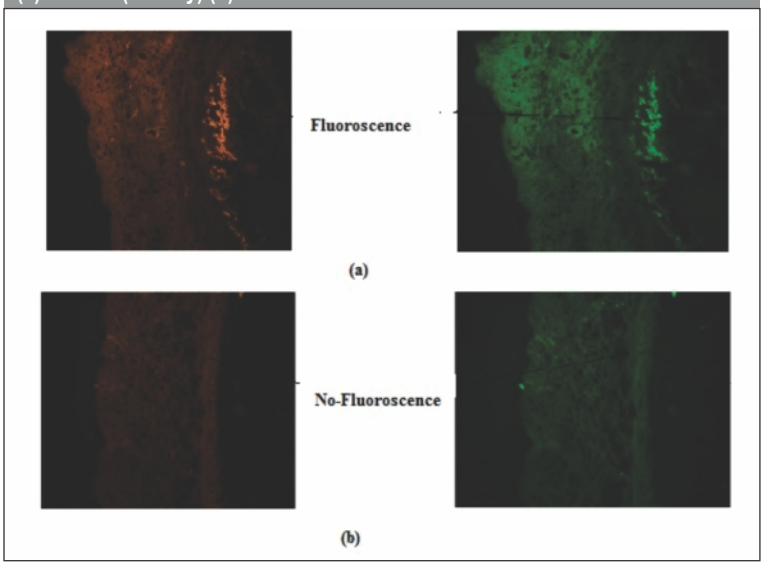


Fig. 9. Fluorescent images showing permeation from SF4, through the skin (a) at 1 hr, (b) at 2 hr, and (c) at 6 hr.

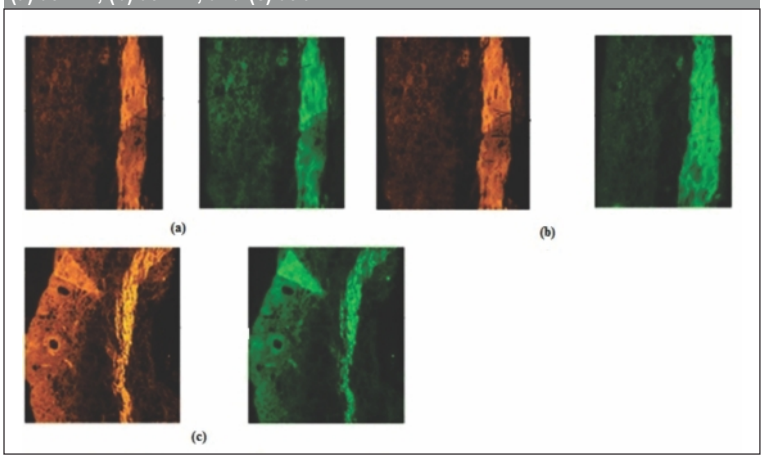


Table 5: Flux and Permeability coefficient of different formulations

S. No.	Formulation code	Flux (µg/cm^2/hr)	Permeability coefficient (× 10^{-3} cm/h)
1.	Sf 1	74.39	2.479
2.	Sf 3	62.075	2.069
3.	Sf 4	56.672	1.88
4.	Sf 6	73.05	2.435
5.	Sf 7	76.778	2.559
6.	Sf 8	76.233	2.54
7.	Sf 10	104.21	3.473
8.	Sf 13	176.258	5.875
9.	Sf 14	168.719	5.62
10.	Sf 15	181.78	6.0595
11.	Sf 16	193.18	6.44
12.	Sf 17	184.80	6.16

3.8 Evaluation of the nano-emulsion gel : The nanoemulsion gel NEG was subjected to study of various parameters for its characterization. The spreadability of gel was found to be 7.4 cm. viscosity of the gel measured was 498±0.014 cPs. The pH of the formulation was found to be 6.7±0.115. Ex-vivo skin permeation study was also performed and the cumulative amount of drug permeated amounted to 1231.214 µg. The comparative study of Sf4 and NEG suggested that the cumulative amount of drug permeated in the case of nanoemulsion was higher (56.67 µg cm^{-2} h^{-1}) as compared to nanoemulsion gel (Sf4- NEG) which was found to be 52.90 µg cm^{-2} h^{-1}. The reduction in permeation is actually attributable to the gel formation and thickening action of carbopol 934, which enmeshed the drug and precluded the release. The thickening effect of carbopol 934 gel had lent the formulation a better spreadability and

sensorial aesthetic value. Further the drug was observed to be uniformly distributed in the nano emulsion gel system.

3.9 The Skin Permeation study by fluorescence microscopy: On comparison of the permeation study of different formulations, it was observed that the marketed preparation permeated quickly and no fluorescence was observed after 1 hour (Fig 8 B) while the optimized formulation (SF4-NEG) permeated very slowly and even after the 6 hours the fluorescence was evident in deeper layers of skin (Fig 9 A- 9C). On the other hand the permeation of the oily solution was found to be intermediate as fluorescence was observed for 1 hour (Fig 8A). As describe earlier, the effective treatment of onychomycosis will need a continued retention of the drug inside the skin layers and hence it can be concluded that the prepared optimized formulation Sf4-NEG is suitable for delivery of the drug for longer duration for effective treatment of infection with the reduced time of therapy and considerably fewer side effects.

CONCLUSION

The current study has therefore successfully formulated a thermodynamically stable antifungal nanoemulsion gel containing ciclopirox olamine which can be retained for a prolonged period of time. Such formulation achieves better local concentration of the drug with low systemic absorption of potentially toxic anti-fungal drug. Hence, the formulation has proved to be a promising approach for treatment of onychomycosis.

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