Original Research

NANOEMULSION GEL AS NOVEL OIL BASED COLLOIDAL NANOCARRIER FOR TOPICAL DELIVERY OF BIFONAZOLE

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ABSTRACT

Background: Nanoemulsion (NE) based topical gel of Bifonazolewas developed by converting NE comprising caproyl oil as an oil phase, Tween 80 as surfactant and Isopropyl alcohol (IPA) as a co-surfactant into gel using carbopol 934.

Method: NE was prepared by aqueous titration method using oil, blend of surfactant and co-surfactant (Smix = 1:1) and distilled water which thereafter converted into gel using different concentrations of carbopol-934.

Results: Optimized NE revealed spherical morphology under TEM analysis having globule size 85.3 ± 1.65 nm and the highest *in- vitro*release 93.12% within8 h in a medium containingPhosphate buffer pH 6.8 and methanol in ratio 3:1. Optimized NE based gel revealed pH, viscosity andspreadibilityas 6.3 ± 0.03, 4726 ± 23.69 cps and 5.01 ± 0.06 g.cm/sec respectively. %Cumulative release of gel by *ex- vivo* permeation studies was found to be 81.23 % in 24 h as compared toNE (84.18%).

Conclusion: Nanoemulsion served as a better contrivance to provide better traversing of drug through skin layer due to very small sizeby incorporatingeven hydrophobic drugand itsconversion into gel provides sustained effect to overcome the shorter half-life of the drug.

KEYWORDS: Nanoemulsion, Nanoemulgel, Bifonazole, Topical drug delivery, aqueous titration

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1.0 Introduction

The incidence of superficial fungal infections of skin, hair and nails has been increased in worldwide. It has been estimated that about 40 million people have suffered from fungal infections in developing and under developed nations[1]. The progression of fungal infections can be rapid and serious in immunocompromised persons [2]. Dermatophytesare one of the most frequent causes of tineaand onchomycosis[3].

Topical treatment of fungal infections has several superiorities including, targeting the site of infection, reduction of the risk of systemic side effects, enhancement of the efficacy of treatment and high patient compliance[4].

The efficiency of the topical antifungal treatment depends on the penetration of drugs through the target tissue. Hence, the effective drug concentration levels should be achieved in the skin [5]. Development of alternative approaches for topical treatment of fungal infections of skin encompasses new carrier systems for approved and investigational compounds.

Nanoemulsions (NE's) are non-equilibrium, thermodynamically stable optically transparent, metastable dispersion of nano-sized particles [6] having defined surface tension formed by certain shear, comprises of a suitable oil and definite blend of surfactants and co-surfactants and having capacity to dissolve large quantities of hydrophobic drugs [7]. They have ability to protect the drugs from hydrolysis and enzymatic degradation makes them ideal vehicles for drug delivery. Moreover, the lack of flocculation, creaming and high kinetic stability offers obvious benefits over emulsions of larger particle size. NE's are often misinterpreted as lyotropic liquid crystals or microemulsions. Although nanoemulsions are non-equilibrium structures which don't form spontaneously without any shear as compared to lyotropic crystals [8]. There was so many conflicts regarding suitable method of preparation of nanoemulsions and later on it was proved that nanoemulsions can be formulated easily even by "low- energy emulsification method" along with high shear method [9]

A dermally applied nanoemulsion is expected to penetrate the stratum corneum and to exist intact in the whole horny layer which has been proved by double labeling studies for liposomes that resembles nanoemulsion in structure [10]. The lipophilic domain of NE interacts with stratum corneum and the drug dissolved in the lipid domain can directly partition into lipids. The hydrophilic domain of the NE can hydrate the stratum corneum to the greater extent which plays an important role in the percutaneous uptake of the drug [11].

Nanoemulgels (NEGs) are the nanoemulsions, either of the oil-in-water or water in-oil type, which are gelled by mixing with a suitable gelling agent [12]. They have a high patient acceptability, show higher penetration across the skin due to nano sized small droplets since; they possess the advantages of both nanoemulsions and gels. Therefore, they have been recently used as vehicles to deliver various drugs across the skin [13].

Gels are that class of dosage form which is created by entrapment of large amounts of aqueous or hydro alcoholic liquid in a network of colloidal solid particles [14]. Gels for dermatological use have several favorable properties such as being thixotropic, greaseless, easily spreadable, easily removable, emollient, non-staining, compatible with several excipients, and water-soluble or miscible [15].

In spite of many advantages of gels, a major limitation is in the delivery of hydrophobic drugs. So, to overcome this limitation nanoemulgels are prepared and with their use even a hydrophobic drug can enjoy the unique properties of gels. When gels and nanoemulsions are used in combined form the dosage forms are referred as nanoemulgels[16]. Nanoemulgelsoffers so many advantages such as incorporation of hydrophobic drugs easily, avoidance of hepatic first pass metabolism, medication can be self-applied, improved patient compliance, medication can be terminated when needed and suitable for drug with shorter half-life and site specific delivery etc. [17].

Bifonazole (BIF) is a broad spectrum azole anti-fungal drug and effective against dermatophytes, moulds and several other fungi. It is topically administered once a day (100 mg/day) to treat athlete's foot, 1% shampoo is recommended in saeborrhic dermatitis etc. Due to its lower solubility and permeability it shows very less absorption i.e. 0.6 % of an applied dose. Hence the aim of this study was to develop nanoemulsion based gel of BIF in order to enhance permeability and provide sustained effect to increase the time of contact [18].

2.0 Materials and methods

2.1 Materials

Bifonazole was obtained as a gift sample from Vital Laboratories Pvt. Ltd., Vapi. Caproyl oil was also obtained as a gift sample from Gattefosse SAS, Saint-Priest, and France. Tween 80, isopropyl alcohol and Carbopol-934 was obtained from Central Drug House, Delhi, India. Cellulose membrane – 70, Code LA393 having molecular weight cut off 12,000-14,000 and pore size of 2.4 nm was used and Candida albicans NCTC 3179 (clinical isolate grown at 25°C for 24 h on Sabouraud's agar). All other chemicals used were of laboratory reagent grade except methanol (Analytical grade).

2.2 Screening and selection of oils, surfactants (S) and co-surfactants (CoS)

Solubility of Bifonazole was determined in different oils viz. Oleic acid, Caproyl 90, Olive oil, Castor oil, Labrafac, Coconut and Soyabean oil, surfactants such as Tween 20, Tween 80, Labrasol, Span 20, and Labrafil and cosurfactants such as Ethanol, Propylene glycol (PG), Polyethylene glycol-400 (PEG-400); Isopropyl alcohol (IPA) by taking 2 ml of different substances in small vials and excess amount of the drug was added. The vials were tightly stoppered and were continuously stirred for 72 h at 37 ± 0.5˚C, and samples were centrifuged at 10,000 rpm for 10 min. The supernatant was separated, filtered and after appropriate dilution with methanol, solubility was determined by ultraviolet-visible (UV) spectroscopy at 254 nm.

Based on the solubility data, caproyl oil, Tween 80 and IPA were selected as oil, surfactant and cosurfactant respectively.

2.3 Construction of pseudo-ternary phase diagram to optimize S/CoS ratio (Smix)

Blend of S/CoS specified as Smix in a specific ratio had to be optimize by construction of pseudo-ternary phase diagram using aqueous titration chart [19]. The ratio which provide maximum NE zone had been selected for placebo formulations.For determination of existence of NE zone, Pseudo-ternary phase diagram was constructed. Surfactant (Tween 80) and cosurfactant (IPA) were mixed in different volume ratios (1:0, 1:1, 1.2, 1:3, 1:4, 2:1 and 3:1) to give Smix as shown in Table 1.

Table 1: Preparation of Smixratio

Sl. NO.	Volume of surfactant (Tween 80) ml	Volume of co-surfactant (IPA) ml	Smix ratio
1	100	0	1:0
2	50	50	1:1
3	33.3	66.7	1:2
4	25	75	1:3
5	66.7	33.3	2:1
6	75	25	3:1
7	80	20	4:1

These combinations were in the order of increasing CoS with respect to S and vice versa. For each phase diagram, oil phase and Smix were mixed thoroughly in different volume ratios from 1:9 to 9:1 in different vials and aqueous phase was added accordingly with continuous vortexing to delineate the boundaries of NE zone [20]. After every 5% addition of aqueous phase, visual observation was recorded on the following basis

- ✓ Transparent & easily flowable o/w or w/o nanoemulsion
- ✓ Transparent gel; Nanoemulgel
- ✓ Milky/ cloud appearance; phase separation, emulsion
- ✓ Milky gel; emulgel

2.4 Placebo formulations

From the phase diagram, ratio of Smix showing maximum NE region was selected and number of NE formulations with different formula were selected. Almost entire range of NE region was covered and different oil composition with minimum surfactant concentration and maximum water concentration were selected. For formulation, required amount of oil was taken and mixed with required amount of surfactant and cosurfactant and slow titration was performed till a transparent clear NE was obtained. Compositions had been shown in Table 2.

Table 2: Placebo Formulations

Formulations	Smix	Oil: Smix	Oil %	Smix %	Distll. H_2O%
F1	1:1	1:9	5.00	50.00	44.44
F2	1:1	1:9	4.55	45.00	50.00
F3	1:1	1:9	4.00	45.91	54.55
F4	1:1	1:8	5.00	40.00	55.00
F5	1:1	1:8	3.88	31.07	65.05
F6	1:1	1:7	5.00	35.00	60.00
F7	1:1	1:7	4.35	30.43	65.22
F8	1:1	1:6	5.00	30.00	65.00
F9	1:1	1:6	4.26	25.53	70.21
F10	1:1	1:5	5.00	25.00	70.00
F11	1:1	1:5	4.17	20.83	75.00
F12	1:1	1:4	5.00	20.00	75.00
F13	1:1	1:4	4.00	16.00	80.00

2.5 Formulation of Drug loaded NEs

Bifonazole (10mg/ml) required to be added in formulations for which 0.5 ml (5%) oil was enough to solubilize it completely (according to solubility of drug). Hence required amount of oil and Smix were mixed and water was added during slow titration. Six formulations were developed after selection from the placebo formulations as mentioned in Table 3.

Table 3: NEs Formulations

Formulations	Smix	Oil: Smix	Oil %	Smix %	Distll. H_2O%
F1	1:1	1:9	5.00	50.00	44.44
F4	1:1	1:8	5.00	40.00	55.00
F6	1:1	1:7	5.00	35.00	60.00
F8	1:1	1:6	5.00	30.00	65.00
F10	1:1	1:5	5.00	25.00	70.00
F12	1:1	1:4	5.00	20.00	75.00

2.6 Thermodynamic Stability Studies

To check that the stability under stress conditions, the NEs were subjected to thermodynamic stability testing, which comprises of 1) Heating cooling cycle, during which nanoemulsions were kept at 37±0.5°C for 24 h and after that they were kept at room temperature. Signs of turbidity, cracking, creaming was observed during the entire cycle. 2) Freeze thaw cycle during which NEs were kept in deep freezer (at -20°C) for 24 h and after 24 h they were removed and kept at room temperature. The thermodynamically stable NEs returned to their original form within 2-3 minutes. 2-3 such cycles were repeated and 3) Centrifugation tests in which NEs were subjected to undergo centrifugation for 30 minutes at 5,000 rpm in a centrifuge. Phase separation or turbidity was observed.

2.7 Physical Evaluation of NEs

Selected NEs were evaluated for various physical and morphological characters as color, transparency and phase separation by naked eye visually.

Determination of Particle size

Average particle size and Polydispersity index of oil droplets were determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to brownian motion of the particles using a Zetasizer (1000 HS, Malvern Instruments, U.K.) at JamiaHamdard, New Delhi, India. The formulation (0.1 mL) was dispersed in 50 mL of water in a volumetric flask, mixed thoroughly with vigorous shaking and light scattering was monitored at 25°C at a 90° angle.

Transmission Electron Microscopy (TEM) analysis

Morphology of the NEs was studied using TEM (FEI Philips Morgagni 268D) at Sophisticated Analytical Instrument Facility (SIF), AIIMS, and New Delhi operating at 100 kV and magnification upto 2,80,000x 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 NE. In order to perform the TEM observations, the diluted nanoemulsions were deposited on the holey film Cu grid with 2% phosphotungestic acid (PTA) and observed after drying.

***In-vitro* release studies**

Five formulations F1, F4, F6, F8, and F10 which passed the thermodynamic stability tests were selected for in-vitro release studies using phosphate buffer pH 6.8 in combination with methanol in ratio 3:1 by dialysis membrane method. The release studies were compared with the aqueous suspension of pure Bifonazole in same amount as that in NE formulations. Samples were analyzed using UV spectrophotometric analysis at 254 nm.

The receptor compartment was filled with the media (phosphate buffer pH 6.8: methanol in ratio 3:1). The membrane was trimmed to the appropriate size and mounted on the diffusion cell with the selected nanoemulsion formulation and suspension (Control) in such a way that the lower portion of the membrane was in contact with the receptor medium throughout. The donor compartment was filled with 1 ml of the selected nanoemulsions and suspension. The whole assembly was placed on the magnetic stirrer and stirred magnetically using magnetic beads placed within the receptor compartment at 600 rpm. Drug release study was carried out for 24 h at a temperature of 37°C. The aliquots of 1 ml was withdrawn from receptor compartment at predetermined time intervals of 0.5, 1,2, 4, 6, 8, 10, 12 and 24 h and were replaced by the same volume of

the media. The samples were analyzed at 254 nm after appropriate dilutions.

2.8 Formulation of Nanoemulgel (NEG)

Selected NE formulation (F1) was converted into gel using Carbopol in 1, 1.5, and 2% concentration (Modi, 2011) to obtain F1-A, F1-B, and F1-C NEGs respectively. Composition of NEGs had been mentioned in Table 4.

Table 4: Formulation of NEGs

Compositions	F1-A % w/w	F1-B % w/w	F1-C % w/w
Bifonazole	1	1	1
Caproyl oil	5	5	5
Smix	45	45	45
Distilled water	50	50	50
Carbopol-934	1	1.5	2

F1 = selected NE and A, B and C = 1, 1.5 and 2% Carbopol

2.9 Evaluation of Nanoemulgels (NEGs)

NEGs were evaluated for various physical characters, basic properties of a gel like viscosity, spreadability, ex-vivo permeation studies and color, clog and phase separation by naked eye visually.

2.9.1 Determination of pH

The pH was determined using digital pH meter. Nanoemulgel was weighed accurately and dispersed in few ml of distilled water. The pH meter was calibrated before each use with buffer solution of pH 4, 7 and 9. The measurement of pH of formulation was done in triplicate and mean values were calculated.

2.9.2 Determination of Viscosity

Viscosity was determined by using Brookfield viscometer (DV-I). The spindle number 6 was dipped in the preparation and rotated at 5, 10, 15, 20 and 50 rpm at room temperature.

2.9.3 Determination of Spreadability

Excess formulation was placed between two glass slides and 100 g weight was placed on the upper glass slide for 5 minute to compress the formulation to uniform thickness. Weight (100 g) was added to the pan. The time in seconds required to separate the two slides was taken as a measure of spreadability. It was calculated by using the following formula

$$s = (m \times l) \div t$$

Where *s* is spreadability; *m* is the weight tied to the upper slides; *l* is the length of glass slide and; *t* is the time taken in seconds.

2.9.4 Determination of % Drug content

100 mg of nanoemulgel was taken in 100 ml volumetric flask containing 50 ml methanol and stirred for 30 min. Volume was made up to 100 ml with methanol. From the above solution, 0.1 ml was further diluted with 10 ml methanol to get 10 mcg/ ml. The resultant solution was filtered through Whatman filter paper and absorbance of the solution was measured at 254 nm using UV spectrophotometer.

2.9.5 *Ex-vivo* skin permeation study

This study was carried out by using excised skin samples (dorsal side) of rat. The skin was excised from the abdominal region of male albino rats weighing 110-125 g and covered with aluminum foil and then was frozen to -20°C until further use. On the day of experiment the skin was thawed to room temperature. Hairs and fat were removed using hair removing cream and adhering subcutaneous fat layer was removed by using isopropyl alcohol (IPA) and immediately kept in isotonic phosphate buffer solution to flatten and smooth.

This study was carried by using Franz diffusion cells with an effective diffusion area of 3.7994 cm^2. The excised skin samples of rat were clamped between

the donor and the receptor compartment with the stratum corneum facing the donor compartment. Then, 1 g of nanoemulgel containing 1% (w/w) Bifonazole was applied on the donor compartment. The receptor compartment was filed with phosphate buffer of pH 6.8 in combination with methanol in ratio 3:1 and maintained at 37°C with stirring at 100 rpm. At predetermined time intervals, 1 ml receptor medium was withdrawn and the same volume of pure medium was immediately added into the receptor compartment. The procedure was repeated up to 8 h. All samples were filtered through Whatman filter paper and analyzed by UV spectrophotometer at 254 nm.

2.9.6 Skin Irritation Studies

Skin irritation test was carried out on wistar male or female rats weighing 200-250 g. (approved by the Committee for the purpose of control and supervision on experiments on animals (CPCSEA) of PDM/ approval no. PDM/CPCSEA/RES/2013/1/2) were used. The study was carried out on three groups comprising 6 rats each (Genno, 1998).

- Group 1 – Negative Control (without any application)
- Group 2 – Positive Control (Standard irritant)
- Group 3 – Test (Optimized formulation)

Small amount of these were applied on the shaved skin of the rats and the applied area was covered with gauze and adhesive bandage. The exposed skin of rats was graded for formation of edema and erythema after 24 h according to Draize's score chart.

After giving respective scores to the skin reaction, Primary Irritation Index (PII) was calculated using the following equation:

$$\textit{Primary Irritation Index} = \frac{(\textit{Erythematic reaction scores} + \textit{Edema reaction scores})}{\textit{Time intervals (h)}}$$

2.9.7 Anti-fungal activity

Anti-fungal activity was carried out by cup plate method (Carrilo, 1996). The overnight grown culture of *Candida albicans* was inoculated into the sterilized agar media plates. After solidification, wells were cut into the media and fixed with 100 mg of the optimized nanoemulgel formulation and marketed cream. The plates were incubated at room temperature and the widths of zone of inhibitions resulting after diffusion into media were measured (Hugo, 1977).

2.10 Stability studies of optimized formulation of Bifonazole as per ICH guidelines

The optimized nanoemulgel formulation (F1-A) was stored at 4°C, room temperature and 45°C for 3 months and samples were evaluated for physical parameters like turbidity, color change, pH and physicochemical parameters like particle size and drug content at one month interval. Shelf-life was also calculated using stability data by Sigma plot.

3.0 Results and discussion

3.1 Screening and selection of Oils, Surfactants (S) and Cosurfactants (CoS)

Oils: : The most important criterion for screening is the solubility of poorly soluble drug in oils. Solubility of drug in oil is the most important criteria as the ability of NE to maintain the drug in solubilized form is greatly influenced by solubility profile of drug in oils[20]. Taking different oils viz. Oleic acid, Caproyl, Olive oil, Castor oil, Labrafac, Coconut and Soyabean oil, the solubility data is tabulated in Table 5. It revealed that Bifonazole possessed maximum solubility in Caproyl oil (200 mg/ml). Hence it was selected as an oil phase for NE formulation. The other advantage with the use of caproyl oil is that it has an anti-microbial activity against fungi, bacteria etc.

Table 5: Solubility of bifonazole in various oils

S.NO	Oils	Solubility (mg/ml) (n = 3 ± S.D.)
1	Oleic acid	162.00 ± 0.120
2	Caproyl 90	200.28 ± 0.141
3	Castor oil	26.69 ± 0.213
4	Labrafac	4.57 ± 0.112
5	Soyabean oil	0.50 ± 0.124
6	Coconut Oil	0.40 ± 0.091
7	Olive Oil	4.00 ± 0.322

Surfactants (S) and Cosurfactants (CoS)

Surfactants are required to decrease the interfacial tension between the particles and cosurfactants are required to allow more penetration of oil (carrier vehicle of drug) and lowering down the concentration of surfactants along with aiding in lowering surface tension to improve solubilization of drug[20]. Hence screening is essential for S and CoS for which solubility of bifonazole was carried out in in various surfactants such as Tween 20, Tween 80, Labrasol, Span 20, Labrafil and cosurfactants such as Ethanol, Propylene glycol (PG), Polyethylene glycol-400 (PEG-400), and Isopropyl alcohol (IPA) and results are tabulated in Table 6. From the presented data, Tween 80 and IPA were selected as surfactant and cosurfactant respectively due to maximum solubility profile of drug. Solubility profile of bifonazole in oils, surfactants and cosurfactants is as shown in Figure 1.

After selection of Caproyl oil as oil phase, Tween 80 as surfactant and IPA as cosurfactant, miscibility was checked for the mixture of S and CoS with Caproyl oil so as to get clear transparent mixture of all the three components.

Addition of S/CoS is required to be added in a definite ratio which has been optimized by plotting ternary phase diagram giving arise to NE zone of existence. Ratio providing maximum zone has to be selected.

Table 6: Solubility of Bifonazole in Surfactants and Cosurfactants

S.NO	Surfactants and Cosurfactants	Solubility (mg/ml) (n = 3 ± S.D.)
1	Tween 20	49.60 ± 0.150
2	Tween 80	56.66 ± 0.231
3	Labrasol	40.7 ± 0.130
4	Span 20	2.33 ± 0.182
5	Labrafil	1.5 ± 0.202
6	Isopropyl Alcohol (IPA)	94.96 ± 0.191
7	Ethanol	68.25 ± 0.213
8	PEG 400	36.94 ± 0.243
9	Propylene glycol	19.72 ± 0.262

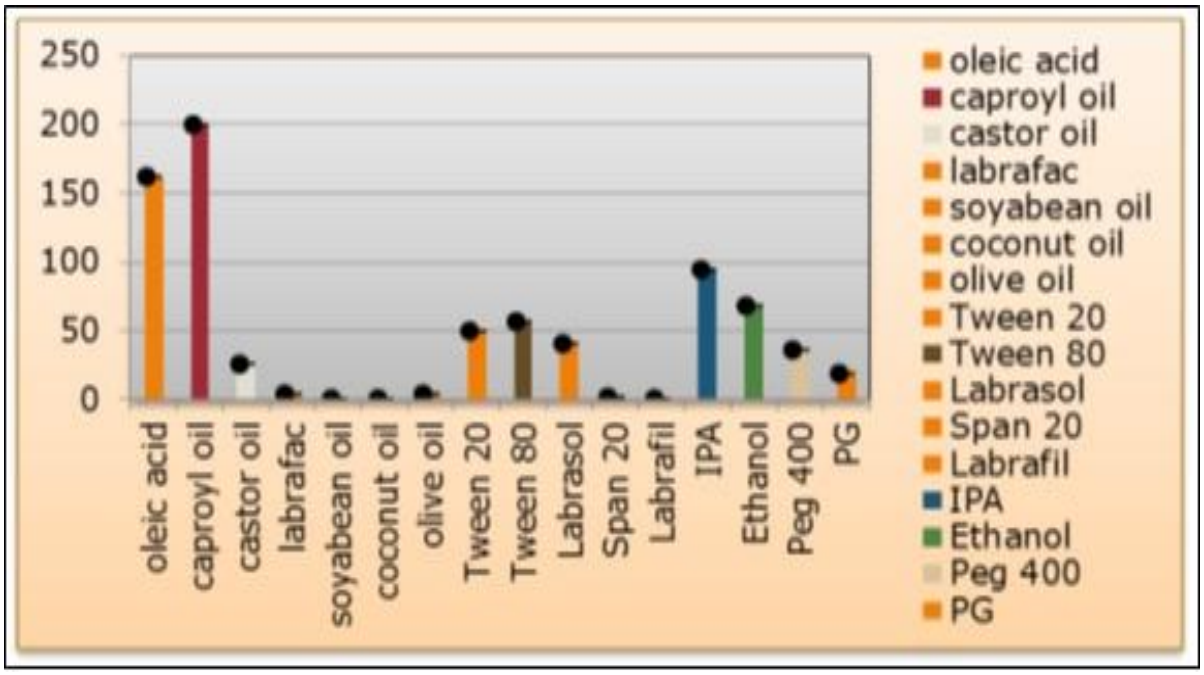

Figure 1: Solubility of Bifonazole in oils, surfactants and cosurfactants

3.2 Optimization of surfactant and cosurfactant through Ternary Phase diagrams

S/CoS mixture is required to be added in a definite ratio representing effect of increased conc. of CoS with respect to S and vice versa in each formulation which has been optimized by plotting ternary phase diagram by taking different ratios of S/CoS.

For each Smix ratio (1:0, 1:1, 1:2, 1:3, 2:1, and 3:1) separate phase diagrams were constructed using aqueous titration method to obtain highest nanoemulsion region as shown in Figure 2a - 2f.

It was observed that S/CoS mixture in ratio 1:1 yields the highest NE zone and hence it was selected as an optimized Smix ratio.

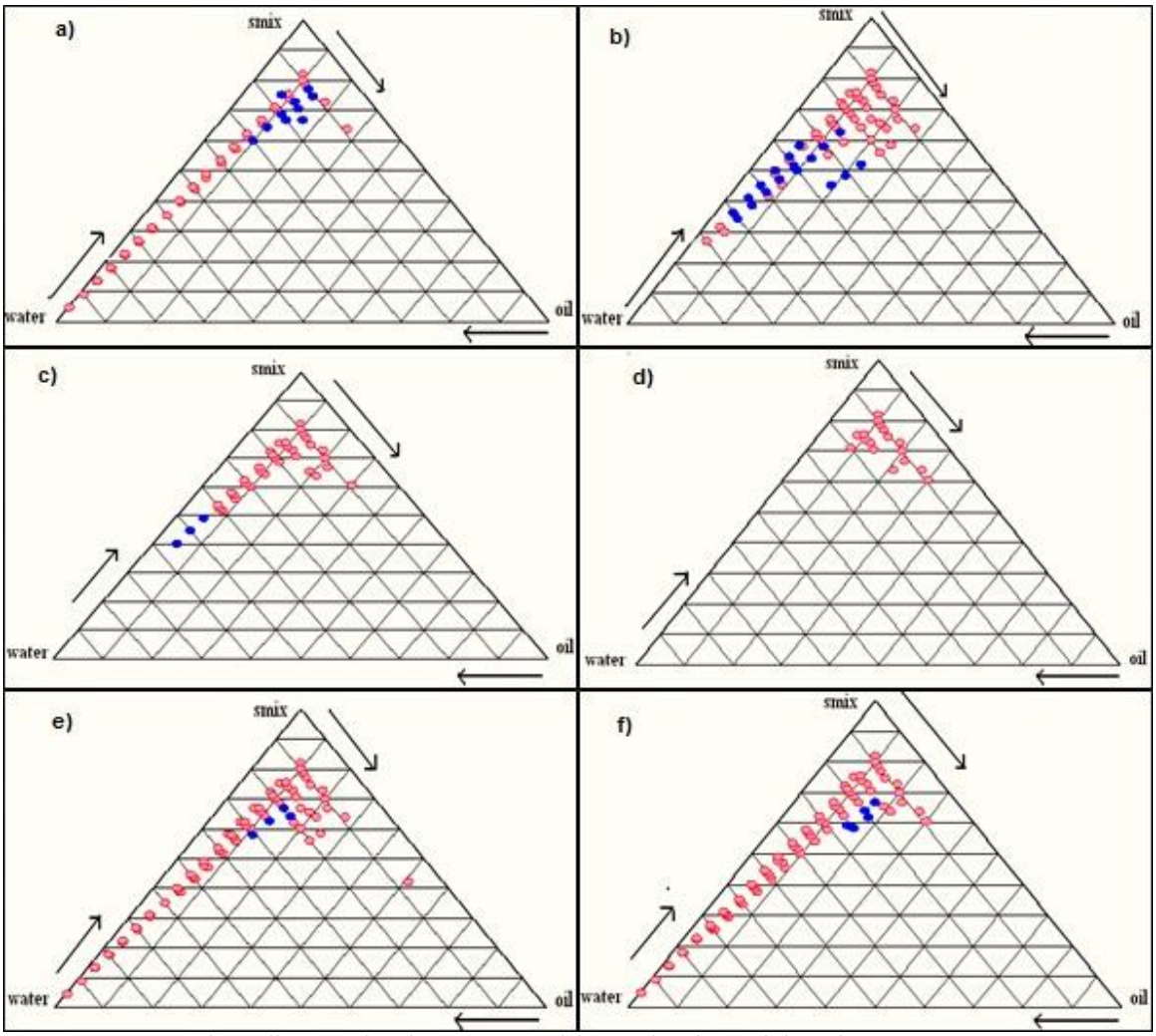

Figure 2: Ternary phase diagram showing nano-emulsion region for Smixin ratios **a)** 1:0**, b)** 1:1**, c)** 1:2**, d)** 1:3**, e)** 2:1**, f)** 3:1.

3.3 Placebo Formulations

By taking Caproyl oil, Tween 80, IPA in ratio 1:1 placebo formulations were developed by slow addition of aqueous phase (distilled water) to obtain clear transparent NE formulations (F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, F12, and F13). During this different oil: Smix ratios were taken. Placebo formulations give an idea of selecting conc. Range of all the components to incorporate drug without any hindrance.

Table 2: Placebo Formulations

3.4 Drug loading

Among the placebo formulations, six (F1, F4, F6, F8, F10, and F12) were selected for successful drug incorporation to get desired formulation. Dose of drug was decided according to the solubility of in the carrier i.e. oil.

3.5 Thermodynamic Stability Studies

Thermodynamic stability testing was done to ascertain that the prepared nanoemulsions were stable

when subjected to centrifugation studies (to check stability at high shear) and freeze thaw cycle (to check stability at low temperature). All the formulations were subjected to above studies came back to their original form and did not show any turbidity or phase separation on high speed centrifugation, freeze thaw and heating cooling cycle(Sheikh, 2007). Observations of the test are as shown in Table 7. The data revealed that F12 failed the thermodynamic stability test. It could be due to less amount of surfactant as compared to water in it which caused the phase separation or turbidity in it. Inference: Hence, five formulations (F1, F4, F6, F8, and F10) were selected for further evaluation parameters.

Table 7: Thermodynamic Stability studies

Formulations	Centrifugation studies	Freeze thaw cycle	Heating-cooling cycle	Inferences
F1	Clear stable	Clear stable	Clear stable	Passed
F4	Clear stable	Clear stable	Clear stable	Passed
F6	Clear stable	Clear stable	Clear stable	Passed
F8	Clear stable	Clear stable	Clear stable	Passed
F10	Clear stable	Clear stable	Clear stable	Passed
F12	**Clear stable**	**Turbid**	**-**	**Failed**

3.6 Physical Evaluation of NEs

Formulations were characterized on the basis of physical state that is color, transparency, and presence of any crystal growth or clogs as shown in Table 8.

This data revealed that the formulations were stable as there is no sign of any non-uniformity in color of all formulations as all are slightly yellowish in color. All the formulations were found to be transparent which means that the particle size is small enough that they are not able to scatter the light passing through it and appeared to be clear. In case of conventional emulsions, there is no such transparency as globule size in that case are not in nano range. Despite being in such a small sized range, particles doesn't show presence of any clogs or crystal growth (Ostwald ripening, a major problem associated with nanoemulsions). Hence all the formulations are complied with physical evaluation parameters(Mason, 2006).

Table 8: Physical characterization of NEs

Formulations	Color	Transparency	Crystal growth
F1	Light yellow	Transparent	Absent
F4	Light yellow	Transparent	Absent
F6	Light yellow	Transparent	Absent
F8	Light yellow	Transparent	Absent
F10	Light yellow	Transparent	Absent

3.6.1 Particle size analysis

PdI is a significant parameter to show "monodispersity" of the formulation. Average diameter (nm) of particles and Polydispersity index (PdI) of all the formulations are shown in Table 9. Size statistical report by intensity and graph of the droplets generated by the instrument is as shown in Figure 3a. Results showed that the average particle sizes of the formulations are in "nano size range" i.e. between 20 – 200 nm. Apart from average globule

size another parameter called Polydispersity Index (PdI) of all the formulations are less than 0.2 which confirmed the "monodispersity" of the formulations (Mason, 2006).

3.6.2 Surface morphology using Transmission Electron Microscopy (TEM)

TEM analysis was carried out to observe the shape of the droplets in the formulations. Some droplet size can also be measured through TEM as it is capable of point-to-point resolution. Shape of droplets by TEM is shown in Figure 3b. The droplets in the nanoemulsion appear dark, and the surroundings are bright; a "positive" image was seen using TEM. All the oil globules are spherical in shape.

Table 9: Average diameter (nm) and Polydispersity index (PdI) of NEs

Formulations	Average diameter (nm)	Polydispersity index (PdI)
F1	85.3	0.212
F4	99.6	0.242
F6	120.3	0.261
F8	148.6	0.282
F10	156.0	0.297

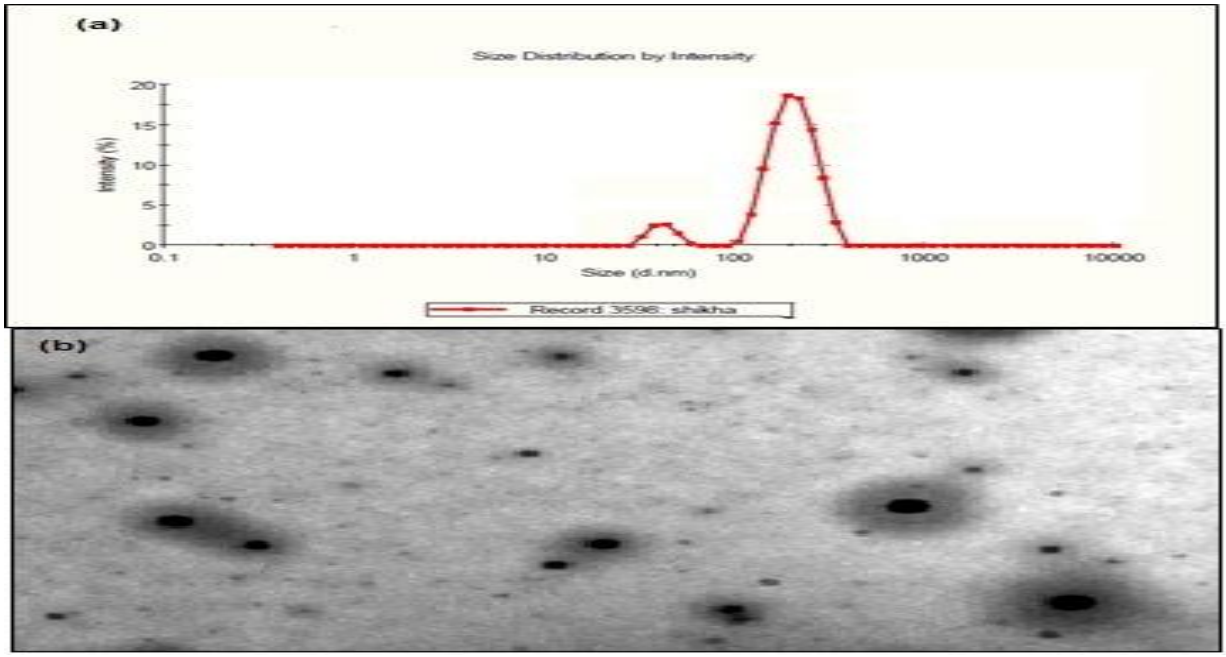

Figure 3a: Hydrodynamic size distribution graph of the droplets and **3b:** TEM micrograph showing Nanoemulsion (NE).

3.6.3 In-vitro Release Study

The development of suitable in vitro release models (for quality control as well as formulation development purposes) is a critical activity, which, should ideally lead to the establishment of an in vitro–in vivo correlation (IVIVC). This usually requires that drug release from the depot be the rate-limiting step in the absorption process and that the drug release mechanism is the same in vitro and in vivo. In vitro release data showing % Cumulative release (CR) of the formulations are shown in Figure 4. Results showed that F1 NE formulation showed maximum release of drug i.e. 93.12 % which was achieved within 8 h. It revealed enhanced or burst effect of NE of the smallest particle size to release the drug in the medium more efficiently than the

other NEs. Moreover data revealed that aqueous suspension of drug Control showed minimum release among all the formulations i.e. 90.80 % proving NEs formulations superior.

Hence F1 NE was selected to convert it into nanoemulgel by addition of carbopol as a gelling agent in varying concentration. It possessed average diameters of globules 85.3 nm in spherical shape, and 93.12 % drug release in 8 h.

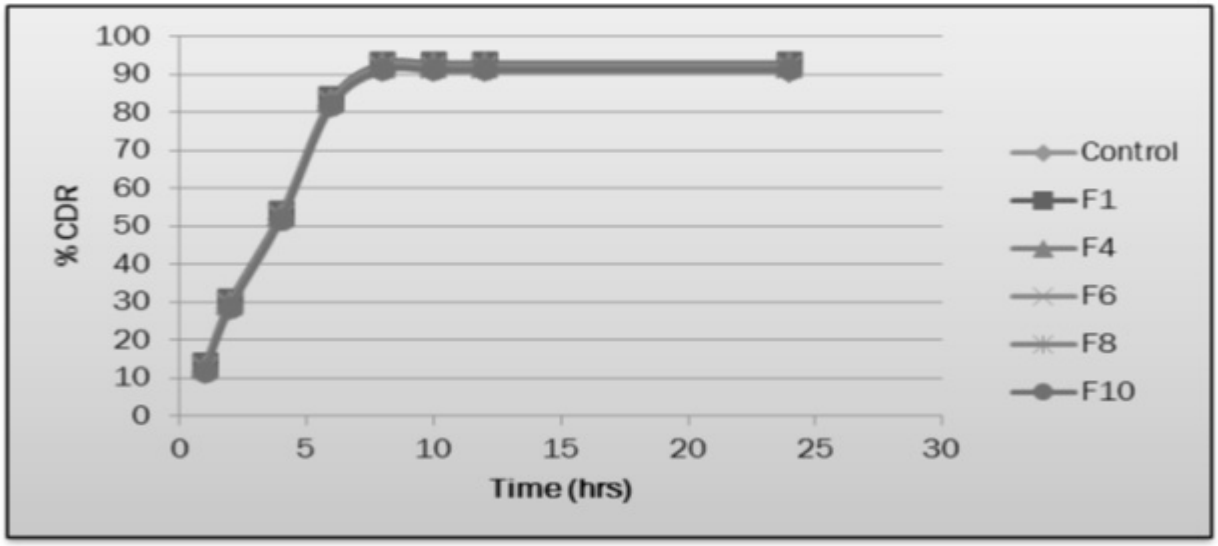

Figure 4: In-vitro release profile of Nanoemulsions (NEs).

3.7 Formulation of Nanoemulgel (NEG)

The selected nanoemulsionF1was formulated into three gels (F1-A, F1-B, and F1-C) by adding different concentration 1, 1.5, and 2 % of Carbopol-934 respectively. To provide local targeted action of this antifungal agent, gelling of the nanoemulsion was chosen as an appropriate approach to provide sustained effect by increasing the time of contact rather than NEs which are fluid in nature, hence less contact time and also immediate release effect of drug comparatively.

3.8 Physical Evaluation of Nanoemulgel (NEGs)

F1-A, F1-B, and F1-C were characterized for their physical appearance to determine color, clogs, phase separation and consistency which is tabulated in Table 10. Results revealed that all NEGs were almost white in color and there were no signs of clogs and phase separation found. In terms of consistency, formulations show F1_A < F1-B <F1-C, which indicates that on increasing the polymer concentration, consistency of the formed nanoemulgel also increases.

Table 10: Physical evaluation of F1-A, F1-B,and F1-C

Formulations	Physical evaluation parameters			
	Color	**Presence of clogs**	**Phase separation**	**Consistency**
F1-A	White	No	No	Less uniform
F1-B	White	No	No	Uniform
F1-C	White	No	No	More uniform

3.8.1 Determination of pH, viscosity, spreadability and drug content:

Being a gel, formulations were evaluated for spreadability and viscosity which are the

characteristic parameters of a semi-solid dosage form. It is clear that for patient compliance gel has to be easily applicable without any stickiness. Viscosity is inversely proportional to spreadability i.e. as viscosity increases, spreadability decreases (Banker 1996). pH of any topical formulation has to be in compliance with skin pH to avoid any unnecessary skin irritation. pH, viscosity, Spreadability, and drug content determined for all the three NEGs are as shown in Table 11.

The pH values of all 3 formulations, F1-A, F1-B, and F1-C ranged from 6.3 to 6.7, which is near to the skin pH and hence considered to be acceptable to avoid the risk of any irritation upon application to the skin. The Spreadability plays an important role in uniform application of gel on the skin. The Spreadability values of formulations show F1-A, F1-B, and F1-C , which clearly indicates that with increase in the concentration of the polymer, spreadability decreases due to increase in the cross linking between the polymer chains. Viscosity was found to be increase as increase in the polymer concentration to provide much better consistency. Drug content of all three formulations was found to be 94 % indicating uniform distribution of drug in the nanoemulgel.

Table 11: pH, viscosity, Spreadability, and drug content of NEGs

Formulations	Evaluation Parameters (n=3 ± S.D.)			
	pH	Viscosity (centi poise) cps	Spreadability (g.cm/sec)	% Drug Content
F1-A	6.3 ± 0.03	4726 ± 23.69	5.01 ± 0.06	94.19 ± 0.06
F1-B	6.5 ± 0.02	7917 ± 14.38	4.12 ± 0.02	94.23 ± 0.13
F1-C	6.7 ± 0.03	9529 ± 13.19	3.31 ± 0.03	94.18 ± 0.12

3.8.2 *Ex-vivo* Skin Permeation Study

The skin mimics our biological membrane to analyze the amount of drug permeated through this skin into the releasing medium. Data will present an idea of release pattern of drug from the formulation if used in humans. Permeation study was carried out for optimized NE i.e. F1 and NEGs, F1-A, F1-B, and F1-C and permeation profile of all the formulations is tabulated in Table 12. Results revealed that F1-A shows the best drug release profile than the other formulations. F1-A containing 1 % carbopol 934 shows 81.23 % cumulative release in 24 h as compared to F1-B (1.5 % carbopol) and F1-C (2 % carbopol) showing 79.28 and 74.76 % cumulative release respectively in 24 h. This might be attributed to the cross linking meshwork of the polymer which is in much more concentration in later two formulations thereby hindering or slowing the release of the drug from the nanoemulgel. *Ex-vivo* permeation profile with respect to time is as shown in Figure 5.

F1 nanoemulsion showed 84.18 % cumulative release which is greater than the F1-A nanoemulgel. It was due to gelling nature of formulation resulting in slow release of drug. F1-Ananoemulgel containing 1% carbopol-934 showed best permeation profile with efficient Spreadability and viscosity. Hence it was selected as an optimized formulation which was later on compared with marketed formulation of Bifonazole (Mycospor cream) to justify this formulation as a better choice to provide effective, long lasting local action against fungal disease.F1-A nanoemulgel assured its significance after analyzing various concluding evaluation parameters and showed an edge over marketed formulation also. Being a topical antifungal formulation, it should also comply with applicant's skin. Hence to assure no

significant skin irritation by this formulation, skin irritation test has to be carried out on shaved skin of rats taking this preparation as a "test sample".

Table 12: Ex-vivo permeation of F1, F1-A, F1-B, and F1-C

Time (h)	% Cumulative drug release (CDR) (n=3 ± S.D.)			
	F1 (NE)	F1 - A	F1 – B	F1 - C
1	11.26 ± 0.11	8.06 ± 0.05	6.80 ± 0.10	5.06 ± 0.11
2	20.23 ± 0.25	14.60 ± 0.02	13.69 ± 0.32	9.52 ± 0.14
4	44.15 ± 0.31	21.06 ± 0.15	20.15 ± 0.086	15.07 ± 0.17
6	59.21 ± 0.25	29.45 ± 0.19	27.05 ± 0.34	19.52 ± 0.18
8	72.23 ± 0.27	35.15 ± 0.12	33.79 ± 0.16	24.59 ± 0.25
10	84.05 ± 0.31	44.03 ± 0.10	41.25 ± 0.18	31.04 ± 0.19
12	84.12 ± 0.22	57.80 ± 0.17	46.09 ± 0.27	38.45 ± 0.23
24	84.18 ± 0.25	81.23 ± 0.22	79.28 ± 0.12	74.76 ± 0.31

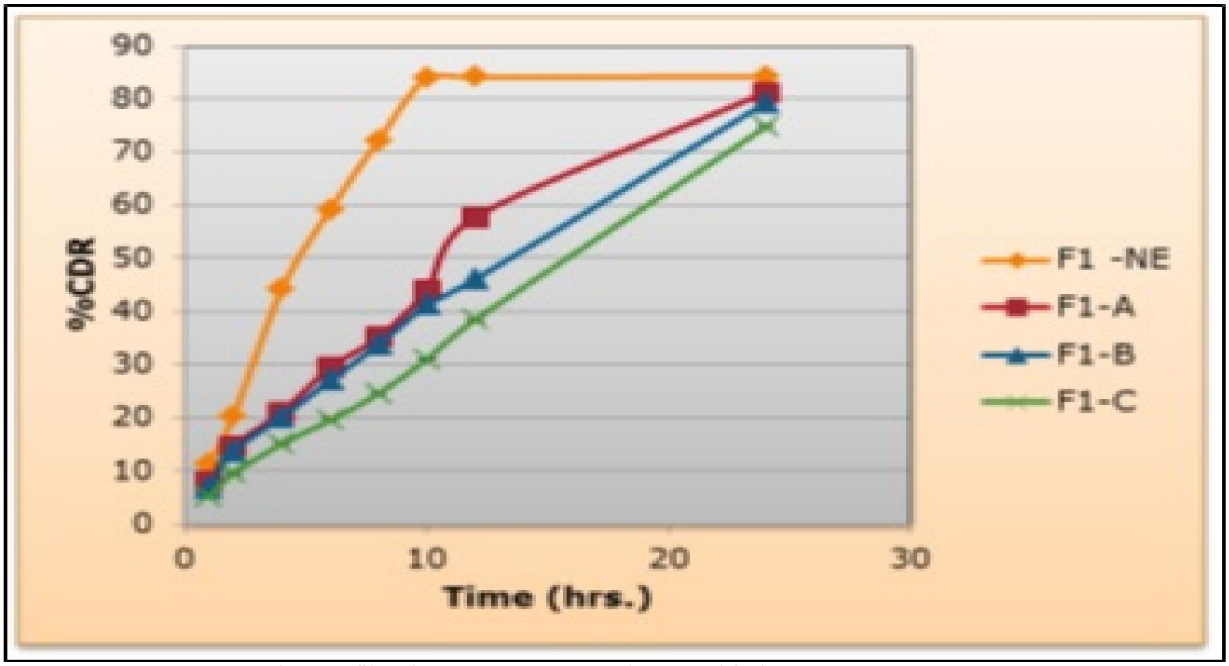

Figure 5: *Ex-vivo* permeation profile of F1, F1-A, F1-B, and F1-C with time.

3.8.3 Skin irritation studies

Images of rat's skin after application of nanoemulgel and standard irritant were taken to analyze any significant skin irritation. The resultant images (I) Control (without any application), (II) standard irritant (Formalin) and (III) nanoemulgel (F1-A) are as shown in figure 6a, 6b and 6c. Skin irritation test was carried out accordance with Draize's scoring criteria to calculate Primary Irritation Index (PII). Averages results are as shown in the Table 13. According to the score chart nanoemulgel showed PII in between 0.04 - 0.99 after 72 h. This showed that the formulation did not produce any skin irritation up

to 48 h. After 72 h, irritation occur but very less as compared to standard irritant which showed PII between 0.99 - 1.99, and responsible for producing "slight irritation".

Table 13: Average responses scores of skin irritation

Groups	Primary Irritation Index (PII)		
	Time (h)		
	24	48	72
Group I: Negative Control (no application)	0.00	0.00	0.00
Group II: Positive Control (Standard irritant)	0.99	1.33	1.89
Group III: Optimized formulation (Nanoemulgel)	0.00	0.0	0.04

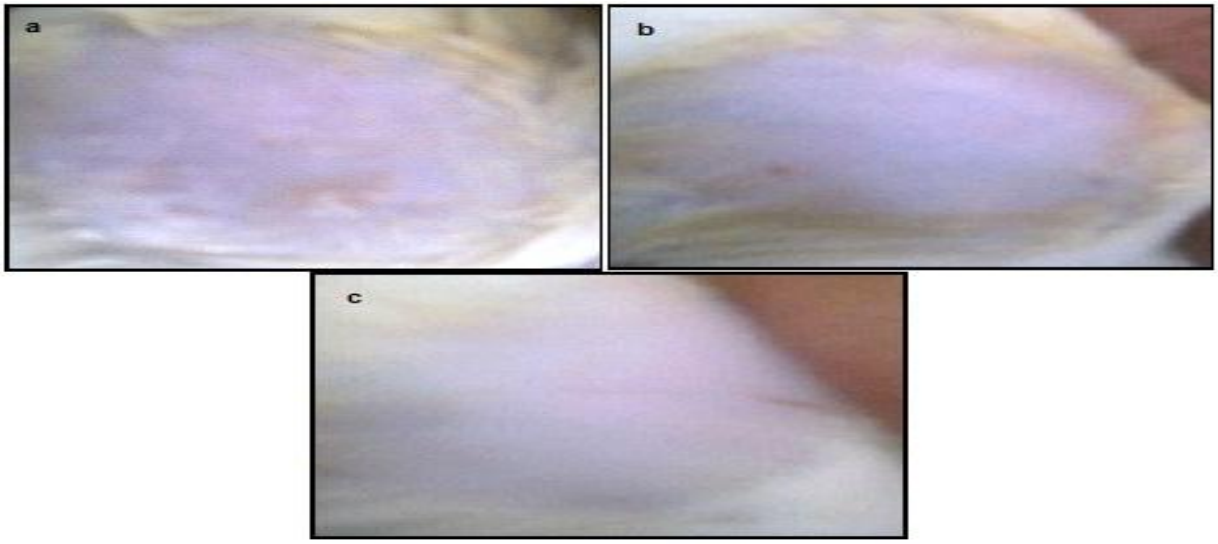

Figure 6: Skin irritation studies **a)** control, **b)** standard irritant and **c)**Nanoemulgel F1-A

3.8.4 Antifungal Activity

The aim of the proposed work was to formulate an anti-fungal nanoemulgel of Bifonazole to provide local targeted action against fungal infections. In vitro antifungal activity of all nanoemulgel F1-A, F1-B, and F1-C was carried out and compared with that of already established marketed cream to know the comparison of both the formulation in respect of their activity against fungus strain*Candida albicans*. The antifungal activity of the nanoemulgel was quite effective for inhibition of fungi with desired concentration. The formulated nanoemulgels as compared to marketed cream gave almost similar efficacy represented as "zone of inhibition" as shown in the Figure 7. The mean of zone of inhibition of A5-A, A5-B and A5-C are found to be 1.45, 1.39 and 1.35 cm respectively as compared to marketed cream 1.5 cm after 24 hr. as shown in the Table 14. It was found that F1-C showed least zone of inhibition which could be due to slow release of drug due to presence of more amount of polymer.

Table 14: Zone Of Inhibition of NEGs and marketed cream

Time (hrs.)	Zone of inhibition (cm)			
	Marketed Cream	Nanoemulgel Formulations		
		F1-A	F1-B	F1-C
24	1.5	1.4	1.3	1.3

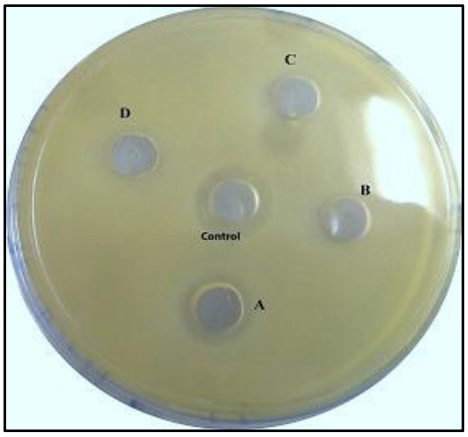

Figure 7: Zone of inhibition.
Control = Marketed cream; A= F1-A,
B = F1-B and C = F1-C

3.9 Stability studies as per ICH guidelines

Formulation has to be remained stable for sufficient period of time. Accelerated stability studies have to be carried out according to ICH guidelines to ensure stability of the optimized formulation at even adverse, variable conditions. Results of stability study of optimized nanoemulgel F1-A formulation for parameters like clog formation, phase separation, viscosity, drug content and pH after keeping at 5°C, room temperature and 45°C for 3 months are tabulated in Table 15. There was no significant change in the viscosity, drug content and pH found during the stability study. It shows that the formulation passed all the tests and efficiently stable up to 3 months at variable temperature conditions. Shelf life was calculated on the stability data as shown in Figure 8. It was found to be 1233 days or 3.3 years approximately.

Table 15: Stability studies of optimized nanoemulgel F1-A

Parameters	Storage conditions											
	5^{o}C/ 75% RH ± 5%				25^{o}C /75% RH± 5%				45^{o}C/75% RH ± 5%			
	Months											
	0	1	2	3	0	1	2	3	0	1	2	3
Clog	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Phase separation	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
pH	6.3	6.3	6.3	6.3	6.4	6.4	6.1	6.2	6.5	6.4	6.4	6.3
Consistency	G	G	G	G	G	G	G	G	G	G	G	G
Viscosity	4726	4727	4727	4728	4726	4726	4726	4726	4726	4726	4725	4725
% Drug content	94.19	94.19	94.19	94.18	94.19	94.19	94.19	94.19	94.19	93.99	93.90	93.90

G = Good

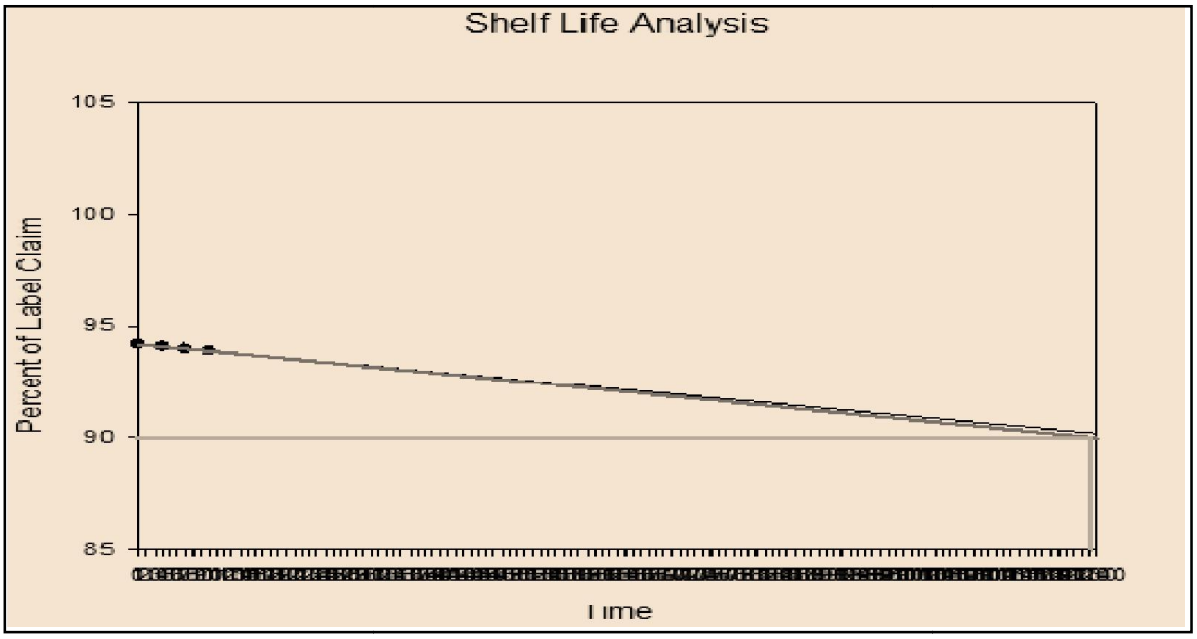

Figure 8: Shelf life of F1-A.

Conclusion

Aim of the present study was to formulate anti-fungal Nanoemulgel (NEG) of Bifonazole for topical delivery to provide efficient local targeted action with better permeation and sustained effect in spite of its low permeability and aqueous solubility, which was achieved. Nanoemulsionprovide higher loading of this hydrophobic drug in an aqueous environment, higher permeation and thermodynamic stability. Conversion of this nanoemulsion formulation into gel provided sustained effect with increase in the retention capacity. No selective permeation enhancers were being used during the formulation providing an advantage over the other formulation approaches which utilizes various permeation enhancers to increase the traversing of drug across the skin. Formulation, being nano-sized provides efficient larger interfacial area to penetrate into the skin tissues in higher amount and less greasy, easy to spread than other topical forms such as ointments, creams etc. Hence it is concluded that Nanoemulgel; a multi-purpose technology can be exploited in delivery of poorly soluble and even poorly permeable drugs efficiently.

References

[1] Ameen. M. Epidemiology of Superficial Fungal Infections. Clinical Dermatology. 2010; 28(2): 197- 201.
[2] Havlickova B, Friedrich M. Epidemiological trends in skin mycoses worldwide. Mycoses. 2008; 51(4): 2-15.
[3] ZhangA Y, CampW L, ElewskiB E. Advances in Topical and Systemic Antifungals. Clinical Dermatology. 2007; 25(2): 165-183.
[4] Gungur S, Erdal M S, Aksu B. New formulation strategies in Topical antifungal therapy. 2013; 3: 56-65.
[5] Lee M, Maibach H I. Deep percutaneous penetration into muscles and joints. Journal of Pharmaceutical Science. 2006; 95(7): 1405-1412.
[6] Jain N K. Controlled and novel drug delivery system. 1st edition. New Delhi: CBS Publications; 1997. P. 100-128.

[7] Shah P, Bhalodia D, Shela P T. Nanoemulsion: A Pharmaceutical Review. Systemic Reviews in Pharmacy. 2010; 1(1): 24-32.
[8] Mason T G, Wilking J N, MelesonK,Chang C B, Grant S M. Nanoemulsion: formation, structure, physical properties. Journal of Physics: Condensed Matter. 2006; 18.
[9] Solans C, Izquierdo P, Nolla J, Zemar N A, Garcia M J. Nanoemulsions. Current Opinion in Colloids and Interface Sciences. 2005; 10: 102-110.
[10] Naguib G, Halidy E L, Magdi H K, Mohamed I, Farid M. Microemulsion as vehicles for topical administration of voriconazole: formulation and *in vitro* evaluation. Drug Development & Industrial Pharmacy. 2012; 38(1): 64-72.
[11] Campos F F, Naveros B C, Serrano O L, Merino C A, Campmany A C. Evaluation of novel Nystatin nanoemulsion for skin candidiasis infections. Mycoses. 2013; 56(1): 70-81.
[12] Mohamed M I. Optimization of ChlorphenesinEmulgel Formulation. AAPS Pharmaceutical Sciences and Technologies. 2004; 6(3).
[13] BaryAA EL, Shalaby S, AalA S. Formulation and Stability of Chloramphenicol gel and Emulgel. Bull facPharma. 2001; 39: 89-99.
[14] Zhang X L, Zhao R, Qian W. Preparation of an Emulgel for treatment of aphthous ulcer on the basis of Carbomers. Chin Pharm Journal. 1995; 30: 417-418.
[15] Ansel H C, Allen N G P, Loyd V. Pharmaceutical Dosage Forms and Drug Delivery System. 8th edition. B I Publications Pvt. Ltd.; 2005.
[16] Baibhav J, Gurpreet S, Rana A C, Seema S, Singla V. Emulgel: A Comprehensive Review on the recent advances in topical drug delivery. International Research Journal of Pharmacy. 2011; 2(11): 66-70.
[17] Khullar R, Saini S, Seth N, Rana A C. Emulgels: A surrogate approach for topically used hydrophobic drugs. International Journal of Pharmacy and Biological sciences. 2011; 1(3): 117-128.
[18] Carrilo AJ, Munoz, Tur C, Torres J. *In-vitro* Antifungal activity of Bifonazole, Miconazole, Sertoconazole against yeast of Candida genus. Journal of Anti-microbial Chemotherapy. 1996; 37: 815-819.
[19] Shakeel F. Criteria for Excipients Screening in the development of Nanoemulsion formulation of three Anti-inflammatory drugs. Pharmaceutical Development and Technology. 2010; 15(2): 131-138.
[20] Sheikh S, Faiyaz S, Sushma T, Farhan J A. Development and Bioavailability Assessment of Ramipril Nanoemulsion Formulation. European Journal of Pharmaceutics and Biopaharmaceutics. 2007; (66): 227-243.
[21] Modi J D, Patel J K. Nanoemulsion based gel formulation of Acelofenac for topical delivery. International Journal of Pharmacy and Pharmaceutical Sciences and Research. 2011; 1(1): 6-12.
[22] Genno M, Yamamoto R, Kojma H, Konishi H, Klausner M. Evaluation of new alternative to Primary Draize Skin Irritation testing using Epderm skin model. Alternative Animal Test Experiments. 1998; (5).
[23] Hugo W B, Russell A D. Pharmaceutical Microbiology. Oxford, UK: Blackwell Scientific Publication; 1977:190.
[24] ICH Guidelines.
[25] Banker G S, Rhodes C T. Modern Pharmaceutics. 2nd edition. New York: Marcel Dekker Inc.; Madison Avenue; 1990.
[26] Morrow D I J, Caron P A MC, Woolfson A D, Doonley R F. Innovative strategies for enhancing Topical and Transdermal drug delivery. The open Drug Delivery Journal. 2007; 1: 36-59.

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