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ORIGINAL ARTICLE

Nanostructured lipid carrier system for topical delivery of terbinafine hydrochloride

Bharti Gaba, Mohammad Fazil, Saba Khan, Asgar Ali, Sanjula Baboota, Javed Ali *

Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, New Delhi 110062, India

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Abstract The main aim of the present study was to develop and evaluate Terbinafine HCl (TH)-loaded nanostructured lipid carrier (NLC) for the treatment of fungal infection via topical administration. Fungal infections are tremendously widespread and the treatments are effective but associated toxicities restrict their use. TH-NLC was prepared using high pressure homogenization technique using Glyceryl Monostearate (GMS) as solid lipid, Labrasol as liquid lipid and Pluronic F-127 as surfactant, binary lipid phase was selected in the ratio 6:4 w/w (solid:liquid lipid ratio). The mean diameter of optimized TH-NLCs was found to be 128 ± 4.5 nm. Spherical shape and size were confirmed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses. The *in vitro* release studies showed 92.60 ± 0.87% drug release over 24 h as compared to the marketed formulation which showed only 82.826 ± 0.29%. *Ex vivo* skin permeation study showed about 86.35% permeation however from the marketed formulation it showed 69.41%. The pharmacodynamic studies indicated that TH-NLC (771 ± 41.797 CFUs) gel efficiently reduced the fungal burden in shorter duration of time as compared to marketed formulation (1558 ± 140.524 CFUs) and dispersion (95,582 ± 2316.619 CFUs) (p value > 0.001). Therefore, it can be concluded that the developed NLCs showed a sustained release pattern and reduction of fungal burden in the infected area. Hence, TH-NLC could be a potential alternative for treatment of topical fungal infection after clinical evaluation in near future.

1. Introduction

In the current scenario, fungal infections are tremendously widespread. Over billions of people are diagnosed each year with either topical or severe systemic fungal infections.[1] Although, antifungal drugs are effective in treating fungal infections but they are associated with severe toxicities like liver damage or they may affect estrogen levels or may cause

* Corresponding author at: Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi 110 062, India. Tel.: +91 9811312247; fax: +91 11 2605 9663.
E-mail addresses: javedaali@yahoo.com, jali@jamiahamdard.ac.in (J. Ali).
URLs: http://www.scopus.com/authid/detail.url?authorId=25641028400, http://orcid.org/0000-0001-5308-0655 (J. Ali).

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allergic reactions. For example, the antifungals with azole groups are known to have caused anaphylaxis. Terbinafine Hydrochloride (TH) is a potent antifungal agent of the allylamine class which selectively inhibits fungal squalene epoxidase. (Fig. 1) It has a broad-spectrum activity against yeast, fungi, molds, and dermatophytes and is indicated for both oral and topical treatments of mycoses. It is typically administered orally (250 or 500 mg per day) or topically (1% cream, applied twice daily).[2–4]

TH has very poor water solubility and is highly lipophilic (log P 3.3) in nature. The main advantage of this drug is that it can be used as a topical drug delivery system. The benefits of topical administration of TH include direct delivery and targetability to the affected area of the skin, low dose requirement and minimized drug related toxicities.[5]

Lipid based drug delivery systems are nowadays popular as they are expected to be the promising carriers because of their potential to increase solubility and improve bioavailability of poorly water soluble and/or lipophilic drugs.[6] The introduction of advanced generation of lipid nanoparticles named as NLCs has overcome the general limitations associated with conventional lipid based formulations and solid lipid nanoparticles (SLNs).[7,8] Earlier, SLNs attracted lot of attention as a drug delivery system[9] for they offer the advantages of biocompatibility, drug targeting, modified release and ease of large scale production.[10] However, depending on the drug, various potential problems can occur, such as drug leaking during storage and insufficient drug loading. Later on, NLCs are designed by mixing the solid lipid with the liquid lipid, which leads to special nanostructures with improved properties for therapeutic loading, alteration of the drug release profile and stability.[11,12] The major advantage of this type of carrier/delivery system is its ability to incorporate large quantities of drugs as a result of formation of a less ordered lipid matrix with many imperfections.[12–16] NLC ensures close contact to the stratum corneum owing to its unique lipid composition and smaller particle size, thereby enhancing drug flux through the skin. Also, because of solidified lipid matrix, a controlled release of the therapeutic moiety from these carriers is possible.[17,18] NLCs for the reason that they formed a less ordered lipid matrix with many imperfections, have the ability to incorporate large quantities of drugs.[19] They are also found to significantly increase skin hydration and exhibit occlusive properties due to reduction in the transepidermal water loss.[20] The primary site of action of Terbinafine is the stratum corneum in fungal infections residing superficially on the skin layers.[21,22] It possesses minimum inhibitory concentrations (MIC) of 0.001–0.01 μg/ml and low minimal fungicidal concentrations of 0.003–0.006 μg/ml. The drug should be present in a concentration above its MIC at the site of action for effective therapeutic action.[23] In this context, topical NLC may prove to be a potential option for increasing the concentration of drug by controlled targeting up to deeper skin layers.

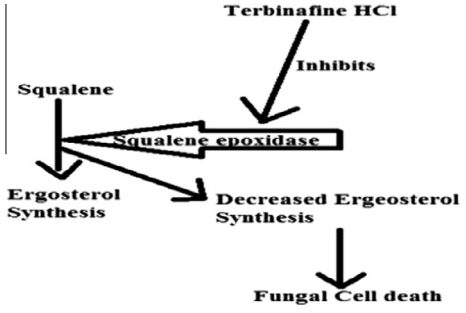

Figure 1 Mechanism of action of Terbinafine Hydrochloride.

SLNs involve the incorporation of the solid lipid whereas NLCs involve the drug entrapment into the solid and liquid lipid mix which may contribute to make it a sustained release formulation and thereby overcoming the limitation of SLNs. Topical NLC formulation aims to reach the target site with required concentration to achieve its therapeutic action with minimal adverse effects[24,25] however stability issues still remained the point of concern. Several attempts have been made to enhance the bioavailability of the drug via topical route. SLN formulations were developed prior to effectively reducing the fungal burden. SLNs produced showed a significant effectiveness when compared to the marketed formulations[26,27] Vaghasiya and coworkers prepared Terbinafine HCl (TH) loaded SLNs using Compritol 888 ATO as lipid matrix, Pluronic F-127 as stabilizer and distilled water as dispersion medium using solvent injection technique.[27] Also, Chen and coworkers prepared the SLN formulation by incorporating drug in the combination of GMS and Compritol® 888.[28] Although above stated study findings highlighted the advantages of SLN, stability and poor drug loading remained to be a serious issue of consideration. Therefore NLCs, the newer generation of SLNs were chosen to overcome these limitations and provide better therapeutic prospects. As a result, the objective of the present study was to prepare and optimize TH loaded NLC for topical administration and to assess the developed formulation for *in vitro* release, *ex vivo* permeation and *in vivo* (pharmacodynamics) studies which may be found to be more effective than the SLNs developed before.

2. Materials and methods

2.1. Materials and components

TH was received as a gift sample from Dr. Reddy's Laboratory (Andhra Pradesh, India). Precirol ATO 5, Compritol 888 ATO and Labrasol were obtained from Gattefosse India Pvt. Ltd. (Mumbai, India), Glyceryl Monostearate was obtained from CDH, Pvt. Ltd, (Delhi, India), Tween 20 and Tween 80 were from SDFCL, (Mumbai, India), Castor oil was obtained from Thomas Baker Pvt. Ltd. (Mumbai, India), Oleic acid, Span 80, HPLC Methanol, HPLC Water and Glacial Acetic Acid were obtained from S.D. Fine Chemicals, Ltd. (Mumbai, India), Pluronic F 188 and Pluronic F 127 were from BASF (Ludwigshafen, Germany).

2.2. Experimental design of nanostructured lipid carrier (NLC)

In the present study, Box–Behnken statistical design with 3 factors, 3 levels, and 17 runs was employed for the optimization study using Design-Expert software (Design-Expert 8.0.0.6, State-Ease Inc., Minneapolis, USA). Percent lipid concentration (A), percent surfactant concentration (B) and number of cycles of high pressure homogenizer (HPH) (C) were selected as independent variables and they were set at high-, medium- and low levels on the basis of the results of initial trials. Table 1 summarizes the coded values of different

Table 1 Variables and their constraints in Box–Behnken design.

Variables	Constraints	
	Lower limit	Upper limit
Independent variables		
A = Quantity of lipid concentration (%)	1	2
B = Quantity of surfactant concentration (%)	0.5	1.5
C = Numbers of HPH cycles	4	6
Dependent variables	*Goals*	
R1 = Particle size (nm)	Minimize	
R2 = Entrapment efficiency (%)	Maximize	
R3 = Drug loading (%)	Maximize	

variables. In accordance with the design, 17 NLC formulations were prepared and characterized for particle size (R1), entrapment efficiency (R2) and drug loading (R3) which were chosen as response parameters Table 1. This design explicates the main effects, interaction effects and quadratic effects of the independent variables on the formulation characteristics.

2.3. Screening of components

As such there is no standard method set for the selection of the lipids; however the procedure and results are in accordance with the previous research articles.[29,30]

2.3.1. Screening of liquid lipids (oils) and surfactants

The solubility of TH in various liquid lipids (Castor oil, Oleic acid, Labrasol, Isopropyl myristate, Cremophore EL) and surfactants (Tween 20, Tween 80, Span 80, Pluronic F 188 and Pluronic F 127) was determined by adding excess amounts of drug in 3 ml of oils in small vials. The vials were tightly stoppered and were continuously stirred to reach equilibrium for 72 h at 25 °C in a mechanical shaker. After that, the mixtures were centrifuged using High Speed Centrifuge (3K30, SIGMA, Germany) at 5000 rpm for 30 min at 37 °C.[31] The supernatant was separated, dissolved in methanol and solubility was quantified by UV Spectrophotometer at 223 nm. The solubility studies were done in triplicate and results reported as ±SD.

2.3.2. Screening of solid lipids

The solubility determination of TH in various solid lipids (Precirol ATO 5, Compritol 888 ATO, Glyceryl Monostearate, Gelucire) was performed by adding TH in increments of 1 mg until it failed to dissolve further in the molten solid lipids (which were heated at 5 °C above their melting point). The amount of solid lipids required to solubilize TH was calculated. The experiment was conducted in triplicate.[32]

2.4. Selection of a binary lipid phase

The solid and liquid lipid with the best-solubilizing potential for TH were mixed in different ratios viz., 95:5, 90:10, 85:15, 80:20, 70:30, and 60:40 in order to establish the miscibility of the two lipids. Lipid mixtures were agitated at 200 rpm for 1 h at 85 °C using a magnetic stirrer (Remi instruments Ltd., Mumbai, India). The miscibility between the two components was investigated by smearing a cooled sample of the solid mixture onto a filter paper, followed by visual observation to determine the presence of any liquid oil droplets on the filter paper. A binary mixture exhibiting a melting point above 40 °C which did not reveal the presence of oil droplets on the filter paper was selected for the development of TH – loaded NLCs.

2.5. Preparation of NLC

The preparation of aqueous NLC was carried out according to the method reported by Muller and co-workers.[9] Accordingly, the weighed amount of drug was added to the lipid phase which was heated at 10–15 °C above the melting point of solid lipid and simultaneously, aqueous surfactant solution was heated at the same temperature (85 °C). Then the lipid mixture was poured in the hot aqueous surfactant solution using a magnetic stirrer (Remi instruments Ltd., Mumbai, India) at 12,000 rpm for 30 min, to prepare the primary emulsion. This primary emulsion was converted to the NLC system using high pressure homogenizer (Stansted Fluid Power Ltd., Harlow, UK) at 15000 PSI. The obtained NLC dispersion was cooled down to room temperature. The NLC dispersion was lyophilized for long term stability. Mannitol (5% w/v) was added as cryoprotectant. The samples were frozen at -78 °C for 10 h followed by lyophilization for 36 h. The lyophilized formulation was reconstituted with phosphate buffer pH 6.8 as per the requirements for later experiments.

2.6. Optimization and characterization NLC

2.6.1. Particle size and particle size distribution

The particle size analysis of NLC formulations was done by photon correlation spectroscopy (PCS) with a Zetasizer (Malvern Instruments, Worcestershire, UK). The PCS provides the mean particle size (z-average) and the polydispersity index (PDI) as a measure of the width of the distribution. The analysis was performed after dilution with double distilled water followed by filtration through Whatman filter paper.

2.6.2. Entrapment efficiency (EE) and drug-loading capacity (DL)

For EE and DL, the drug-loaded NLC dispersion was uniformly mixed by gentle shaking. 1.0 ml of this dispersion was diluted with 9.0 ml methanol, centrifuged using High-Speed Refrigerated Centrifuge (3K30, SIGMA, Germany) for 45 min at 15,000 rpm and then filtered using Millipore® membrane (0.2 µm). The analytical method employed was as per the method reported by Baboota and coworkers.[33] Accordingly, UV absorption spectra of stock solution (10 µg/ml) were scanned for absorbance in the region of 400–200 nm at 223 nm. Serial dilutions of standard solutions were prepared and absorbance was recorded at 223 nm. The calibration curve was prepared and the method was validated. The filtrate was collected and appropriately diluted with methanol and measured spectrophotometrically (Shimadzu, model UV-1601, Kyoto, Japan) at λ_{max} of 223 nm. The percent entrapment efficiency (EE%) was calculated using the following equation.[34]

$$EE\% = \frac{W(\text{Total}) \times W(\text{Free})}{W(\text{Total})} \times 100$$

$$DL\% = \frac{W(\text{Total}) \times W(\text{Free})}{W(\text{Lipid})} \times 100$$

W_{total}, W_{free} and W_{lipids} are the weight of drug added in system, analyzed weight of drug in supernatant and weight of lipid added in system, respectively.

2.7. Evaluation of NLC

2.7.1. Transmission electron microscopy (TEM)

The Particle Size (PS) was determined for the NLCs using TEM (TECNAI-G2, 200 kV, HR-TEM, FEI, The Netherlands). A drop of NLC was placed on a paraffin sheet and carbon coated grid was put on sample and left for 1 min to allow NLC to adhere on the carbon substrate. The remaining NLC was removed by adsorbing the drop with the corner of a piece of filter paper. Then the grid was placed on the drop of phosphotungstate (1%) for 10 s. The remaining solution was removed by absorbing the liquid with a piece of filter paper and samples were air dried and examined by TEM.

2.7.2. Scanning electron microscopy (SEM)

The shape and surface characteristics of NLCs were determined by SEM using gold sputter technique (ZEISS EV40, Carl Zeiss NTS, North America). Samples of NLC were dusted onto a double-sided tape on an aluminum stub. The stubs containing the sample were coated with gold using a cool sputter coater (Polaran E 5100) to a thickness of 400 A^0. Photomicrographs were taken at the accelerated voltage of 20 kV and chamber pressure of 0.6 mmHg.

2.7.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) and XRD were employed to identify the crystal form of TH dispersed in the lipid matrix. Thermograms were recorded by means of DSC for the identification of crystallinity. For calorimetric measurements, standard aluminum pans with accurately weighed 1–2 mg samples were tightly sealed. Samples were heated at a scanning rate of 10 °C/min over a temperature range between 40 °C and 400 °C. An empty pan was used as reference. An inert atmosphere was maintained by purging with nitrogen.

2.7.4. X-ray diffraction study (XRD)

XRD pattern of drug, and freeze dried NLC were obtained using X-ray diffractometer (Bruker D 8 ADVANCE, Bruker, USA) and Cu as a radiation source. The range of scan used was 0–50° of the diffraction angle 2θ. XRD pattern was measured with a voltage of 40 kV and a current of 30 mA. Equivalent amounts of samples were used in the XRD study.

2.8. Preparation of TH NLC gels

The gels were prepared by dispersing 1% w/w Carbopol® 940 in the selected NLC formulations and subsequently neutralizing the Carbopol® dispersion using triethanolamine (TEA). The final concentrations of TH in the NLC gels were maintained at 0.5, 1 and 1.5% w/w and were coded as NLC1, NLC2 and NLC3 respectively.

2.9. Characterization of TH NLC gels

2.9.1. Viscosity

The viscosity of the different NLC samples was obtained by Brookfield viscometer (Brookfield engineering laboratories, Inc., MA, USA) with spindle No. 6 at 10 rpm at temperature of 37 ± 0.5 °C.

2.9.2. Measurement of pH

100 mg of the gel formulation was weighed in a 50 mL volumetric flask and then volume was made up with distilled water to 50 mL (0.2% strength). The pH of the dispersion was measured using pH meter (pH Tutor Bench Meter, EUTECH Instruments, Singapore).

2.9.3. Spreadability

0.5 g of gel was placed within a circle of 1 cm diameter pre-marked on a glass plate, over which second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate for 5 min. The increase in the diameter due to gel spreading was noted.

2.9.4. Extrudability

The test involves measuring the quantity of gel extruded from collapsible tube on application of constant weight. A closed collapsible tube containing 20 g of gel was pressed by applying a constant load of 1 kg at the crimped end. When the cap was removed, gel extruded until pressure dissipated. The extruded gel was collected and weighed.

2.10. In vitro drug release studies

In vitro drug release studies of NLC were performed using dialysis bag technique. The activation of dialysis membrane was carried out. The experiments were carried out under sink conditions. 10 mg of each formulation i.e., TH dispersion, NLC, marketed formulation was loaded into a cellulose membrane dialysis bag (molecular weight cut-off 12,400, Sigma–Aldrich Co., St. Louis, MO, USA), immersed in 200 mL of pH 7.4 phosphate buffer containing 0.8% tween 80 solution magnetically stirred at 32 °C at pH 7.4. Samples were taken at predetermined intervals from the receiver solution, replaced with equal volumes of fresh solvent, and spectrometrically assayed for drug concentration at λ_{max} 223 nm. The correction for the cumulative dilution was calculated. The release studies were performed in triplicate.[35,36]

2.11. Ex vivo permeation studies

Ex vivo study was carried out using full thickness rat abdominal skin. In this work due to easy availability, the skin of albino rat was used. The species used was Wistar Albino Rats of 18–25 weeks and weight of 150–200 g of either sex. The abdominal skin was removed and dipped into phosphate buffer saline (PBS) pH 7.4. Hairs were removed from the skin by hair removal cream. The subcutaneous fat was removed with a scalpel. The skin was mounted on the Franz diffusion cell and the receptor chamber was filled with 20 ml diffusion medium. The diffusion medium consisted of PBS pH 7.4 containing 0.8% v/v of Tween-80. The skin was positioned

on the receptor chamber with the stratum corneum facing upward in the receptor chamber and then the donor chamber was clamped in place. The excess skin was trimmed off and the whole assembly was put on a magnetic stirrer to continuously stir the medium present in the receptor compartment. The diffusion cell was placed in the diffusion apparatus to stabilize at 32 °C. The test formulations (NLCs based gel and commercial formulation i.e., 1% cream) equivalent to 20 mg drug were applied to the skin. Samples were withdrawn from the receptor compartment at predetermined time intervals, and immediately replaced with fresh diffusion medium. The studies were performed for 12 h according to the clinical application time and samples were analyzed spectrophotometrically at λ_{max} 223 nm. Drug permeation, Flux (μg/cm^2/h) and permeability coefficient (Pb) [cm^{-2}h^{-1}] studies were calculated using the formulas mentioned.[33,37]

$$\text{Cumulative amount of drug permeated} = \frac{\text{concentration}\left(\frac{\mu g}{ml}\right) \times \text{dilution factor}}{\text{Area of permeation cell}} \times \text{volume of receiver chamber}$$

$$\text{Flux} = \frac{\text{cumulative amount of drug permeated}}{\text{time(hrs)}}$$

$$P_b = \frac{\text{Flux}}{\text{drug concentration in donor cell}}$$

2.12. In vivo pharmacodynamic studies

The pathogen free 18-–25 week old male albino rats weighing 180–200 g were used for the experiment. All animal experiments were approved by IAEC (Jamia Hamdard, New Delhi, India). Animals received a normal, pathogen free diet and water. Animals were divided into four groups and each group consisted of three animals. Group 1 was control (untreated rats), groups 2, 3 and 4 consisted of rats induced with fungal infection and treated with TH loaded NLCs based Carbopol® 940 gel, commercial TH cream (commercial formulation i.e., 1% cream) and TH dispersion in water, respectively. All the materials used were sterilized by autoclaving at 121 °C and 15 psi for 30 min.

2.12.1. Test organism and preparation of fungal inoculum

Culture of *Candida albicans* ATCC 10231 was obtained from Microbiology Department, HIMSR, Jamia Hamdard, New Delhi, India. This strain was stored on Sabouraud dextrose agar (SDA) slant during the study and subculture was used to induce infections. The parent culture of *C. albicans* was subcultured on 3% Sabouraud dextrose broth and allowed to grow for 2 days at 27 °C. The final conidial suspension of *C. albicans* was prepared in sterile saline containing 0.05% w/v Tween-80. The conidial suspension was adjusted to the density of 1×10^6 CFU/ml by counting under hemocytometer and it was used as innoculum.[38]

2.12.2. Induction of cutaneous candidiasis infection

Hairs were removed by hair removal cream from an area (2 cm^2) on the back of the albino rats to make a hairless square. On the following day, the skin was slightly abraded with sandpaper. The naked skin of the rat was disinfected with ethyl alcohol and sterile cotton impregnated with 0.1 ml of conidial suspension was fixed to the animal skin by adhesive tape and kept for 3 days.[39] The skin tissue of the infected site was excised using sterile scalpel at various time intervals, implanted onto 3% sabouraud dextrose broth (containing 4% agar powder) plate and incubated at 27 °C for 2 days.[40] Suitable dilution was done to achieve countable colony forming unit (CFU) growth. CFU was counted using a digital colony counter based on countable CFU values. This was continued till induction of fungal infection was confirmed based on the number of CFUs.

2.12.3. Treatment regimen

TH loaded NLC based Carbopol® gel, TH dispersion and commercial formulation i.e., 1% cream (equivalent to 2 mg drug on daily basis) were applied topically and the results in terms of reduction in fungal burden were compared. Treatment was started after confirmation of induction of fungal infection by counting the number of CFUs. After initiation of treatment, quantitative analysis of fungal burden was performed by the above mentioned procedure.

3. Results and discussion

3.1. Screening of components

The criteria for selection of excipients for developing TH-NLC include pharmaceutical acceptability, nonirritant and non sensitizing to the skin and that they fall under GRAS (generally regarded as safe) category. Solubility of various solid lipids, liquid lipids and surfactants in TH is given in Fig. 2 and Table 4. As per the results of solubility studies, TH exhibited maximum solubility in Glyceryl Monostearate (GMS) (69.8 ± 1.57 mg/ml), Labrasol (124 ± 0.35 mg/ml) and Pluronic F-127 (92.1 ± 2.09 mg/ml). Therefore, TH-NLC was prepared using GMS as solid lipid, Labrasol as liquid lipid and Pluronic F-127 as surfactant. Based on the visual observation of smear test, binary lipid phase was selected in the ratio 6:4 w/w (solid: liquid lipid ratio) for designing NLC.

3.2. Optimization and characterization of NLC

On the basis of defined constraints for each independent variable, the Design Expert software® automatically generated the optimized formula, Table 2. The experimental values were obtained by preparing 17 batches of drug loaded NLCs and simultaneously evaluating them for response parameters (PS, EE and DL) according to the optimized formula generated by the software. Calculation of percentage prediction was done to determine the accuracy of prediction by the software and the utility of the experimental design for modifying the NLCs with desirable parameters.

When the experimental values of responses for 17 runs were fitted to different models of Box Behnkehn design, it was observed that the best-fitted model for all of the three dependent variables was the quadratic model with coefficient of correlation (R^2) nearly equal to 1. The summary of results of regression analysis for the three responses is given in Table 3. The "Predicted R^2" was in reasonable agreement with the "Adjusted R^2" values for all response parameters (as shown

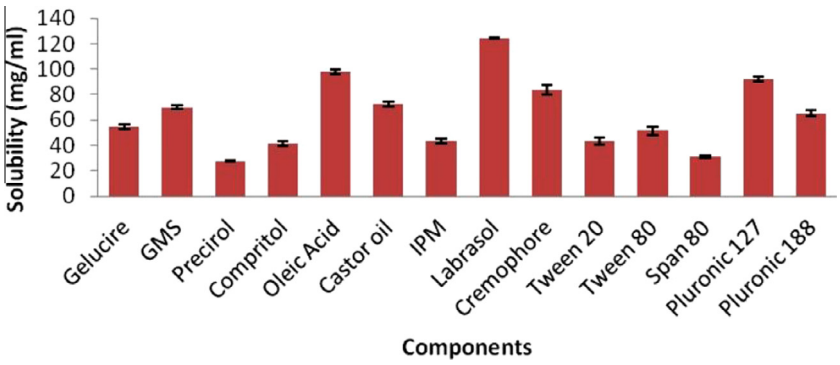

Figure 2 Solubility of various components with ±S.D.

Table 2 The Box–Behnken experimental design matrix for the formulation and optimization of NLCs.

Exp. run	Coded values			Actual values			Dependent factors		
	Lipid conc. (A)	Surfactant conc. (B)	No. of HPH cycles (C)	Lipid conc. (A) (%)	Surfactant conc. (B) (%)	No. of HPH cycles (C)	PS (nm) $n = 3$ (±SD)	EE (%) $n = 3$ (±SD)	DL (%) $n = 3$ (±SD)
1	+1	−1	0	2.00	0.50	5.00	319.62 ± 15.21	74.32 ± 6.53	4.35 ± 2.34
2	0	−1	0	1.50	1.00	5.00	126.01 ± 8.67	84.32 ± 8.21	17.84 ± 1.38
3	−1	0	−1	1.00	1.00	4.00	208.56 ± 12.24	74.21 ± 6.52	7.38 ± 3.86
4	0	+1	+1	1.50	1.50	6.00	198.31 ± 8.92	72.16 ± 4.98	10.77 ± 3.12
5	0	0	+1	1.50	1.50	4.00	310.64 ± 9.53	66.33 ± 6.45	1.98 ± 1.29
6	0	0	0	1.50	1.00	5.00	124.72 ± 7.86	83.93 ± 5.82	11.32 ± 2.96
7	0	0	−1	1.50	0.50	4.00	276.1 ± 12.34	70.45 ± 6.34	5.73 ± 2.37
8	+1	0	0	2.00	1.00	4.00	175.65 ± 12.65	77.84 ± 4.46	5.19 ± 2.14
9	0	−1	0	1.50	1.00	5.00	127.08 ± 6.92	80.42 ± 6.21	11.75 ± 3.12
10	−1	−1	−1	1.00	0.50	5.00	238.65 ± 14.86	73.1 ± 3.45	10.02 ± 2.75
11	−1	+1	0	1.00	1.50	5.00	344.23 ± 11.23	63.25 ± 5.67	3.5 ± 4.23
12	0	+1	+1	1.50	0.50	6.00	282.25 ± 7.52	73.12 ± 6.72	9.56 ± 1.59
13	+1	+1	0	2.00	1.50	5.00	190.42 ± 4.98	72.91 ± 6.53	10.98 ± 2.34
14	0	0	0	1.50	1.00	5.00	129.24 ± 6.54	79.89 ± 8.21	11.21 ± 1.38
15	−1	0	0	1.00	1.00	6.00	154.1 ± 8.43	77.27 ± 6.52	10.86 ± 3.86
16	0	0	0	1.50	1.00	5.00	121.34 ± 6.55	82.16 ± 4.98	12.65 ± 3.12
17	+1	0	0	2.00	1.00	6.00	132.12 ± 11.72	79.02 ± 6.45	11.42 ± 1.29

Table 3 Optimization constraints selected.

Variables	Constraints		
	Lower limit	Upper limit	Goal
Independent variables			
A = Quantity of lipid concentration (%)	1	2	In range
B = Quantity of surfactant concentration (%)	0.5	1.5	In range
C = No. of HPH cycles	4	6	In range
Dependent variables			
R1 = Particle size (nm)	121.34	344.23	Minimize
R2 = Entrapment efficiency (%)	63.25	84.32	Maximize
R3 = Drug loading (%)	1.98	17.84	Maximize

in Table 3). The Model *F*-value implies the significance of the model which was significant for all response parameters (Table 3).

The following polynomial equations were generated by the statistical analysis of the results:

$$PS = +125.68 - 15.97A - 9.13B - 25.52C - 58.70AB + 2.73AC - 29.62BC + 24.17A^2 + 123.38B^2 + 17.76C^2.$$

$$EE = +82.14 + 2.03A - 2.04B + 1.59C + 2.11AB - 0.47AC + 0.79BC - 2.34A^2 - 8.91B^2 - 2.72C^2.$$

$$DL = +12.95 + 0.023A - 0.30B + 2.79C + 3.29AB + 0.69AC + 1.24BC - 2.02A^2 - 3.72B^2 - 2.22C^2.$$

where A, B and C represent the coded values of the percent lipid concentration, percent surfactant concentration and number of HPH cycles respectively.

Only statistically significant ($p < 0.05$) coefficients are included in the equations. A positive value in polynomial

Table 4 Summary of results of regression analysis for responses.

Response	Model	R^2	Adjusted R^2	Predicted R^2	F-value	SD (±)	% CV	Mean
PS	2F1	0.9815	0.9704	0.9099	88.28	4.44	3.57	124.52
DL	Quadratic	0.9822	0.9593	0.7209	42.87	1.02	11.74	8.65
EE	Quadratic	0.9805	0.9553	0.7513	39.03	1.42	1.72	80.58

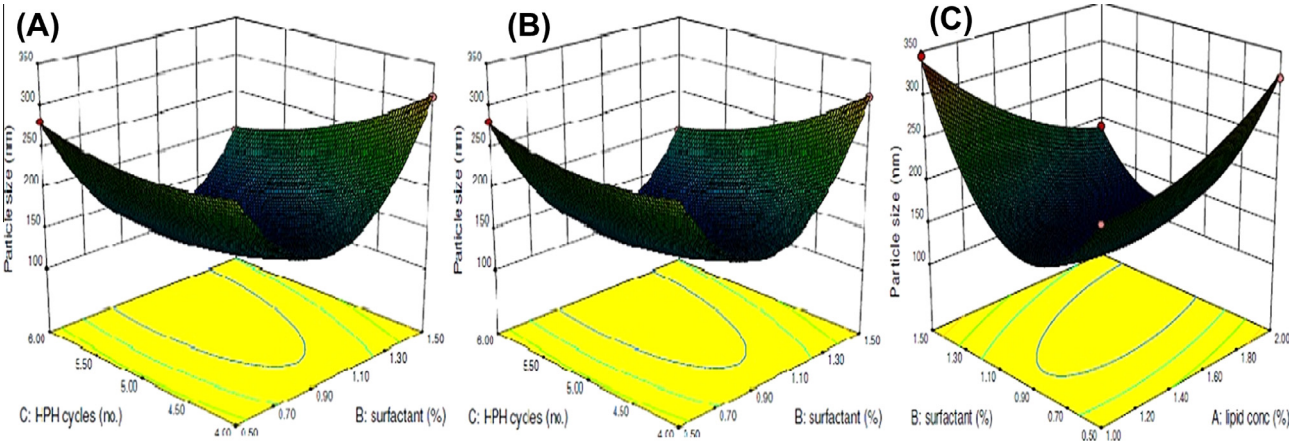

Figure 3 (A) Response surface plot of factor C vs. A against PS; (B) response surface plot of factor B vs. A against PS; (C) response surface plot of factor C vs. B against PS.

equations corresponds to an effect that favors the optimization, while a negative value corresponds to an inverse relationship between the factor and the response. In accordance with the above stated equation for PS, an increase in the number of HPH cycles led to a very significant decrease in particle size of NLC formulation. Also, as the concentration of total lipid and surfactant increases, the particle size decreases. In the case of EE, it was noticed that an increase in concentration of total lipid resulted in increased EE. The possible reason behind this could be that higher lipid content prevents the escape of drug to outer milieu by effectively enclosing it.[41] However the increase in surfactant concentration decreases the %EE of drug in NLC which might be due to the decrease in particle size of NLC by surfactant resulting in a decrease in overall accommodation space for drug. Moreover, an increase in the number of HPH cycles led to a significant decrease in EE which might be again due to the decrease in particle size. For DL, an increase in the amount of total lipid causes a non-significant increase in % drug loading whereas an increase in surfactant concentration decreases drug loading non significantly. An increase in HPH cycles was found to have a significant effect on DL.

The three-dimensional (3D) response surface graphs which depict the interaction effects of the independent variables on the responses are shown in Fig. 3–5. The selection of optimization constraints is represented in Table 5. The predicted and experimental values obtained based on optimized formula generated by Design Expert® software are seen in Table 5.

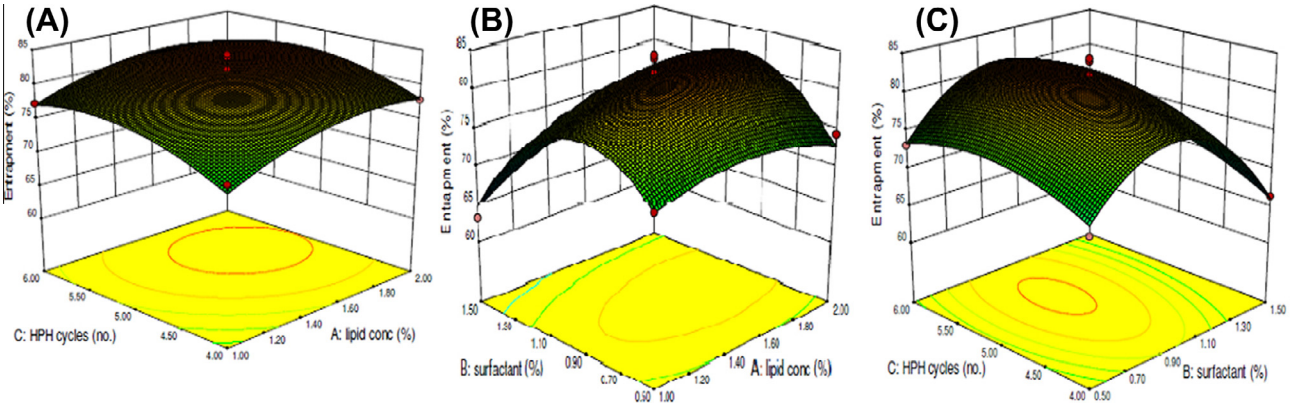

Figure 4 (A) Response surface plot of factor C vs. B against EE; (B) response surface plot of factor B vs. A against EE; (C) response surface plot of factor C vs. A against EE.

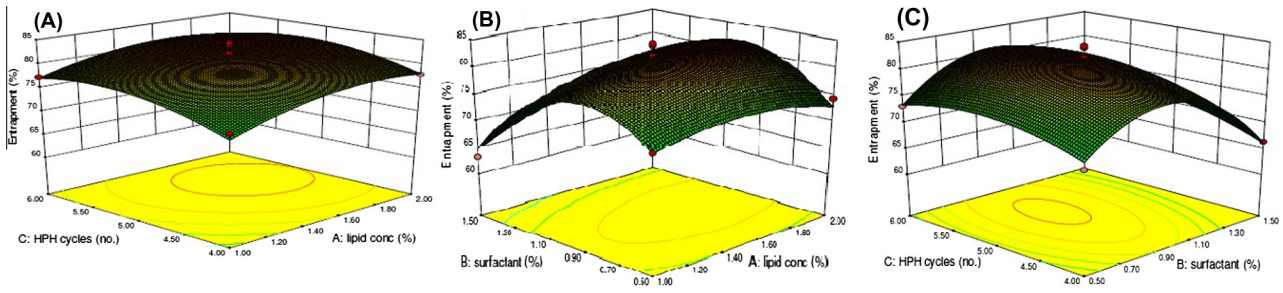

Figure 5 (A) Response surface plot of factor C vs. B against DL; (B) response surface plot of factor C vs. A against DL; (C) response surface plot of factor B vs. A against DL.

Table 5 Predicted and experimental values obtained based on optimized formula generated by Design Expert® software.

Factors			Responses		
Predicted values based on optimized formula					
Quantity of lipid concentration (%)	Quantity of Surfactant concentration (%)	No. of HPH cycles	Particle size (nm)	Entrapment efficiency (%)	Drug loading (%)
1.613	1.009	5.501	114.660	82.521	13.793
Experimental values based on optimized formula					
Quantity of lipid concentration (%)	Quantity of surfactant concentration (%)	No. of HPH cycles	Particle size (nm)	Mean Entrapment efficiency (%) (±S.D)	Mean drug loading (%) (±S.D)
1.5	1.00	6.00	126.00	80.24 ± 4.56	14.26 ± 1.82
Percentage prediction error =			9.89	2.764	3.386

3.2.1. Particle size and polydispersity index

The optimized NLCs were in the nanometric size range (128 ± 4.5 nm) with low polydispersity index (0.211 ± 0.012) (Fig. 6). The presence of 40% oil in the NLC lipid matrix led to a small mean diameter of NLC. It was usually observed that on increasing the content of oil in NLC the mean particle size decreases. Furthermore, the surfactant also greatly influences the particle size of formulation by causing stabilization.[9]

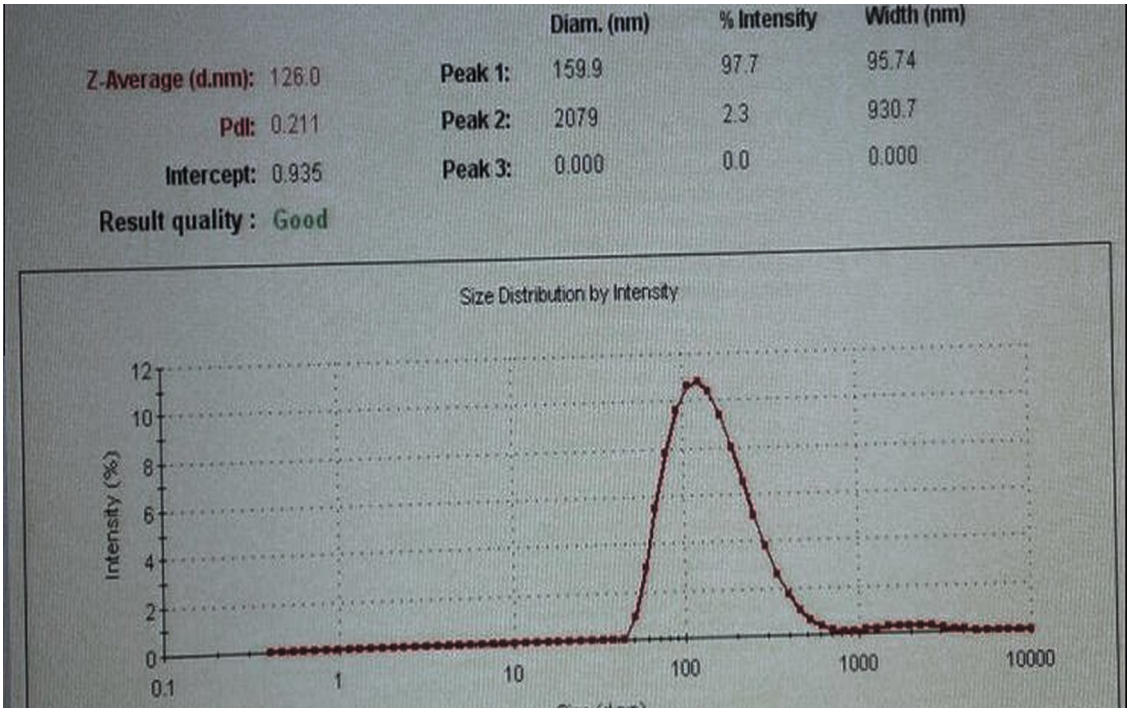

Figure 6 Particle size and particle size distribution of optimized drug loaded NLC formulation.

The size before freeze-drying was 128.33 ± 4.50 nm; whereas after freeze-drying the size was 131.33 ± 3.60 nm; similarly there was a slight increase in PDI from 0.203 ± 0.012 before freeze-drying to 0.323 ± 0.022 after freeze-drying.

3.2.2. Entrapment efficiency (%) and drug loading (%)

The EE and DL of optimized TH-NLC were found to be 80.24 ± 4.56 and 14.26 ± 1.82 which were attributable to the exclusive matrix structure of NLCs. In NLC, the solid lipid matrix encloses tiny oil section in which drug solubility is considerably higher which increases their total drug loading capacity. Therefore the liquid lipids present in NLCs affect their entrapment efficiency to a great extent by creating imperfections in a highly ordered crystal matrix and consequently providing sufficient space for a large amount of drug to lodge successfully.[9]

3.3. Evaluation of NLC

3.3.1. Transmission electron microscopy (TEM)

The TEM studies were carried out to get more insights into the morphology of the NLC systems. From the study it was observed, that after loading the drug into placebo, the particle size of the formulation increased. This might be due to the accommodation of the drug in sufficient space in the lipid matrix. The TEM images (Fig. 7(a)) show the drug enclosed in the lipid matrix. The TEM images of TH-NLC show uniform size distribution of lipid nanoparticles having coarsely spherical shape. The uniformity in particle size distribution correlates with the small PDI (0.2) obtained via photon correlation spectroscopy. The particle size after the TEM study was found to be in the range of 100–140 nm.

3.3.2. Scanning electron microscopy (SEM)

The SEM photomicrograph (Fig. 7(b)) revealed that the NLCs were spherical with more or less smooth surface. Occasional clumps were also observed in some of the images which might arise from problem associated with the shrinkage of NLCs during drying or concentration of dispersion medium.

3.3.3. Differential scanning calorimetry (DSC)

The DSC thermogram of pure drug and patterns of freeze-dried TH-NLC are shown in Fig. 8(a) and (b). A shift in the peak was seen (Fig. 8(b)) which may be due to the interaction of the drug with the lipid matrix. The DSC thermogram demonstrates the disappearance of drug peak in the formulation (NLC) which suggests that the drug is completely enclosed inside the lyophilized drug-loaded NLC. DSC curves of the excipient were compared to the already reported curves in the literature.[42–44]

3.3.4. X-ray diffraction studies (XRD)

TH powder was highly crystalline as evident from sharp peaks seen at the 2θ value of TH in the X-ray scan (Fig. 9 (b)). Fig. 9 (a) shows the XRD patterns of freeze-dried TH loaded NLC. XRD of TH-NLC confirmed that the individual components have partially lost their crystalline nature when incorporated into NLC. The X-ray diffraction of the excipients of blank NLC which were available in the literature was accordingly compared.[42–44] These results are also in accordance with the results established by earlier workers.[45]

3.4. Preparation of TH-NLC gel

The optimized NLC gel was formulated into gel by the use of 0.5%, 1% and 1.5% carbomer 940. Gel (1%) was found to be suitable for gelling the NLC because of desirable consistency. Table 6.

3.5. Characterization of TH-NLC gel

3.5.1. Spreadability

Spreadability means area to which the gel readily spreads on application to the skin or affected part. Formulation (0.5 g) was placed within a circle of 1 cm diameter pre-marked on a glass plate over which a second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate for 5 min. The increase in the diameter due to spreading of the test formulation was 6.7 ± 0.07 cm. The obtained value indicated a good spreadability of the obtained gel preparations. Spreadability is an essential property of topical formulations from the point of view of the patient compliance. In fact, gel application on the inflamed or diseased skin would be more comfortable if it can be spread easily.

3.5.2. Viscosity

The viscosity of the formulations was determined using Brookfield viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). The viscosity of the optimized formulation was found to be 501 ± 0.57 cps.

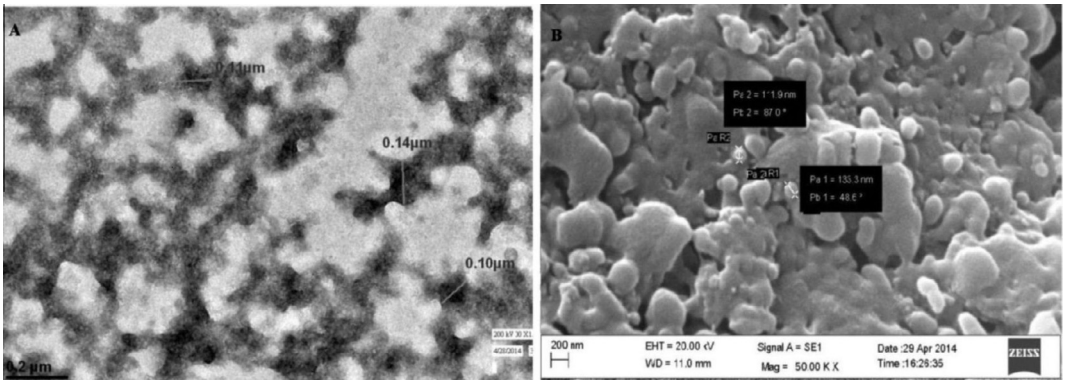

Figure 7 TEM image (A) of optimized formulation and (B) SEM image of drug loaded formulation.

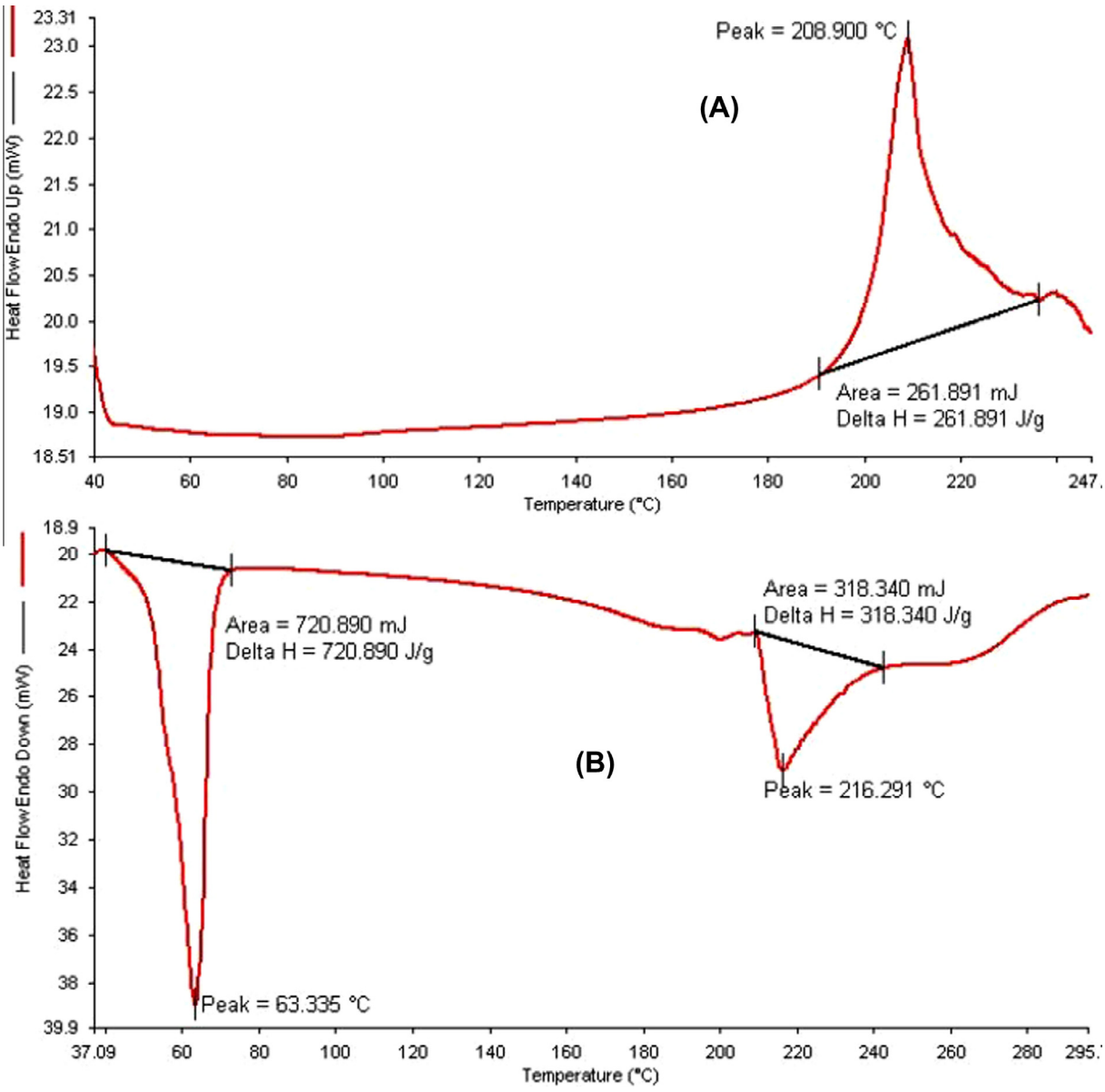

Figure 8 DSC Curve (A) Terbinafine Hydrochloride; (B) optimized formulation.

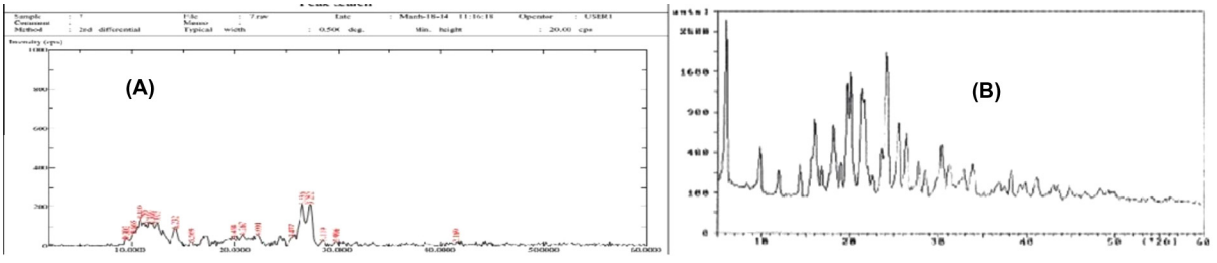

Figure 9 Xrd (A) drug loaded NLC; (B) XRD of pure drug.

Table 6 Characteristics of gelling agents for NLC gel.

S. No.	% of Carbomer	Gel formation	Spreadability
1.	0.5	No gel formed	–
2.	1	Formed	Good
3.	1.5	Formed	Poor

3.5.3. Measurement of pH

The apparent pH of the gel was determined using pH meter (pH Tutor Bench Meter, EUTECH Instruments, Singapore) in triplicate at 25 °C. pH of the optimized NLC was found to be 6.3 ± 0.04. The pH of the NLC-loaded gel was within the acceptable range for topical formulations and compatible with the pH of the skin.

3.6. In vitro release study

The *in vitro* release profile curves obtained by the dialysis method from TH loaded NLC gel, drug dispersion and commercial topical marketed product of TH which was used as reference for comparison were investigated over 24 h and comparative results are shown in Fig. 10.

It was apparent that TH released *in vitro* showed an initial rapid release followed by slow drug release. The initial rapid release of drug may be due to the release of TH from the NLC surface, while, at a later stage TH was constantly released from the solid lipid core of NLCs. Fig. 10 shows the comparative release profile of optimized NLC, dispersion and commercial topical product of TH where the dispersion showed the faster release in comparison of the NLC gel and commercial formulation. However, the release of the NLC is greater (92.60 ± 0.87%) and faster than the release of the marketed formulation cream (82.2 ± 0.69%) which might be due to the presence of liquid lipid (oils) in the NLC system with improved drug solubilizing and release potential.

NLC showed a higher *in vitro* drug release and therefore a higher cumulative amount of permeation than market preparation because of its occlusive properties. During the preparation of NLCs, cooling from high temperature to room temperature favors the enrichment of the drug in the outer layers of the particles resulting in superficial entrapment causing initial burst release.[46,47] TH entrapped deep within the nanoparticles sustains the release to more than 24 h. Also, after lipid crystallization, the solubility of oil in solid lipid exceeded; hence, oil precipitates leading to formation of fine droplets of oil incorporated in solid lipid thereby providing prolonged release.[48]

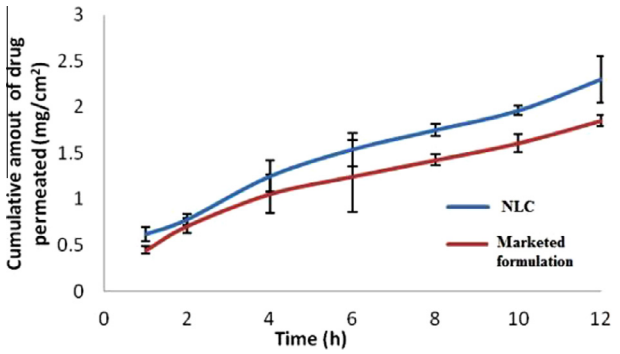

Figure 11 Comparative *ex vivo* permeation study of NLCs and drug solution.

3.7. Ex vivo permeation studies

The purpose of the present study was to target the drug into the skin with controlled release effect. Drug penetration into certain layers of the skin can be achieved using NLCs as a consequence of the creation of a supersaturated system.[15] *Ex vivo* drug penetration studies were performed for NLC based gel and commercial formulation i.e., 1% cream. The cumulative amount of drug permeated through the skin was 23.16 ± 2.33% from NLC based gel as compared to 16.72 ± 3.67% from marketed preparation (Fig. 11). After 12 h, 69.41 ± 1.85% of the drug was retained in the skin from marketed preparation as compared to 83.65% ± 2.51 from NLC based gel. The drug was dispersed within the lipid matrix

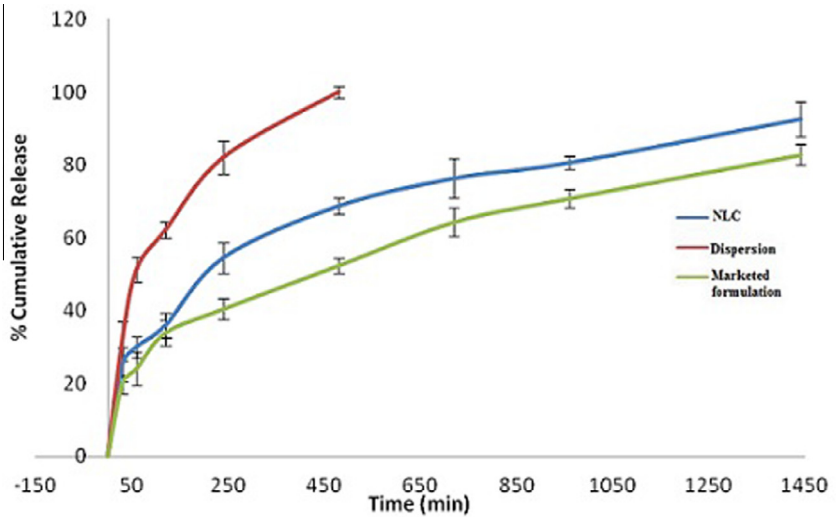

Figure 10 Comparative *in vitro* release of TH loaded optimized NLC, dispersion and marketed cream.

Table 7 Quantitative analysis of fungal burden ($n = 3$).

Group	Formulation	CFU on different days (CFUs)			
		0	1	3	5
Group-I	Control	–	–	–	–
Group-II	TH loaded NLC	2,56,000 ± 3,605.551	94,270 ± 2,825.678	5,542 ± 169.02	771 ± 41.797
Group-III	Marketed formulation	2,56,000 ± 3,605.551	1,08,855 ± 4,852.473	87,581 ± 2,012.106	1558 ± 140.524
Group-IV	TH Dispersion	2,56,000 ± 3,605.551	1,77,308 ± 2,179.077	1,05,597 ± 1,792.152	95,582 ± 2316.619

which was further incorporated into carbopol® gel which adhered to the skin, further increasing the contact time.[17] Therefore, the enhanced TH retention in the skin is mainly attributed to the NLCs, their size and bioadhesive properties. Also, the fact that the drug diffusion through the skin was less in the case of NLC based gel than the marketed formulation indicated its skin targeting ability, which is desirable for effective therapy. By formulating NLC based drug delivery system, the drug can be targeted to the skin with its reduced systemic access and reduced side effects. NLCs are formulated using lipids which are solid at ambient temperature and application of NLCs incorporated into gel may induce structural change of particle structure due to evaporation of water resulting in the transition of lipid matrix into a highly ordered structure causing drug expulsion.[47] From this, it could be concluded that NLCs may play an important role in controlling the release of TH from NLCs as well as targeting of drug to the skin.

The amount of drug retained in the skin for NLC based gel was found to be significantly higher as compared to marketed formulation (p value > 0.001). This dermal retention of TH was attributed to the increased contact with corneocytes, skin occlusion and sustained release owing to the properties of NLCs. Due to their small particle size, NLCs make closer contacts with superficial junctions of corneocyte clusters and furrows present between corneocyte islands and favor accumulation for several hours, allowing sustained drug release which is already studied by Roman and coworkers and Cevc and associates.[49,50]

3.8. In vivo pharmacodynamic studies

Fungal burden was quantitatively analyzed in terms of colony forming units (CFUs) after initiation of the treatment. CFUs were counted using a colony counter (Microbiology lab, Jamia Hamdard, New Delhi, India). Table 7 gives the quantitative analysis of fungal burden.

Control group did not show any growth, as infection was not induced to this group. Group treated with NLCs showed a significant decrease in fungal burden after 5 days (771 ± 41.797 CFUs) (p value < 0.001) as compared to CFUs before initiation of treatment (2,56,000 ± 3,605.551 CFUs) (p value < 0.001). Group treated with marketed formulation also showed a significant decrease in fungal burden after 5 days (1558 ± 140.524 CFUs) (p value < 0.001), but it was higher as compared to the group treated with developed formulation. Also, the TH dispersion in water showed an initial reduction in fungal burden after which it almost came to a steady state. These results showed that the NLC reduced the fungal burden in a shorter duration of time as compared to marketed formulation and dispersion. Thus, TH was found to be more effective when formulated as NLC based gel because of improved contact, adhesion, occlusion and sustained release.

4. Conclusion

In the present study, TH loaded NLCs were successfully prepared using GMS as solid lipid, Labrasol as liquid lipid, Pluronic F-127 as stabilizer using HPH technique. The topical delivery of TH to the skin by means of NLCs could possibly minimize its systemic access and side effects. Furthermore, TH loaded NLCs were capable of reducing the fungal burden. Therefore, it can be concluded that TH-NLC demonstrated to be potential formulation approach for treating fungal infection in contrast to commercially available product.

5. Conflict of interest

The authors report no declaration of interest.

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