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Formulation and Evaluation of Butenafine Hydrochloride Incorporated Solid Lipid Nanoparticles from Novel Excipients for the Treatment of Superficial Fungal Infection

Short Title: Butenafine Nanolipid Gel for Fungal Infection

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Abstract
Objectives: The objective of present research was to develop natural excipients based solid lipid nanoparticles (SLN) of butenafine hydrochloride (BUTE) by using modified solvent emulsification technique and to evaluate the competence of *aloe vera* nanolipidgel in enhancing the penetration of BUTE.
Materials and Methods: The BUTE-SLNs were prepared by 2^3 factorial design to correlate the effect of formulation components on the BUTE-SLN. Particle size, polydispersity index, zeta potential, entrapmentperformance, and drug loading were assessed in the formed SLNs. The average particlesize and polydispersity index were observed to be 261.25±2.38 nm and 0.268±0.01 respectively. Fabricated BUTE-SLN was evaluated for TEM, FTIR, DSC and XRD study and revealed the encapsulation of butenafine hydrochloride in lipid in amorphous state. BUTE-SLN based *aloe vera* gel was formulated and evaluated comparatively with the marketed product with respect to primary skin irritation, hydration, skin permeation and antifungal activity.
Result: The BUTE-SLN promisingly showed no irritation, higher hydrating potential, slow and sustained release and enhanced antifungal activity. With an aim to target the deeper skin strata, to minimize the side effects of drugs and symptomatic effects of fungal infection and to shorten duration of therapy BUTE-SLN hasbeen successfully prepared.
Conclusion: BUTE-SLN gel offer improved topical delivery of BUTE with significantly higher compatibility and the antifungal activity as compared to marketed formulation.
Key words
Modified solvent emulsification technique, factorial design, butenafine, *aloe vera* gel, Natural origin lipid and surfactant, *In Vitro* antifungal activity.

INTRODUCTION

Fungal infections are one of the most common skin diseases among major population of world. According to the unpublished survey of International foundation of dermatology, superficial mycosis was usually reported as one of the three commonest diseases among the community pattern of skin diseases in nine different countries across the world. Human skin has favorable conditions forthe growth of dermatophytes[1]. Dermatophyte fungi invade the stratum corneum. Dermatophyte areenriched with keratinolytic, proteolytic and lipolytic activity[2]. Dermatophytes also contain serine proteinases which play a major role in breaching of skin barrier. Invasion of skin includes two basicmechanisms - Colonization and Host-parasite interaction. During the colonization phase the host begins to respond immunologically and the first detectable immune response is cell-mediatedimmunity (CMI), which is characterized in colonized skin as an intense inflammatory process. Duringthe host-parasite interaction phase, CMI produces most of the pathology as an acute inflammatory type of dermatophytosis which results into erythema and edema of the dermis and epidermis leadingto breaching epidermal integrity[3].
Current medications for the superficial fungal infections include variety of antifungal agents. Oral toxicity of antifungal

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drugs and treatment of fungal infection residing in stratum corneumfocuses the need of topical delivery of antifungal agents. Topical treatment has several superiorities compared to oral and systemic delivery but still have some pitfalls such as side effects of drugs, diffusion of drug across biological tissues, drug and biological cell interaction, residence time of conventional dosage form. Various novel colloidal drug delivery systems overcome the mentioned limitation of conventional route. Amongst various colloidal carrier's solid lipid nanoparticles (SLN)have shown promising healing ability in skin infection.

Butenafine hydrochloride (BUTE) is a synthetic allylamine antifungal agent. The suggested mode of action is it inhibit enzyme squalene monooxygenase responsible for converting squalene to2,3-oxydo squalene. Hence interferes with biosynthesis of ergosterol which results in increased cellular permeability causing leakage of cellular contents. Blockage of squalene monooxygenase causes accumulation of squalene and leads to fungicidal effect[4, 5].

The observed side effects of BUTE include contact dermatitis, erythema, irritation, burning and itching at the site of application[6]. To subside the side effects of BUTE and symptomatic effectof fungal infection as inflammation, stinging and itching, the possible combination of BUTE and agents with anti-inflammatory activity can be applied. The topical steroids provide rapid symptomaticrelief but consist of many steroid related complications like atrophy, purpura and rosacea[7]. Hence to provide better therapeutic and pharmacological effect for fungal infection, the present workis aimed to formulate BUTE solid lipid nanoparticles having natural lipid and surfactant incorporatedinto *aloe vera* gel which has in-built anti-inflammatory, antioxidant and healing property which showed synergistic effect of drug. The present individual research work can be used to develop natural excipients based solid lipid nanoparticles (SLN) of butenafine hydrochloride (BUTE) by using modified solvent emulsification technique and to evaluate the competence of *aloe vera* nanolipidgel for the enhancement of the penetration of BUTE.

MATERIALS AND METHODS

Materials

Butenafine was a kind gift by Cipla PVT LTD, Mumbai (India). OLML and OLMS were generous gift from Chemhouse Marketing, Mumbai. D-α tocopheryl polyethylene glycol succinate (TPGS) was purchased from BASF India Ltd, Mumbai. Stearyl amine was purchased from Sigma Aldrich GmbH (St. Louis, MO) and other chemicals, such as stearic acid, glyceryl monostearate, Tween 80, acetone, Dimethyl sulphoxide and poloxamer 188 were of analytical grade and from Merck ChemicalCompany (Mumbai, India).

Optimization of surfactant, solid lipid and organic solvent concentration/ Effect of variables

To determine influence of concentration of different ingredients on properties of butenafine loaded solid lipid nanoparticles (BUTE-SLN), 3-factor 2-level factorial experimental design was employed using Design Expert software 8.0.1, Stat-Ease, Inc., Minneapolis, MN, USA. On the basis of solubility study and pre-optimization study, various variables at different levels were selected. The effect of three independent variables concentration of surfactant (X1), Concentration of lipid OLML (X2) and concentration of organic solvent (X3) was determined on the formulation of nanoparticles dispersion in terms of five responses particle size (Y1), polydispersity index (Y2), zeta potential (Y3), percent entrapment efficiency (Y_4) and percent drug loading (Y_5). All possible eight combinations ofthree factors at two different levels were performed and evaluated for each response. All nine experimental runs, different independent variables with two levels i.e., low (-1) and high (+1), and obtained responses are depicted in Table 1. By using Design Expert software, the response surface plots were generated to identify the influence of significant variables.

Preparation of butenafine loaded lipid nanoparticles (BUTE-SLN)

A Simple and easily accessible method was adopted for the preparation of BUTE-SLN. A modified solvent emulsification technique was used and on the concrete of the solubility and compatibility studies of lipid, drug and excipients BUTE-SLN dispersion was prepared. BUTE-SLNis composed of the natural excipients from Natural stearin fraction of olive as lipid OLML and surfactant OLMS (Natural fraction of olive). BUTE-SLN was prepared by dissolving butenafine in acetone and DMSO (1:1) and then mixed with stearyl amine and lipid OLML at 80°C. Water phase containing TPGS and surfactant OLMS was maintained at 80°C same as lipid phase. Lipid phase wasadded into aqueous phase with constant stirring at 2000 rpm for 1 h with sudden cooling of dispersion.

Physicochemical characterization of BUTE-SLN dispersion

Particle size analysis (PS) and Polydispersity Index (PDI)

The particle size in nanometric range can be determined by concept of dynamic lightscattering. Particle size and PDI of the optimized BUTE-SLN were determined by photon correlationspectroscopy (PCS) using a Zetasizer (Nano ZS 90, Malvern Instruments, UK) at 20°C, by 900 scattering optics[8, 9].

Zeta Potential (ZP)

The ZP of SLN dispersion was determined by using Zetasizer (Nano ZS 90, Malvern Instruments, UK) at 25°C with electric field strength of 23 V/m 10, 11, 12. BUTE-SLN samples were diluted with double distilled water.

Entrapment Efficiency (EE) and Drug Loading (DL)

% EE of butenafine in BUTE-SLN can be calculated by determining concentration of butenafine in supernatant SLN dispersion. Ultracentrifuge was used to determine concentration of un-entrapped butenafine in aqueous BUTE-SLN dispersion. 1.5 ml of BUTE-SLN dispersion was filled in the Eppendorf tubes and speed of cooling ultracentrifuge (Remi Instruments Ltd., Mumbai, India) was kept at 60000 rpm for 45 min at 4°C. A UV–visible spectrophotometer (UV 1700, Shimadzu, Japan) was used to measure the concentration of butenafine in the

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aqueous phase at a wavelength of 224 nm. Equations. (1) and (2) were used to measure the EE and DL percent values,respectively [10-13].

$$\% EE = \frac{Total\ mass\ of\ Bute - Total\ mass\ of\ Bute\ in\ supernatant}{Total\ mass\ of\ drug} \times 100 \qquad Eq.1$$

$$\% DL = \frac{Total\ mass\ of\ Bute - Total\ mass\ of\ Bute\ in\ supernatant}{Total\ mass\ of\ Lipid} \times 100 \qquad Eq.2$$

Transmission Electron Microscopy Study (TEM):

Particle size, shape and morphology were studied by using transmission electron microscopy study. In this ongoing study, the BUTE-SLN dispersion was mixed with water and a drop of it was mounted on a copper grid coated with a thin film of carbon, which was then dried for 45 min.Transmission electron microscope Philips CM200 (Philips, Netherlands) was used for scanning.

Screening of Cryoprotectant

BUTE-SLN dispersion was freeze dried by using mannitol as cryoprotectant. The frozen (- 25°C) dispersion containing 5, 7.5 and 10 % concentration of mannitol were lyophilized at 0.25 mbarfor 24 h and the temperature was increased from -15 to 0°C. At final step drying was carried out for 2 h at +15°C, 0.01 mbar and then stored at -4°C. BUTE-SLN was further characterized for DSC, XRD and FTIR study [14,15].

Characterization of lyophilized BUTE-SLN

Differential Scanning Calorimetry (DSC)

The thermal behavior and interaction between drug and additives were studied using differential scanning calorimetry. In differential scanning calorimeter (DSC 1 STARe System, Mettler-Toledo, Switzerland), an empty standard aluminum pan was used as reference and scans wererecorded at heating rate of 10°C/min in the range of 30°C to 300°C. DSC studies were carried out on Butenafine pure drug, bulk OLML and lyophilized BUTE-SLNs of the optimized batch.

X- Ray Diffraction (XRD) Study

XRD Study was done to study spacing of the lattice planes in crystal. Crystal structure of Butenafine, pure lipid and butenafine in SLN was determined by X-ray diffraction study. Philips PANanalytical expert PRO X-ray diffractometer 1780 was used to study. A Cu Ka radiation source was used, and the scanning rate was 2°/ min. XRD measurements were carried out on Butenafine pure drug, bulk OLML and lyophilized BUTE-SLNs of the optimized batch.

Fourier Transform Infra-Red Spectroscopy (FTIR) Study

A Jasco FTIR spectrophotometer (Perkin Elmer Jasco FTIR- 401, Japan) was used fordetermination of compatibility between excipients. FTIR studies were carried out on pure OLML, butenafine and SLN loaded with butenafine. The samples were examined in transmission mode over awave number range of 4000 to 400 cm^{-1}, using about 1-2 mg of sample mixed with dry potassium bromide.

Preparation of BUTE-SLN into semisolid formulation

BUTE-SLNs were formulated by using *aloe vera* gel as semisolid base. *Aloe vera* gel was formulated with *aloe vera* extract of plant *Aloe barbadensis* along with highly pure and GRASS gradestabilizer, antioxidant and preservatives. After centrifugation of dispersion, analysis for drug contentwas determined and BUTE-SLN dispersion equivalent to 0.1 g butenafine was added in the *aloe vera* gel to prepare 1% gel.

Evaluation of BUTE-SLN gel

Rheological measurement

Viscosity

The rheological behavior and the characteristic flow property of formulated *aloe vera* gel incorporated with SLN-dispersion were studied. Cone and plate rheometer (Brookfield viscometer AP 2000+2) was used to determine the effect of shear stress and shear rate on SLN gels. To determinethe influence of storage temperature and nature of lipid matrix on the rheological behavior of BUTE-SLN based *aloe vera* gel was investigated by recording the variation in shear stress at pre-defined shear rate from 0 s-1 to 1000 s-1 as up curve and 1000 s-1 to 0 s-1 as down curve at three different temperatures (5°C, 25°C and 40°C).

Spreadability

The BUTE-SLN enriched gel was evaluated on the ground of rheological pattern, film forming ability, degree of consistency and spreadability. Spreadability is an important parameter for evaluation of topical dosage form as it represents the stability and particle-particle interaction. This study includes the use of wooden block and glass slide apparatus in which one rectangular wooden block fixed with the glass slide in it. In the same apparatus another movable glass slide is attached with thread to pan through pulley. The time was noted to separate upper movable slide from fixed slide when a BUTE-SLN gel was placed between the slides. Spreadability was quantitativelydetermined by the ratio of time required to separate two glass slides completely by the weight of sample. Spreadability was computed by the formula [16,17].

$$S = \frac{M \times L}{T}$$

Where,

S = Spreadability

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M = Weight tide to the upper glass slide
L = Length of a glass slide
T = Time taken to separate the slide completely from each other

Occlusive study

In vitro occlusion study was performed to determine the efficiency of BUTE-SLN gel to prevent the water loss from the surface of test assembly which simulates the trans epidermal water loss from skin. In this study the pre-weighed beaker of approximately same dimensions having 50 mlcapacity were filled with 25 ml purified water and covered with Whatman Microfiber Filters 9.0 cm.For comparative study one beaker surface was spread with 0.25 g of BUTE-SLN gel, other with plain gel without BUTE-SLN and one beaker without any application was kept as reference. All the beakers were stored at 32°C and 60±5% RH for 72 h. The occlusion factor F was calculated for the formulation according to formula[18].

$$F = 100 \times \left(\frac{A - B}{A}\right)$$

Where,
A represents the water loss in the absence of sample,
B represents the water loss in the presence of sample.

Ex-vivo skin hydration study

Ex-vivo skin hydrating effect of BUTE-SLN was studied and compared with conventional cream. The BUTE-SLN *aloe vera* gel and marketed cream were applied to the prepared human cadaver skin. The skin under study was isolated after 24 hours of gel and cream application. The skinwas vertically sliced using microtome, stained with carbol fuchsin and observed under optical microscope for thickness of stratum corneum. An optical microscope with an image analyzer wasused to take the photomicrographs (Magnus MLX) [19].

In-Vitro drug diffusion study

In-Vitro drug release of BUTE-SLN was studied on the Franz diffusion cell using cellulose acetate membrane. Phosphate buffer pH 7.4 and methanol (60:40) was used as diffusion medium andthe membrane was soaked in diffusion medium for 30 min before placing the sample. Phosphate buffer pH 7.4 and methanol (60:40) (10 ml) of was placed in receptor compartment of Franz diffusioncell. The diffusion medium in receptor compartment was continuously stirred using magnetic bar with maintaining the temperature at 37°C. The experiment was started with even application of 0.5 g of BUTE-SLN gel on the surface of cellulose acetate membrane from donor compartment side. Sampling was performed after 0, 1, 2, 5, 7, 9, 12 and 24 h and the fresh diffusion medium was addedwith each withdrawal of sample. The samples were diluted and analyzed using UV spectrophotometrically at 224 nm.

Drug permeation study

In vitro skin penetration and permeation experiments were studied using Franz diffusion cellson pig ear skin. Amongst rodents the most relevant animal model for human skin is the pig. Pig ear skin have been proved to be similar in histological and biochemical properties, the vascular anatomyand contents of stratum corneum with human skin, hence give comparable results with human skin. Fresh pig ears were obtained from a local abattoir. Pig ears were washed with distilled water and adipose subcutaneous tissue was removed. The skin was placed between the donor and receptor compartment of Franz diffusion cell, exposing dermal side to receptor compartment and stratum corneum in contact with donor compartment. Sink conditions were maintained in the receptor compartment with diffusion fluid. The temperature of cell was maintained at 37.0±0.1°C. The BUTE-SLN gel equivalent to 1% of drug was applied on the membrane in donor compartment ensuring an intimate contact with the skin. Sampling was done at 0, 1, 2, 5, 7, 9, 12 and 24 h. At each point, 1 mlaliquots were drawn from the receiver compartment and simultaneously an equivalent volume of receptor fluid was replaced. Amount of butenafine in diffusion medium was determined by UV method. A graph of cumulative % drug release Vs time and another graph of amount of BUTE diffused per unit area (Q/A) versus time (h) was plotted. The excess amount of BUTE found in surfaceof the skin and from the entire dosing area was determined. The unabsorbed drug on the skin was also quantified [20,21].

In-vivo skin retention studies

BUTE-SLN gel (0.25 g) was applied on the shaved skin area of albino rat. After 24 h, the animal was humanely sacrificed and the skin was collected. The applied formulation was removed, and the stratum corneum layer was stripped away with adhesive tape, whereas the epidermis layer was differentiated through using heat separation technique. Presence of butenafine in different skin stratawas extracted and quantitatively determined [22].

Skin irritation study

The primary skin irritation of the SLN-based butenafine gel was evaluated according to OECD guideline #404 by acute skin irritation test. The protocol for the study was approved by the Institutional Animal Ethical Committee. 2.5-3.0 Kg healthy male New Zealand white rabbits were used for study. Animals were categorized into four groups positive control (formalin), plain butenafine gel, marketed formulation, and BUTE-SLN gel. 0.5 g of the test samples was applied to the hair-free shaved area of skin i.e., right side of the trunk (approximately 6 cm^2) and covered with a gauze patch. The left side of trunk was kept as untreated skin areas which serve as the control. After1 h exposure of test sample, the rabbits were observed for signs of erythema, edema and irritation. The degree of irritation, erythema and edema were read and scored according to Grading of skin reactions of OECD guidelines. Data was recorded at interval of 24 h,

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48 h and 72 h after patch removal[23].

In vitro antifungal activity

In vitro antifungal activity study was performed against *Candida albicans* species using modified agar diffusion method. Fresh *Candida albicans* cultures were prepared then incubated in the dark at 37±2 °C for 48 hours. *In vitro* antifungal activity was studied for blank gel, BUTE-SLN gel, plainbutenafine gel and marketed formulation. The formulations were mixed with potato dextrose agar and poured in the spherical ditch made by sterile borer on agar plate under sterile conditions. The plates were dried and incubated for 48 hours at 37±2°C. At the end of the incubation, the zone of inhibition was measured [23-26].

RESULTS

Effect of different variables on the preparation of BUTE-SLN and optimization of surfactant, solid lipid and organic solvent concentration

To determine the effect of various variables on the optimization of BUTE-SLN 2^3 factorial design was used. The eight formulations were obtained with possible combination of concentration of surfactant X_1, concentration of lipid X_2 and concentration of organic solvent X_3 as independent variables with low and high levels as shown in Table 1 and Table 2. The responses Y_1, Y_2, Y_3, $Y_4$and Y_5 were found to be in the range of 231-278 nm, 0.241-0.369, 11.7-24.9 mV, 87.45-91.65% and 15.05-22.86% respectively. The observed responses were fitted to various models using Design-Expert software. The dependent variables show that the models were important for all measured responses, as determined by the negligible lack of fit ($P<0.05$) in the ANOVA results. It was observed that, independent variables X_1, X_2 and X_3 had a positive as well as negative influence on the selected dependent variables such as particle size, PDI, ZP, EE and DL (see Eq. 3 to 7).

$$\text{Particle size}=+255.625-3.125*X_1+14.375*X_2+3.875*X_3+3.125*X_1X_2+2.125*X_1X_3 \quad \text{Eq. 3}$$

$$\text{Polydispersity index}=+0.302125+0.002875*X_1+0.014875*X_2+0.045625*X_3-0.001125*X_1X_3+0.005875*X_2X_3 \quad \text{Eq. 4}$$

$$\text{Zeta Potential}=+18.5038+2.07875*X_1+3.74625*X_2-0.47125*X_3-0.67875*X_1X_2+0.47875*X_2X_3 \quad \text{Eq. 5}$$

$$\text{Entrapment Efficiency} = +89.7075-0.17*X_1+1.1825*X_2+0.3575*X_3-0.565*X_1X_3-0.2975*X_2X_3 \quad \text{Eq. 6}$$

$$\text{Drug Loading} = +18.8625-0.21*X_1-1.7075*X_2-1.585*X_3+0.175*X_1X_2 \quad \text{Eq. 7}$$

As per the ANOVA analysis near about 100% desirability was found to be with predicted values of 264 nm for Y_1, 0.270 for Y_2, 24.60 mV for Y_3, 91.225% for Y_4 and 18.705% for Y_5 with the concentration of surfactant 4%, concentration of lipid 5% and concentration of organic solvent 0.5%. To confirm and reproduce the predicted model the optimized values of independent variables were applied and the observed responses were measured as shown in Table 4. The observed values ofY_1, Y_2, Y_3, Y_4 and Y_5 were 261.25 nm, 0.268, 23.98 mV, 91.35% and 19.692% respectively which were in close agreement with the predicted values demonstrating the reliability and reproducibility of this method. Effects of various independent variables on the dependent variables were expressed by 3 D graphical presentation shown in figure. 1

- A Effect of lipid OLML and surfactantOLMS on the particle size,
- B Effect of lipid OLML and organic solvent on the polydispersity index,
- C Effect of lipid OLML and surfactant OLMS on the zeta potential,
- D Effect of surfactant OLMS and lipid OLML on the percent entrapment efficiency and
- E Effect of organic solvent and surfactant OLMS on the percent drug loading.

Physicochemical characterization of BUTE-SLN dispersion

Particle size analysis and size distribution

The effect of various formulation components and process variables on the preparation ofsolid lipid nanoparticles can be observed from the results of particle size and PDI of BUTE-SLN gel depicted in Table 3. It is observed that the particle size and polydispersity index of BUTE-SLN increases with increase in lipid content. The range of particle size and PDI of the BUTE-SLN were observed to be 231±2.79 nm to 278±3.19 nm and 0.241 to 0.369 respectively. Particle size and PDI of optimized batch was found to be 261.25±2.38 nm and 0.268±0.01 respectively.

Zeta Potential

Zeta potential values indicate stability of the nanoparticles in SLN dispersion. The zeta potentials of all the nine batches were found to be in-between 11.7±0.16 mV to 24.9±0.41 mV and for optimized batch it was observed to be 23.98±0.27 mV.

Entrapment Efficiency and Drug Loading

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Different concentrations of lipid and organic solvent showed significant effects on EE and DL of butenafine loading in lipid nanospheres. EE of BUTE was found to be higher in the selected formulation. The EE of eight formulations run 1 to run 8 were found to be in between 87.45±2.26% to 91.65±1.69% with the drug loading in between 15.05±0.58% to 22.86±0.46%. The EE and DL of optimized formulation was found to be 91.35±2.35% and 19.692±0.95% respectively 10, 13.

TEM Imaging

With the similar results to particle size analysis, TEM image of BUTE-SLN (figure 2) revealedthe presence of spherical shape nanometric range particles.

Screening of cryoprotectant

The concentration of cryoprotectant was optimized on the basis of aggregation of SLNs, timeof reconstitution and particle size distribution upon reconstitution. From the different concentrationsof cryoprotectant screened for lyophilization, mannitol at 5% w/w resulted in good lyophilized product upon reconstitution with quick reconstitution, no aggregation and with less difference in particle size distribution (PDI) 14, 15.

Characterization of lyophilized BUTE-SLN

Differential Scanning Calorimetry (DSC)

Thermal profiles of bulk lipid, pure drug butenafine and lyophilized drug loaded SLN formulation are given in figure 3(1). The melting point of bulk lipid OLML was determined by a DSC thermogram that showed a high endothermic peak at 63.20°C (curve A). A sharp endothermic peak at 217.35°C was seen on the second thermogram of pure drug butenafine. The endothermic peak wasobtained for BUTE-SLN at 46.91°C representing the transformation of crystalline lipid and drug intoamorphous form with numerous lattice defects in which drug is encapsulated 14.

X – Ray Diffraction (XRD) study

The X-ray diffraction patterns of drug butenafine (a), lipid OLML (b) and BUTE-SLN (c) areshown in figure 3(2). Pure butenafine showed a sharp peak which is specific for its crystalline nature. Lipid OLML also showed a sharp peak revealing crystalline nature of lipid crystals. BUTE-SLN showed broad peaks instead of sharp peak indicating amorphous nature. All the major characteristicpeaks for butenafine and OLML were absent in BUTE-SLN. Presence of broad and small peaks in XRD reveals the existence of butenafine and OLML in BUTE-SLN in amorphous state.

FTIR

The FT IR spectra of the butenafine, pure lipid OLML and BUTE-SLN are compared in figure 4. The FTIR spectra indicated amorphization of drug and lipid in the formulation. BUTE-SLN showed no new peaks, revealing there was no interaction between formulation ingredients with no structural and functional changes in formulation.

Preparation of BUTE-SLN into semisolid formulation

Instead of conventional semisolid dosage form here we have formulated BUTE-SLN incorporated in *aloe vera* gel. *Aloe vera* Gel has been used for external treatment of mild wounds as well as inflammatory skin diseases for a long time, with the aim of speeding up the healing process and reducing inflammation and tissue scarring. Mild skin irritations may be treated with this gel. Hereattempt has been made to formulate such semisolid product that will give synergetic antifungal effectof drug as well as anti-inflammatory, wound healing and reducing skin irritation effects by the *aloe vera* gel 24, 25, 26.

Evaluation of BUTE-SLN gel

Rheological measurement

Viscosity

Viscosity of any semisolid formulation is dependent on adhesion, ease of application and spreadability. The results were recorded for the BUTE-SLN *aloe vera* gel at 5ºC, 25ºC and at 40ºC after the storage of one week to check the consistency and compatibility of gel with pharmaceutical excipients. Slight increase in viscosity of BUTE-SLN gel was observed compared to plain *aloe vera*gel due to existence of nanometric particle size of SLN and polysaccharide cross linking with the lipid component of SLN. The increase in total lipid content and particle size increases the viscosity of gel. BUTE-SLN gel showed non-Newtonian flow particularly pseudoplastic flow at all temperatures conditions as depicted in figure 5 A at 5ºC, figure 5 B at 25ºC and figure 5 C at 40ºC. Because of the nanometric particle size of SLN, the viscosity of BUTE-SLN gel was observed to be significantly higher than that of pure *aloe vera* gel.

Spreadability

The potential application of semisolid topical dosage form with desired consistency and adhesion onto skin was studied by the spreadability values. Nanometric particle size and uniformity in particle size leads to good spreadability forming uniform layer on skin. The presence of BUTE- SLN did not show any changes in spreadability. The spreadability value of BUTE-SLN was found tobe 4.6±0.37 s/g and for plain gel base 4.06±0.41 s/g. Slight increase in spreadability value indicates inclusion of SLN forms a 3D structure with polysaccharide group which leads to increase in viscosityand spreadability.

Occlusive study

The successful topical drug delivery is based on the occlusive and skin hydration ability of semisolid formulation. Figure 6 A shows the comparison of BUTE-SLN versus plain gel effects. After 6 h, 24 h, 48 h and 72 h study, occlusion factor F were found to be 31.362±0.65 %, 41.1372±0.47%,43.2659±0.28% and 45.7±0.44% respectively as shown in

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figure 6 A. Whilst that of plain gel F were found to be 18.2795±0.55%, 23.0020±0.59%, 31.2094±0.21% and 31.5209±0.29% when compared to control. BUTE-SLN showed higher occlusion factor F values in comparison to plain gel owing tohigh lipid content which forms an impermeable layer on the surface of the skin.

***Ex-vivo* skin hydration study**

Using an optical microscope and an image analyzer, the skin hydrating response of the chosen SLN formulation has been investigated in human cadaver skin and compared to a marketed cream Fintop® (Magnus MLX). The aim of this data analysis was to compare the effects of SLN gel with marketed cream Fintop® on the stratum corneum, which is linked to skin moisturization and drug penetration. Figure 6 B I shows photomicrograph of untreated human cadaver skin. The photomicrograph's dark brown left side layer reflects the skin's top layer (i.e., stratum corneum). The thickness of the stratum corneum changed significantly after application of the marketed cream Fintop®, as seen in figure 6 B II, whereas application of the gel enriched with SLN resulted in a substantial improvement in the thickness of the stratum corneum, nearly 3-fold when compared to the marketed cream and 3.5-fold when compared to the untreated skin (figure 6 B III).

***In-Vitro* drug diffusion study**

BUTE-SLN had been evaluated against Fintop® cream in an *In-vitro* release of drug study.Release pattern of butenafine from SLN was initially faster which becomes sustained after 2 h. The sustained release from gel was further useful for maintaining the depot for drug release 27. The marketed cream showed comparatively controlled release than the SLN gel (figure 7 A).

Drug permeation study

The ability of BUTE-SLN to permeate through pig ear skin was studied. The plot of cumulative amount of drug permeated showed 5.42 times higher drug release for the BUTE-SLN gel when compared with reference cream. The flux value calculated from the linear portion of graph Q/A vs time (figure 7 B) from SLN gel was found to be 1666.7 ± 0.198 ncg $cm^{-2}h^{-1}$ and 818.181 ± 0.392 ncg $cm^{-2}h^{-1}$ for reference cream [27]. Results of quantification of butenafine after 24 h were expressed in percentage of drug undiffused, deposited and permeated through skin and depicted in figure 8 I.

***In- vivo* skin retention studies**

Content of drug in various epidermal layers was quantitatively estimated as per the procedure or methods provided in literature. Butenafine was extracted into methanol from stratum corneum separated by the tape stripping method. Epidermis separated by the heat separation technique was studied for drug content. Using the UV spectrophotometer as analytical method Butenafine present in different skin strata was depicted in % present in skin as shown in figure 8 II.

Skin irritation study

In a rabbit skin irritation analysis, the formulation did not display any signs of skin irritation, such as redness or inflammation at the application site (erythema). As a result, it can be assumed that all of the formulations tested are safe for topical use.

***In-vitro* antifungal activity**

The antifungal activity of butenafine was measured *in vitro* against *Candida albicans* NCIM 3471 with the goal of evaluating the effect of process parameters on the intensity (potency) of butenafine and studying its antifungal activity in terms of zone of inhibition. In this study the extract of BUTE-SLN equivalent to 500 µg/ml was compared with the marketed cream dilution equivalent to 500 µg/ml and different standard drug dilutions. From the present investigation the test sample with the strength equivalent to 500 µg/ml showed greater zone of inhibition (32 mm) when comparedwith the standard 500 µg/ml butenafine solution (30 mm) as shown in figure 9.

DISCUSSION

The objective of this research is to develop and evaluate antifungal drug butenafine loaded solid lipid nanoparticles for treating topical fungal infections. Novelty of the work is use of novel excipients such as natural surfactant and lipid and use of organic solvent in the fabrication of SLN and very simple and novel modified solvent emulsification method of preparation.

The regression equation, (Y_1=+255.625 - 3.125*X_1 + 14.375*X_2 + 3.875*X_3 + 3.125*X_1X_2 + 2.125*X_1X_3) generated for Particle size was significant with F value of 43.08 ($p < 0.0001$) and correlation coefficient value of 0.9908. The model generated for particle size revealed that the concentration of lipid, surfactant and organic solvent and their interaction has significant impact on the formation of nanometric particles. The response surface plot for the effect of factor X_1 and factor X_2 on particle size (Y_1) of BUTE-SLN is shown in figure 1 A. Changes in lipid concentration, in particular, resulted in increased particles, while increases in surfactant concentration had negligible effect on particle size. The increase in particle size is attributed to an increase in lipid concentration, which allows the primary emulsion's globule size to increase. The smaller droplet goesinto emulsion and redeposit onto surface of nanoparticles in order to reach a more thermodynamicallystable state. The insufficient surfactant concentration was unable to cover the formed new interfaces hence the particle size increases. The involvement of organic solvent in the BUTE-SLN formulation was a plausible explanation for the rise in surfactant concentration contributing to a small increase in the particle size. The surfactant present in SLN formulation is in equilibrium with the organic solventhence the surfactant covers the SLN at interface and reduces the interfacial tension so that the nano lipid particles are formed but the additional rise in surfactant concentration goes into organic solventhence rise in surfactant slightly increases the particle size 28.

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The regression equation, (Y_2=+0.302125+ 0.002875*X_1 + 0.014875*X_2 + 0.045625*X3 - 0.001125*X_1X_3 + 0.005875*X_2X_3) generated for polydispersity index was significant with F value of 240.33 ($p < 0.0001$). The model generated for polydispersity index revealed that the % concentration of lipid and organic solvent and their interaction has significant impact on Polydispersity index. Theresponse surface plots (figure 1 B) illustrate that the Polydispersity index increases as organic solvent and lipid concentration (%) increase. Increase in organic solvent concentration causes non uniform precipitation of SLN (heterogeneous SLN dispersion) leading to deposition of smaller particles on larger one as per the phenomena of Ostwald ripening. This result into variable particles with bi or trimodal size distribution, hence PDI increases.

The regression equation, (Y_3=+18.5038 + 2.07875*X_1 + 3.74625*X_2 - 0.47125*X_3 - 0.67875*X_1X_2 + 0.47875*X_2X_3) generated for Zeta potential was significant with F value of 87.80 ($p < 0.0001$). The model generated for zeta potential revealed that the only concentration of lipid has significant impact on zeta potential. The response surface plot in figure 1 C shows that increasing lipid concentration lowers zeta potential while increasing surfactant concentration has less of an impact on particle charge. Charge on nano lipid dispersion is the prime important factor for stability of formulation of any colloidal system. Generally, particles with zeta potential of ±30 mV could be considered as stable due to electric repulsion between particles 11. The individual zeta potential for BUTE-SLN ingredients were -16.9 mV for butenafine, -4.81 mV for surfactant and 20.5 for lipid OLML. From the given data we can predict that lipid has more influence on zeta potential. The surfactant induces comparatively less negative charge on zeta potential of particles. The surface covered butenafine, surfactant, TPGS and organic solvent collectively induce negative charges.

The regression equations generated for entrapment efficiency and drug loading (Y_4= +89.7075 - 0.17*X_1 + 1.1825*X_2 + 0.3575*X_3 - 0.565*X_1X_3 - 0.2975*X_2X_3) and (Y_5= +18.8625 - 0.21*X_1 - 1.7075*X_2 - 1.585*X_3 + 0.175*X_1X_2) were significant with F value of 848.75 and 12.22 with ($p < 0.0001$) respectively. Figure 1 D and figure 1 E demonstrate the response surface plots of the effect of surfactant and lipid concentrations on entrapment efficiency and the effect of organic solvent and surfactant concentrations on drug loading respectively. Increase in concentration of lipid showedremarkable increase in entrapment efficiency as the solubility profile of drug showed higher solubility of drug in selected lipid. Due to drug distribution into aqueous and lipid phases, an increase in surfactant concentration resulted in a slight increase in entrapment efficiency. The increase in concentration of surfactant causes formation of micelles which have a property to dissolve lipophilicdrug hence reduces the solubility in lipid phase 29. Cosurfactant also helps to dissolve drug into SLNas it has tendency to support the emulsifying property of surfactant and additionally improves the drug solubilization.

Organic solvent and surfactant concentration showed positive influence on the drug loading.Organic solvent used here dissolves more amount of drug and at the time of cooling of SLN dispersion, lipid core encapsulates DMSO in between imperfect crystal lattice structure of lipid. Solidified SLN showed nanostructured lipid carrier like structure which is composed of solid lipid inamorphous form with various lattice defects in which drug dissolved in organic solvent is presented as pockets. Along with independent variables some practical experimentation work has been done for fabrication of modified SLN such as sudden cooling of hot SLN dispersion leading to sudden precipitation of lipid and drug which forms lipid in α form containing maximum amount of drug andorganic solvent.

The nanometric size of BUTE-SLN was demonstrated by transmission electron microscopy (TEM) study of particle structure and surface morphology. Figure 2 illustrates that the BUTE-SLN formulated appeared to be spherical in shape, with narrow particle size distribution which is in goodagreement with the PCS and PDI analysis.

DSC is a powerful investigational tool used to determine the physical properties of compoundsuch as crystalline or amorphous nature of sample which can be drawn on the basis of fact that different lipid modification exhibit different melting point and physicochemical properties. Figure 3 A shows the thermal analysis of pure butenafine, Lipid OLML and BUTE-SLN. A sharp endothermic peak of melting point was observed on a DSC thermogram of pure drug at 217.35°C.Thermogram for pure lipid OLML showed a sharp endothermic peak at 63.20°C indicating lipid is present in pure crystalline form. Further small peaks are attributed to the decomposition of formed small liquid droplets. The curve for BUTE-SLN showed a left side shifted small and broad peak at 46.91°C. The result shows crystalline lipid is converted into amorphous state as the heat flow throughlarger and perfectly ordered crystal requires greater energy resulting in large and sharp peaks while heat flow through small and less ordered particle requires smaller amount of energy to melt the particle. Our solid lipid nanoparticle formulation showed small and broad peaks with the difference of 16.29°C with pure lipid indicating the presence of nanostructure in amorphous form along with surfactant and pockets of organic solvent inside it 30, 31.

From the XRD data, we can clearly observe sharp diffraction peaks of butenafine at 2 θ valueof 16.0768 with d value of 5.4996 and pure lipid 2 θ at 21.664 with d value 4.1124 which indicate highly crystalline nature of the compound as shown in figure 3 B Whereas BUTE-SLN formulation showed broader and shorter peak with no specific diffraction peak for butenafine revealing encapsulation of drug within the lipid matrix which existed in amorphous form 32. The comparativeFT-IR spectra of butenafine, pure lipid OLML and BUTE-SLN shown in figure 4. FTIR spectra for butenafine gives a typical specific absorption character at 3047.63, 1365.65 and 1072.46 cm^{-1} corresponding to –C-H stretch, -C-C and –C-N vibrations respectively, but the BUTE-SLN spectra showed absence of these peaks. The FTIR results reveal that there is no strong interaction and no incompatibility and butenafine is successfully encapsulated into lipid structure.

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For the effective topical delivery, some constrains should be considered which include physicochemical parameters of drug such as log P, pKa values, rheological study of gel enriched SLN, occlusive and hydrating potential of formulation and disease state. Natural origin *aloe vera* gelwith many additive effects such as excellent anti-inflammatory action, natural healing ability and moisturizing effect is beneficial for diseased condition of skin. *Aloe vera* gel contains the building blocks of glucomannan, C-glucosyl chromone, lupeol and 8 enzymes, *Aloe vera* gel is useful and comfortable to treat exaggerated conditions of skin 33. In the evaluation of semisolid formulation viscosity and spreadability parameters are given more attention because the ultimate formulation wasgoing to be used on inflamed part of skin. BUTE-SLN *aloe vera* gel showed pseudoplastic rheologicalflow which is expected for the skin condition. Small nanosized particle showed excellent slip propertyrequired for spreading of gel onto surface of inflamed skin. Entrapment of SLN into gel network slightly increases the rheology of gel due to increase in amphiphilic surfaces upon which water can bind and become immobile.

The process of SLN permeation starts with occlusion and skin hydration. As SLN is composedof solid lipid and stratum corneum also contains intercellular lipids such as cholesterol, phospholipidsand ceramides, SLN have strong affinity towards stratum corneum. Hence, they form impermeable occlusive layer on the surface of skin preventing the trans epidermal water loss. Due to water pore dynamics, pores that exist in uniform spreading of SLN gel attract the permeated vapour which condensates at pores of SLN. Hence, due to negative pressure they attract water than to lose water. This particular mechanism is very useful in the treatment of superficial fungal infections which causesthe scaling, dry skin, irritation etc. 34, 35. Stratum corneum is composed of compactly packed corneocytes and intercellular lipids. Due to occlusive film and reduction in trans epidermal water loss, hydration of stratum corneum takes place which reduces the compact packing between corneocytes and increases the pore size. This is the possible mechanism behind transport of SLN through skin.

Drug release and permeation showed the biphasic release pattern of drug from SLN matrix. This can be due to use of some novel excipients such as surfactant OLMS, DMSO and TPGS whichentraps the drug inside forming nanosized mini pockets in SLNs. The process of fabrication provokedthe α state lipid transformation hence could include more amount of above said excipients. The formation of α state lipid structure slowly convert into β' form and then most stable β form then slowly expel the drug from lipid matrix which accounts for sustained release.

The possible mechanism for diffusion of drug from lipid matrix can be start with possible events like distribution of drug between lipid and DMSO, OLMS and TPGS, diffusion of drug through lipid, transformation of lipid from α state to most stable β form, distribution of drug in between lipid and dissolution medium, release of drug to dissolution medium. Incorporation of TPGSaids in the burst release from SLN matrix. This could occur at the time of sudden cooling of dispersion. The saturated emulsion when cooled down suddenly leads to solidification of drug and lipid and forms the drug lipid matrix while presence of TPGS and DMSO partially solubilizes the drug in aqueous phase from which drug get precipitated at the time of cooling. This form a uniform coat around SLN particle when such formulation was applied to skin show initial burst release. The efficacy of formulation was also depending upon the skin condition like diseased state, type of infection, inflammation, oedema and dry skin. All these pose the problem in permeation of drug in tothe superficial layer of skin. In disease state the superficial layer of skin gets disturbed exposing the epidermal cell along with existence of various metabolites of fungal cell, enzymes secreted by fungalcell and body immune system (cytokines) degenerate the drug into inactive fragments. Marketed cream showed the reduced diffusion of drug in to skin layer as compared to SLN which form a depotby concentrating in to SC and releases drug slowly. Skin retention studies revealed that the BUTE- SLN dominantly concentrates into stratum corneum which form reservoir for further release of drugand natural surfactant OLMS aid in partitioning of drug properly into epidermal cell 35.

The skin irritation study reveals nonirritant nature of BUTE-SLN gel which explains its usefulness in treatment of fungal infection. To examine the comparative efficacy of butenafine with marketed cream and to establish the effect of different method parameters on the formulation of BUTE-SLN, an *in vitro* antifungal analysis was conducted. Zone of inhibition of marketed preparation was found to be 30 mm and for BUTE-SLN was found to be 32 mm which is greater thanthe marketed formulation proving the therapeutic efficacy of SLN formulation.

CONCLUSION

Various formulation ingredients like lipid, surfactant and DMSO have influence on fabrication ofSLN dispersion which directly affects the particle size, entrapment efficiency and zeta potential of SLN. The DSC and XRD studies indicated that the optimized formulation was amorphous in nature, containing the drug, DMSO and surfactant entrapped inside the SLN. Rheological and spreadability study of BUTE-SLN *aloe vera* gel reveals the excellent slip properties beneficial for inflamed skin application. Along with excellent rheological and spreadability BUTE-SLN gel possesses good occlusive property and hydrating potential. For long term drug delivery through stratum corneum BUTE-SLN showed accumulation of the SLN in the stratum corneum with controlled release of drug.BUTE-SLN demonstrated increased skin deposition, improved *in vitro* antifungal action, and a topical formulation that was less irritating. As a result, the BUTE-SLN *aloe vera* gel formulation is cost-effective, novel, and reproducible alternative to the currently available traditional dosage method.

ABBREVIATIONS

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SLN: Solid lipid nanoparticles, BUTE: butenafine hydrochloride, TEM: Transmission electron microscopy. FTIR: Fourier transform infrared spectroscopy, DSC: Differential scanning calorimetry, XRD: X-ray diffraction study, CMI: Cell mediated immunity, TPGS: D-α tocopheryl polyethylene glycol succinate, DMSO: Dimethyl sulfoxide, PS: Particle size analysis, PDI: Polydispersity Index, PCS: Photon correlation spectroscopy, ZP: Zeta potential, EE: Entrapment efficiency, DL: Drug loading, RH: Relative humidity, OECD: Organsation for economic co-operation and development, SD: standard deviation.

Ethics

Ethics approval and consent to participate

Not applicable as no animal or humans are used in this study.

Consent for publication

This work is original and not published or under consideration in any other journal.

Availability of data and materials

Will be made available on request.

Competing interests

The authors declare that they have no competing interests

Funding

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Author Contribution

Investigation, literature research, experimenting, analysis, interpretation of the data, statistics, preparing the study text and writing the original draft was done by AB. Developing a hypothesis, reviewing the text, resources and supervision was done by VK and MA. DB and SA did the literature research, interpretation of the data and statistical analysis.

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Table 1. Independent variables and their levels of 2^3 factorial designfor formulation of BUTE-SLN.

Factors	**Levels**	
	Low (-1)	**High (+1)**
Concentration of Surfactant % (X1)	2	4
Concentration of lipid % (X2)	3	5
Concentration of organic solvent % (X3)	0.5	1.5

Table 2. 2^3 factorial design for formulation of BUTE-SLN.

Run Code	**Concentration of lipid %(X1)**	**Concentration ofsurfactant % (X2)**	**Concentration of organicsolvent % (X4)**
1	2	5	1.5
2	4	5	0.5
3	4	3	0.5
4	2	3	1.5
5	2	3	0.5
6	4	5	1.5
7	4	3	1.5
8	2	5	0.5

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Table 3. Observed responses of eight experimental formulations (2^3factorial design)

Run Code	Particle size* nm	PDI*	Zeta potential* mV	% EE*	% DL*
1	271±2.74	0.368±0.014	20.2±0.32	91.2±1.82	18.33±0.79
2	258±3.61	0.267±0.009	24.9±0.41	91.65±1.69	18.24±0.86
3	231±2.79	0.254±0.012	17.8±0.43	88.95±2.17	21.05±0.35
4	250±3.65	0.324±0.009	12.3±0.22	88.29±1.73	22.86±0.46
5	245±4.12	0.241±0.005	11.7±0.16	87.45±2.26	21.33±0.78
6	278±3.19	0.369±0.01	22.4±0.26	89.25±1.58	15.2±0.31
7	239±2.37	0.33±0.009	17.23±0.39	88.41±1.42	22.16±0.91
8	269±4.26	0.264±0.009	21.5±0.31	90.46±2.36	15.05±0.58

*Each value represents as mean ± Standard deviation (SD)

Table 4. Observed and predicted responses for optimized formulation parameters.

Reponses	Constraints	Set observed values*	Predicted values	% Error
Particle Size (nm)	Minimize	261.250±2.38	264	1.041
PDI	Minimize	0.268±0.01	0.27	0.7407
ZP (mV)	Minimize	23.98±0.27	24.60	2.5203
EE %	Maximize	91.35±2.35	91.225	1.370
DL %	Maximize	19.692±0.95	18.705	5.2766

*Each value represents as mean ± Standard deviation (SD)

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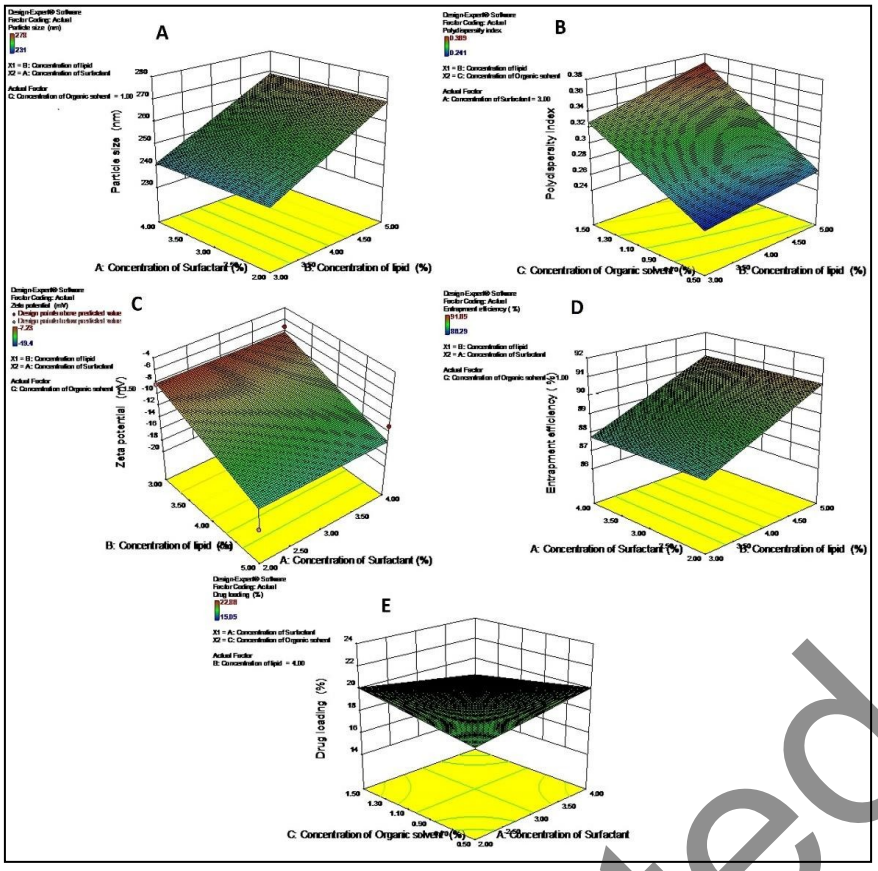

Figure 1. 3D Response surface plot of BUTE-SLN showing

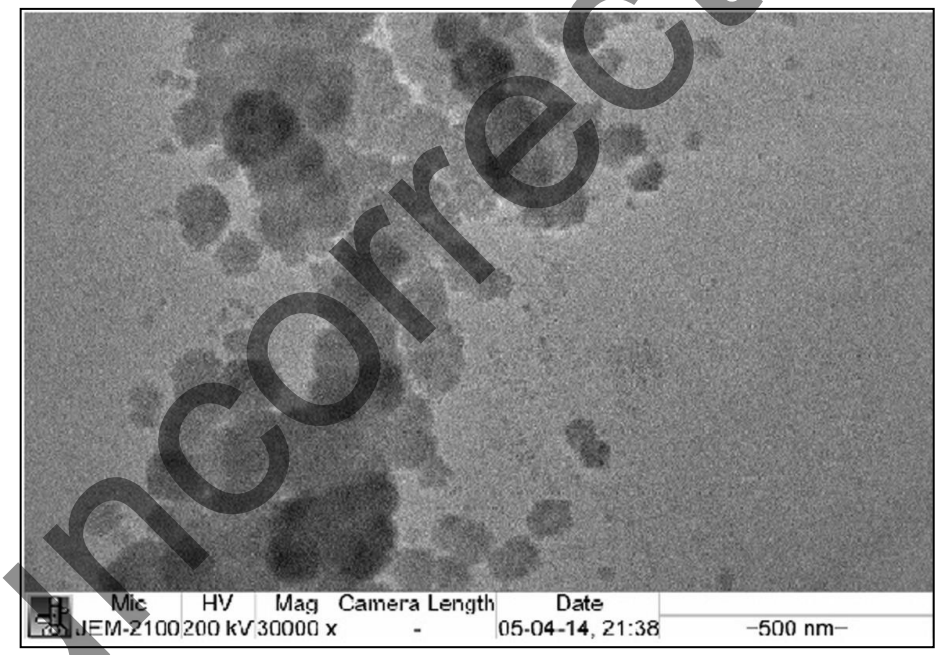

Figure 2. The TEM image of BUTE-SLN.

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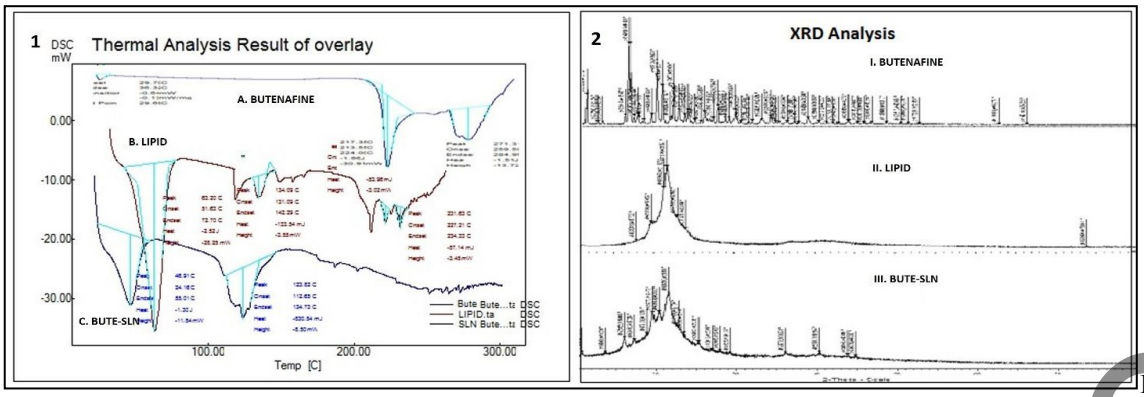

Figure 3. (1) DSC thermogram of A. Butenafine, B. pure lipid OLML, C. BUTE-SLN and (2)Comparison of X ray diffraction pattern: I. Butenafine, II. lipid OLML III. BUTE-SLN.

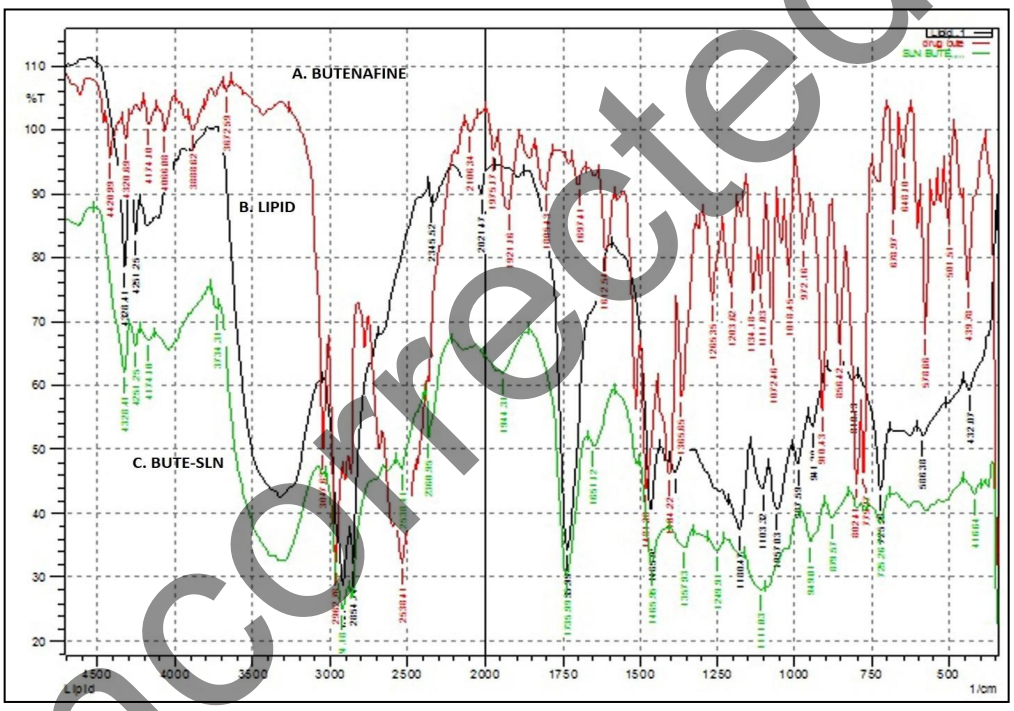

Figure 4. Comparative FTIR spectra of A. Butenafine, B. lipid OLML and C. BUTE-SLN.

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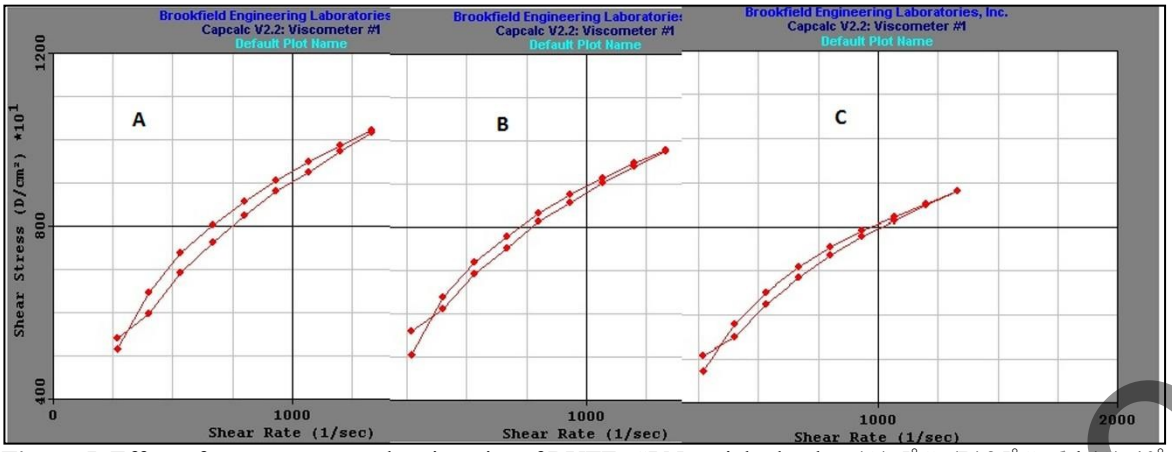

Figure 5. Effect of temperature on the viscosity of BUTE -SLN enriched gel at (A) 5˚C, (B)25˚C and (C) 40˚C.

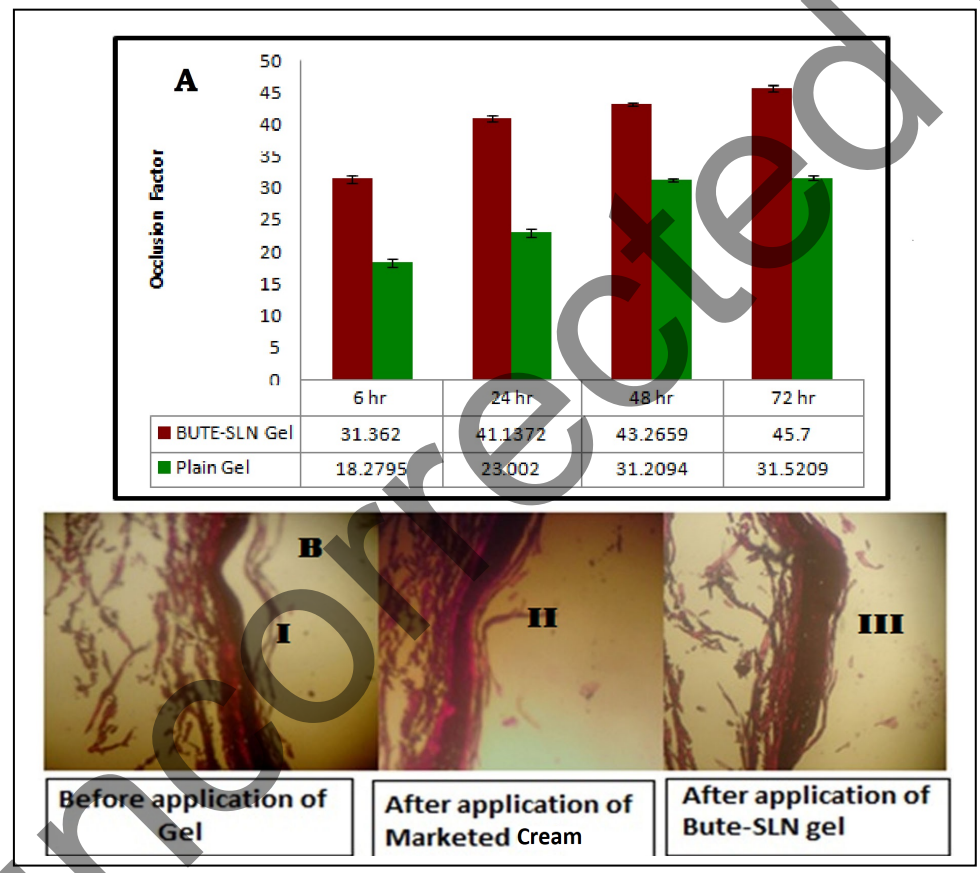

Figure 6. A Comparison of occlusion factor of BUTE -SLN enriched gel and plain gel B Photomicrograph showing skin hydrating potential of BUTE-SLN gel compared with marketed cream and untreated human cadaver skin.

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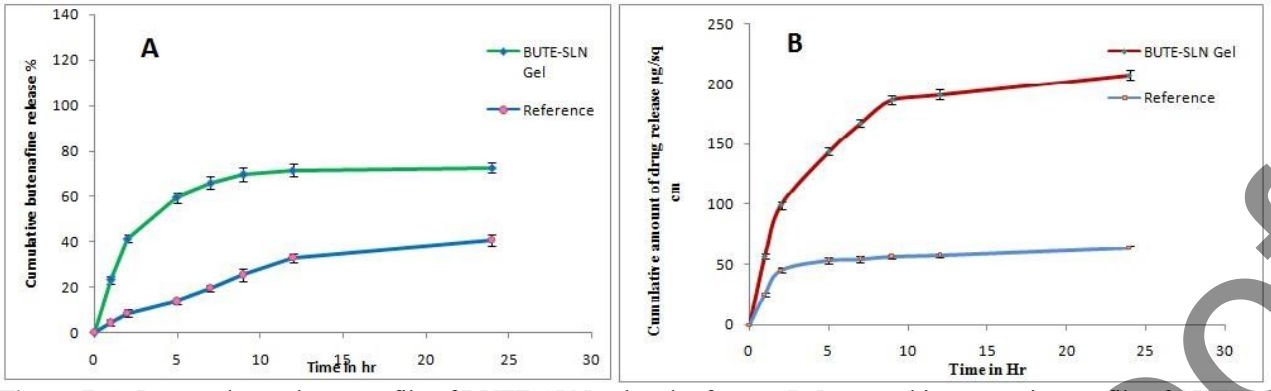

Figure 7. A *In vitro* drug release profile of BUTE-SLN gel and reference B *In vitro* skinpermeation profile of BUTE from SLN gel and reference.

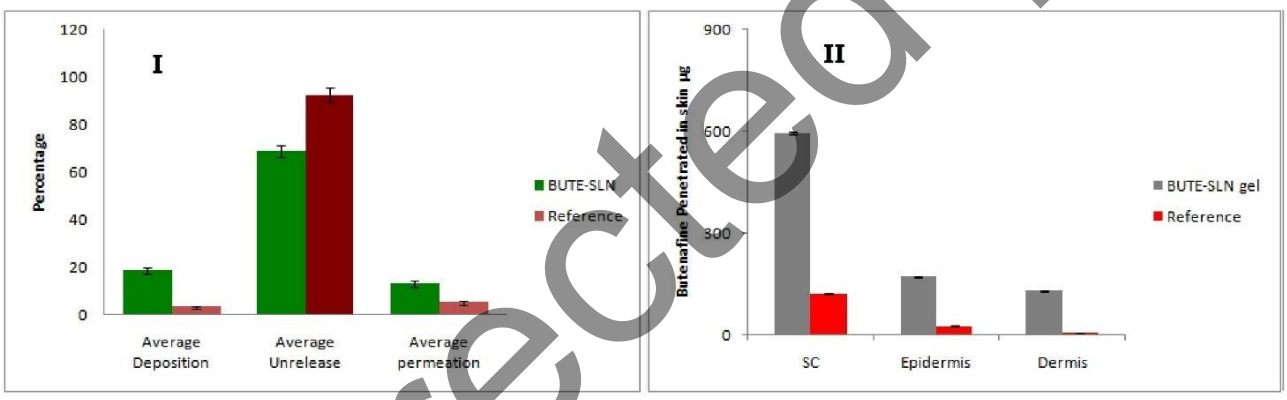

Figure 8. I Percentage of drug permeated, deposited and remain unabsorbed in the skin II *Invivo* skin retention of drug in different skin strata.

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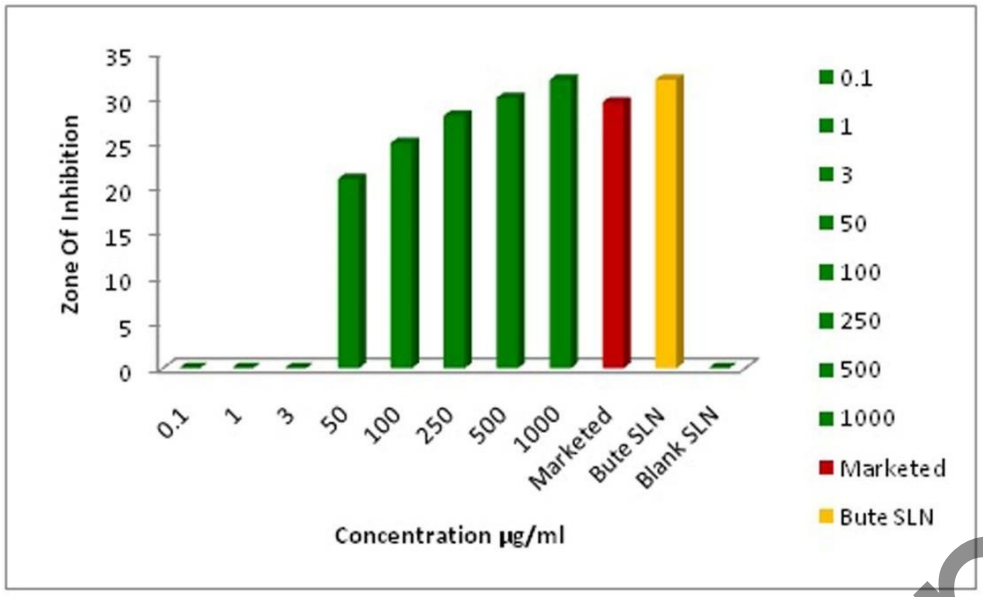

Figure 9. Comparative Antifungal activity of different dilutions of Butenafine with marketed and BUTE-SLN formulation.

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