

DEVELOPMENT AND EVALUATION OF ANTIFUNGAL TOPICAL NIOSOMAL GEL FORMULATION

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ABSTRACT

Niosome are now widely studied as an alternative delivery system to liposome. An increasing number of non ionic surfactant has been found to form vesicles, capable of entrapping hydrophilic and hydrophobic molecules. In our present study we incorporated Nystatin into niosome by using ether injection method by applying 3² factorial design. The niosomes were characterized for size distribution, drug entrapment efficiency, zeta potential and drug release profile. Topical applicability of niosomes was further enhanced by developing niosomal gel formulation using carbomers. Developed niosomal gel was evaluated for pH, spreadability, viscosity, drug content, drug release profile and *ex vivo* deposition study. Microscopic observation and TEM confirmed the uniformity of size and shape and was found to be in the range of 182-219 nm. The entrapment efficiency of the vesicles was determined by ultracentrifugation and was found to be in the range of 62-85%. The release from the niosomal gel was highly prolonged when compared to conventional gel and showed two fold increases in the drug deposition in the skin compared to conventional gel. The stability studies showed that vesicles have greater stability at 4°C followed by 25°C. From the present investigation, it can be concluded that the developed niosomal gel formulation of nystatin has shown great potential in the treatment of fungal infection by providing a prolonged release profile.

Keywords: Niosomes, Factorial design, TEM, Entrapment efficiency, Antifungal.

INTRODUCTION

Non-ionic surfactant vesicles (niosomes) are now widely studied as an alternative to liposomes¹, capable of forming vesicles & entrapping hydrophilic and hydrophobic molecule.² Niosomes are uni or multilamellar vesicles formed from synthetic, non ionic surfactant of alkyl or dialkyl poly glycerol ether class, offering an alternatively to liposome as drug carriers. Niosomes can entrap solutes in a manner analogous to liposomes, are relatively more stable *in vitro* and can improve the stability of entrapped drug as compared to the stability of conventional dosage forms.³ Niosomes characteristics such as lamellarity, lipid composition and structure⁴ surface charge and size⁵ and physicochemical nature of drug itself⁶ may affect follicular deposition of drugs. Niosomes offer several advantages over liposomes such as higher chemical stability, intrinsic skin penetration enhancing properties and lower cost.⁷ In topical preparation it has advantage that increase the contact time with the applied tissue.⁸

Fungal disease is ubiquitous in the world and antifungal medication account for sales of more than US\$ 1 billion annually. Most fungal disorder is relatively benign but can become life threatening in immunocompromised or malnourished population. The mainstay of management of fungal infection and dermatophytes associated with skin and nail injuries has been oral and topical antifungal.⁹ Nystatin is polyene antifungal antibiotic used topically as well as orally. The molecular weight of nystatin is 926.13 and it highly lipophilic drug. Oral bioavailability of nystatin is 0%. Thus, by considering nystatin problems we have to encapsulate the drug into the non-ionic surfactant vesicles (niosomes) and administered topically to deliver drug to the site of action.

Drug deposition, vesicle size and entrapment efficiency were the key parameters involved in formulation of topical niosomes. The number of formulation and processing variables are involved during niosome preparation may affect these parameters and hence the performance of the formulation. Thus it becomes extremely difficult to study the effect of interactions between various variables and preparation of niosome by a conventional method. Factorial design and response surface methodology is an important statistical tool to study the effect of several factors influencing responses by varying them simultaneously by carrying out limited number of experiments.¹⁰

Thus the aim of the present investigation was to prepare niosomal formulation, using factorial design approach and to explore its application for topical delivery of nystatin.

MATERIALS AND METHODS

Materials

Nystatin was supplied by Cipla pharmaceutical ltd. Mumbai. Cholesterol, Span 60 and stearic acid were purchased from Loba chemical Pvt. Ltd., Mumbai. All other chemical used were of analytical grade. The protocol for the animal study was approved by Institutional Animal Ethical Committee.

Method for preparation

The surfactants, lipid and drug were first dissolved in suitable organic solvent. The prepared organic phase was then added dropwise into aqueous phase. In brief thus the surfactant : cholesterol ratio 2:1 along with nystatin and stearic acid were dissolved in 10 ml diethyl ether. Thus the dissolved organic solution containing drug were injected dropwise through 24 gauge needle into preheated 10 ml distilled water, which is magnetically stirred and maintained at 65°C for 45 min. Stirring was continued until all ether evaporating to get drug loaded niosome. Vaporization of ether leads to formation of single layered vesicle.¹¹

Effect of variables

To study the effect of variables on the niosomal formulation 3² factorial design was adopted. Amount of Span 60 and Cholesterol were selected as two independent variables whereas entrapment efficiency & vesicle size were considered as dependent variables. The results of the study are shown in Fig 1, 2. Amount of Stearic acid and nystatin were kept constant.

Preparation of Niosomal gel and conventional gel

Niosomal gel was prepared by adding niosomal dispersion (N7) in to suitable gel base like carbopol U21. Various batches of the gels were prepared using carbopol at varied concentrations (0.1%, 0.5%, 1% and 1.5%). Definite amount of polymer was sprinkled into the vortex created by stirring double distilled water and stirred for 25-30 min. Gelling was induced by neutralization using triethanolamine.

Niosomal dispersion was then added to the hydrated gel with stirring.

A conventional gel (1%) was prepared by adding small portions of gelling agent (Carbopol Ultrez 21) to the dispersion of the drug in

Characterization of niosomes and niosomal gel

Niosomal dispersion of nystatin was successfully prepared by ether injection method. The concentrations of Span 60, cholesterol were optimized by factorial design to obtain stable niosomal dispersion devoid of aggregation, fusion and sedimentation (visual observation). Stearic acid (0.05%) was found to be optimum to maintain required bilayer rigidity, with increased concentration grittiness was observed in the formulation, hence optimum concentration of the stearic acid was used and kept constant in all the batches. Amount Span 60 and cholesterol were found to be critical in preparation and stabilization of niosomes and hence selected as variables in the 3² factorial design (Table 1).

Table 1: Shows 3² Factorial Design for Niosomal batches

Batch no.	Span 60	Cholesterol
N1 (-1,-1)	40	20
N2 (-1, 0)	40	30
N3 (-1,+1)	40	40
N4 (0,-1)	50	20
N5 (0, 0)	50	30
N6 (0,+1)	50	40
N7 (+1,-1)	60	20
N8 (+1, 0)	60	30
N9 (+1,+1)	60	40

Organoleptic properties

Niosomal dispersion and niosomal gel were characterized for appearance color, odour, and pH.

Mean vesicle size and zeta potential measurement

Mean vesicle size and size distribution of nystatin loaded niosomes were determined using Zeta sizer 300HSA (Malvern instrument, Malvern, UK) based on photon correlation spectroscopy at room temperature by keeping angle of detection at 90°. Responses of different batches obtained were subjected to multiple regression analysis using 'PCP Disso V 3' software (IIPC, PCP, Pune, India). The data were fitted in Eq. (1)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{12} X_1 X_2 \text{ ----- Eq. (1)}$$

Transmission electron microscopy (TEM)

The surface appearance and shape of nystatin loaded niosomes was analysed by taking TEM photographs using Hitachi S-7500 transmission electron microscope (Hitachi, Japan). The nanovesicles were dispersed in water and one drop of the diluted dispersion was placed on a 200-mesh carbon coated copper grid. The photographs were taken at 30,000X magnification and 100 kV voltages.¹²

Percentage drug entrapment (PDE)

Free nystatin was separated from entrapped nystatin in niosomes by ultracentrifugation at 40,000 rpm at 4°C for 30 minutes using an ultracentrifuge model LE-80K with rotor type 70Ti (Beckman, California, USA). Supernatant containing free nystatin was collected and analyzed by UV spectrophotometer at 304 nm. Percent drug entrapment was calculated by using following formula,

$$PDE = \left(\frac{\text{Total amount of nystatin} - \text{free amount of nystatin}}{\text{Total amount of nystatin}} \right) \times 100$$

Assay

Niosomal gel (500 mg) containing 10 mg nystatin was taken and dissolved into 50 mL PBS pH 6.8. The sample was stirred at 200 rpm

water under stirring. After complete addition of the polymer, gelling was induced by neutralization using triethanolamine.

with addition of 1% isopropanol. Drug content was then determined using UV spectrophotometer at 304 nm.

Drug content uniformity

Drug content uniformity of niosomal gel was determined by analyzing the drug concentration in the sample taken from four different points. The gel samples were dissolved in 50 ml PBS (pH 6.8) and stirred at 100 rpm to facilitate rupture of the vesicles. Drug (nystatin) content was determined using UV spectrophotometer at 304 nm.

Viscosity of gel

Viscosity of conventional gel containing nystatin (2 %w/w) and niosomal gel (containing niosomal dispersion-N7) was measured using Brookfield LV model viscometer with spindle no. 52 at 25°C. Spindle was lowered into cup containing gel and rotated at speeds 0.3–12 rpm. At each speed the corresponding dial reading on the viscometer was recorded. Direct multiplication of the dial reading with the factors gave the viscosity in centipoises.

Spreadability of Niosomal gel

Spreadability is one of the important characteristics of any topical preparation as far as patient compliance is concerned. It was determined according to the method described by Riffin et. al. About 1gm of gel was placed between the two glass slides onto which weights were allowed to rest. The top slide was then subjected to pull of 100 gm. The time in seconds required for the top slide to travel 100m distance gave an idea of the relative spreadability of gel.

Drug release from niosomal dispersion and niosomal gel

In-vitro release studies were performed using Franz diffusion cells. Phosphate buffer pH 6.8 was used as receptor fluid. Nylon membrane (0.22 µm) was soaked in phosphate buffer pH 6.8 for 1 hrs before carrying out the study. 500 mg of niosomal gel (N 7) containing 10 mg drug was placed onto the donor compartment. Samples were collected at 1, 2, 3, 4, 5, 6, 7, 24 and 48 hrs intervals and analyzed by U.V. And drug release from niosomes was studied by employing the dialysis method.

For *ex vivo* diffusion study, porcine skin was used. Porcine skin from the ear was shaved and skin was carefully separated. Subcutaneous fat was removed using a scalpel & the skin section was mounted on Franz Diffusion cells having surface area of 3.14 cm². The protocol for the preparation of whole skin was approved by Institutional Animal Ethics Committee. Previously stabilization of the skin was done in order to prevent any interference from the skin in absorbance. The release study was carried out as described above. The results are shown in Fig 5.

Deposition study

For determination of drug deposited in the skin, cell was dismantled after 48 hrs and skin was carefully removed from the cell. Drug present on the surface of the skin was removed. After stripping the skin was cut into small pieces and drug present in the skin was extracted in methanol under bath sonication for 1 hr. The drug content in the extract was estimated by using UV spectrophotometer at 304nm. Similarly the above described procedure was used to calculate the drug depositions from Nystatin niosomal formulations, conventional gel & nystatin dispersion.

Antifungal study

Antifungal activity of selected nystatin niosomal dispersion (N7) and niosomal gel (containing N7 niosomal formulation) were carried out by cup plate method. Niosomal dispersion (N7) and respective niosomal gel were studied against *Candida albicans* 36082 using

sabourad dextrose agar media. The conventional dispersion and gel were also evaluated for antifungal activity.

Primary skin irritation studies of niosomal gel

Primary skin irritation studies of the selected formulation were performed using albino rabbits in accordance with the guidelines of the Consumer Product Safety Commission.¹³ Formalin was taken as positive control and plain gel was used as negative control in the study.¹⁴ The study was approved by the Institutional Ethics Committee (IAEC).

Stability of niosomal dispersion and niosomal gel

Stability of selected niosome dispersion (N7) and niosomal gel containing N 7 niosomal formulation were carried out at $4\pm 2^\circ\text{C}$ and $25\pm 2^\circ\text{C}/60\% \text{RH}\pm 5\% \text{RH}$. Effects of temperature and RH on the vesicle size and entrapment efficiency for niosomal dispersion were studied. The drug content, content drug uniformity, spreadability were studied for niosomal gel during stability period.

RESULTS AND DISCUSSION

Evaluation of Niosomal dispersion

Organoleptic properties

The niosomal dispersion was off-white in color, odourless, and fluid in nature. It was stable and did not show sedimentation. pH was found to be in the range of 4.7-5.2.

Mean vesicle size

The mean vesicle size of drug loaded niosomes of the different batches according to the factorial design ranged between 182–219 nm. The polydispersity index (PdI) was in the range of 0.207 – 0.341 for drug loaded niosomes which indicates a narrow vesicle size distribution. The mean vesicle size and PdI of all the nine batches of factorial design is shown in table 2.

It was observed that the relative amount of span 60 and cholesterol was found to play an important role in the determining of vesicles size. Vesicle size of drug loaded niosomal batches were found to decrease as concentration of span increases. Hydrophobic nystatin intercalate into the lipid bilayer leading to appreciable cohesion among a polar portion of the membrane, causing reduction in the vesicle size. Typical profile of particle size distribution in the prepared niosomes was showed in Fig 1.

A good correlation was observed for both variables X1 (Span 60) and X2 (Cholesterol) in vesicle size of drug loaded niosomes ($r^2 = 0.9986$). To understand the effect of lipid concentration on vesicle size of the drug loaded niosomes, observed coefficient values for the drug loaded niosomes was fitted in Eq. (1) to generate Eq. (2).

$$Y = 215 - 4.6667 X_1 + 3.6667 X_2 - 24.6667 X_1 X_1 \text{ ----- Eq. (2)}$$

Table 2: Shows responses obtained for studied parameters from experimental batches

Batch no.	Polydispersibility index	Drug content (%)	Entrapment efficiency (%)	Particle size (nm) \pm S.D.	Zeta potential (Mv) \pm S.D.
N1	0.325	99.23	70.56	192 \pm 0.84	-27.77 \pm 1.55
N2	0.289	98.62	66.32	194 \pm 0.86	-24.84 \pm 0.79
N3	0.341	98.13	62.74	199 \pm 0.97	-23.35 \pm 1.87
N4	0.285	101.41	78.54	211 \pm 0.90	-25.44 \pm 1.86
N5	0.225	98.76	75.09	215 \pm 0.77	-28.07 \pm 0.75
N6	0.207	99.52	74.04	219 \pm 0.80	-31.57 \pm 0.16
N7	0.267	97.29	85.80	182 \pm 0.59	-29.69 \pm 1.03
N8	0.304	97.93	83.10	186 \pm 0.61	-28.27 \pm 0.28
N9	0.259	99.45	80.25	189 \pm 0.55	-30.55 \pm 0.28

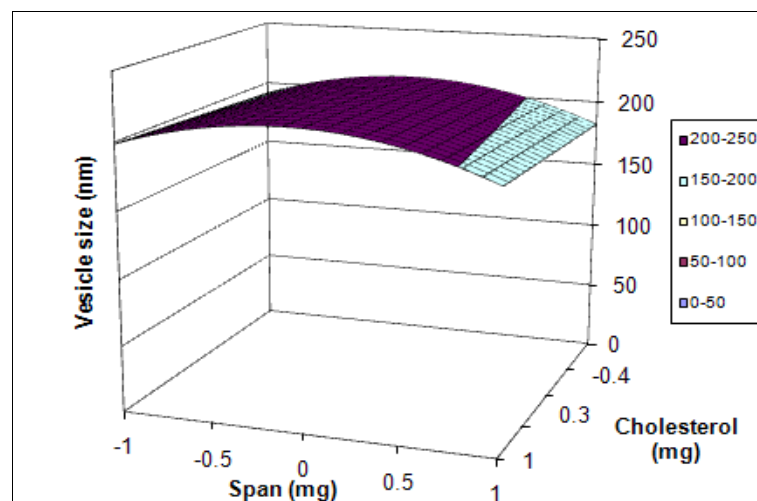


Fig. 1: Shows the effect of variables on vesicle size of niosomes

Zeta (ζ) Potential Determination

The values of ζ potential of the optimized drug loaded niosomal formulation was - 23 to - 31 mV. The effect of process variables was found to be insignificant in case of zeta potential. Zeta potential

value $> \pm 30$ mV is essential for effective stability and to inhibits aggregation. The values of zeta potential showed prepared niosome have sufficient charge to inhibit aggregation of vesicles due to electric repulsion.

Percent drug entrapment

PDE was expressed as fraction of drug incorporated into niosomes relative to total amount of drug used. In the present study the observed percentage entrapment efficiency for all batches were in the range of 62%-85%. percentage entrapment efficiency it was observed that it was significantly affected by the applied processing variables such as concentration of span 60 as well as cholesterol. It was clearly indicated that with increase in concentration of span 60 percentage entrapment efficiency was increased where as concentration of cholesterol was inversely related to the percentage entrapment efficiency.(Fig 2)

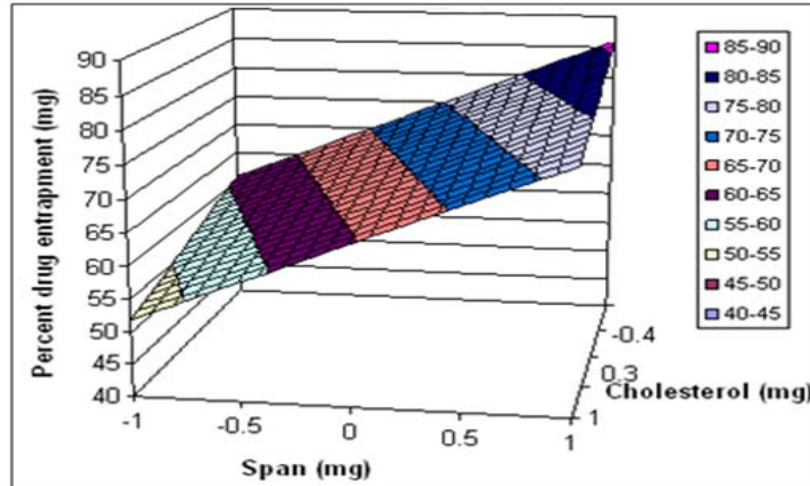


Fig. 2: Shows the effect of Variables on % Entrapment Efficiency of niosome

Transmission electron microscopy (TEM)

TEM image of nystatin loaded niosomes was shown in fig 3. The niosomes were spherical in shape. The vesicle size was in the range of 182-219 nm supplementing the results of particle size analysis.

Evaluation of Niosomal Gel

Assay and content drug uniformity

Drug content for niosomal gel was found to be 97.75% and content uniformity obtained was 99.19 % + 0.96%.

Viscosity

Viscosity of niosomal gel and conventional gel were found to be 11,550 cps and 25,000 cps respectively (Table 3).

Spredability

Spredability of developed niosomal gel was found to be 22.72gm cm/sec.

Organoleptic properties

Carbopol gels prepared with 0.5% w/w carbopol was not of good consistency, they were fluidy / watery in nature. Whereas gel with 1.5 % w/w carbopol was stiff. Carbopol gel (1% w/w) was found to be of good consistency and acceptable feel. Hence 1 % carbopol gel was used to make niosomal gel. The niosomal gels were off-white in

When regression was applied to Significant effect of process variables was observed in PDE. To understand the effect of Span 60 and CH concentration on PDE, the coefficient observed for the drug loaded niosomes was fitted in Eq. (1) to generate Eq. (3). A good correlation was observed for both variables X1 (Span 60) and X2 (CH) in % drug entrapment of niosomes ($r^2 = 0.9865$).

$$Y = 75.16 + 8.225 X1 - 2.978 X2 \text{ ----- Eq.(3)}$$

color, opaque, odorless, with smooth appearance devoid of any aggregation. Gel pH was found to be in the range of 6.4-6.6.

Drug release

The *in vitro* and *ex vivo* release study were done by using nylon membrane (0.22 μm) and porcine skin respectively. The *ex vivo* diffusion data indicates that the released is diminished to a great extent as compared to the *in vitro* release. This could be because of accumulation of drug in the skin layers. This result was probably due to the release retarding effect of the polymeric matrix of gelling agents.¹⁵ The release profile of the developed formulation data is shown in Fig 4 & 5.

Skin deposition study

Skin deposition studies performed by using porcine skin. Results indicated that 43.78 ± 3.71 of drug was accumulated in the skin layers from niosomal gel formulation.(Fig 6) Thus niosomal gel formulations enhanced the permeation of the drug in epidermal layer but did not enhance the penetration of the drug through the skin layers into the diffusion medium.¹⁶

Antifungal activity

Antifungal activity showed that niosomal dispersion showed greater zone of inhibition compared to niosomal gel because drug release from gel formulation was retarded compared to niosomal dispersion. The zones of inhibition of different formulations were measured. The results are shown in fig 7.

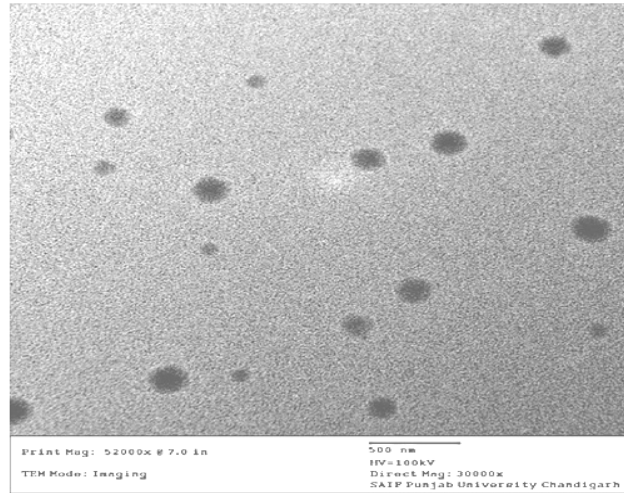


Fig. 3: Shows the TEM image of drug loaded niosomes

Table 3: Shows evaluation of Conventional gel and Niogel

Parameter	Conventional gel	Niogel
Apperance	Transparent homogeneous gel	Opaque, homogeneous gel
Colour	Colourless	Off-white
pH	6.9-7.1	6.4-6.7
Viscosity	25000 cps	11500 cps
Drug content	98%	97.75%
Spredability	21 gm cm/sec	22.72 gm cm/sec
Microscopic evaluation	No aggregation or lump were seen	No aggregation or lump were seen

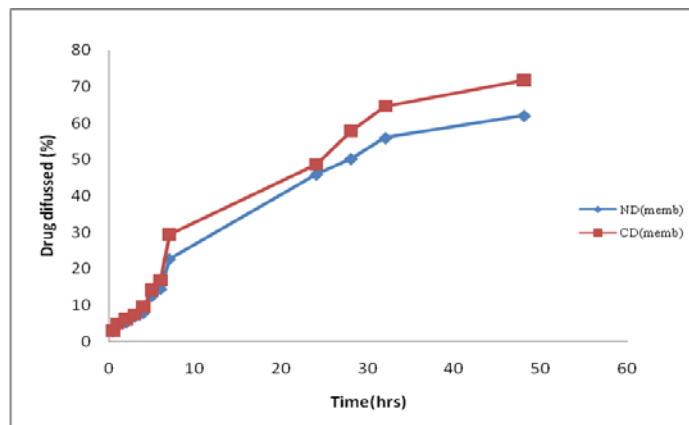


Fig. 4: Shows *In vitro* drug release study

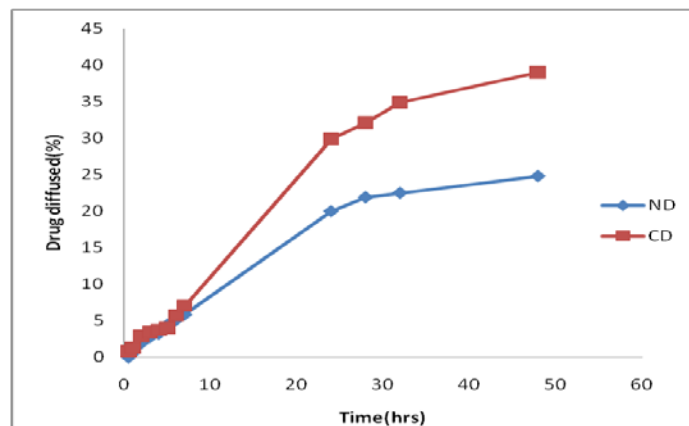


Fig. 5: Shows *ex vivo* drug release study

ND: Niosomal dispersion, CD: Conventional dispersion

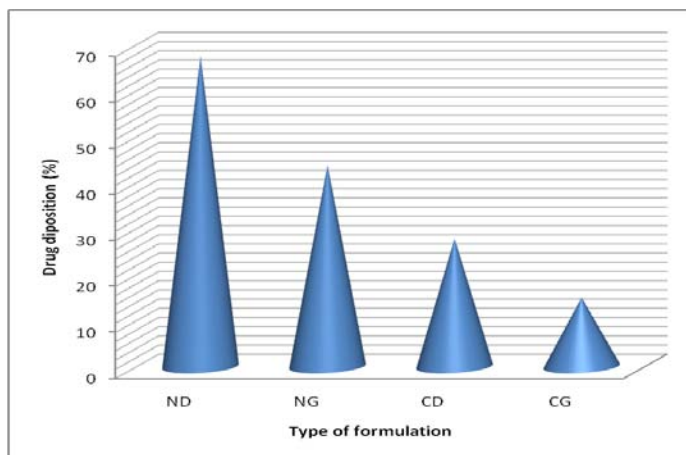


Fig. 6: Shows Drug deposition study on excised rat skin

NG: Niosomal gel, CG: Conventional gel

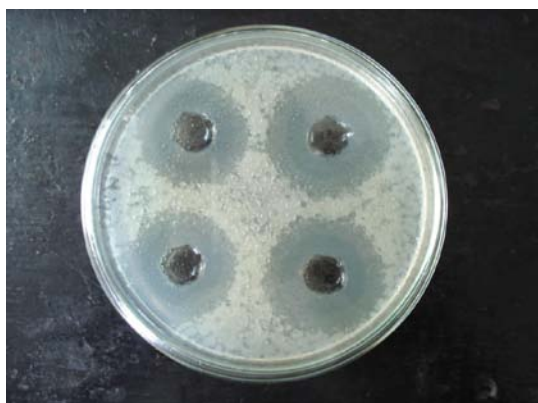


Fig. 7: Shows Antifungal activity by zone of inhibition

Skin irritation study (Draize patch test)

Primary Skin Irritation Studies of niosomal gel was done as per Draize test. The scores for erythema and edema were totaled for intact and abraded skin for all rabbits at 24 and 72 hours. The primary irritation index was calculated based on the sum of the scored reactions divided by 24 (2 scoring intervals multiplied by 2 test parameters multiplied by 6 rabbits). The developed formulation showed no erythema or edema on the intact and abraded rabbit skin Fig 8. The Primary irritation index of the formulation was calculated to be 0.00. Thus the formulation can be classified as a nonirritant to the rabbit skin.

Stability studies

Stability studies of all prepared niosomes were performed by storing at $4^{\circ}\text{C} + 2^{\circ}\text{C}$, $25^{\circ}\text{C} + 2^{\circ}\text{C}$ $60\% \text{RH} \pm 5\% \text{RH}$ for a period of three months. The residual drug content was determined at the end of three month. It was observed that the drug leakage from the vesicles was least at $4^{\circ}\text{C} + 2^{\circ}\text{C}$ followed by $25^{\circ}\text{C} + 2^{\circ}\text{C}$. This may be attributed to phase transition of surfactant and lipid causing vesicle leakage at higher temperature during storage. Hence, it is concluded from the obtained data that the optimum storage condition for niosomes was found to be 4°C as shown in Fig 9, 10.

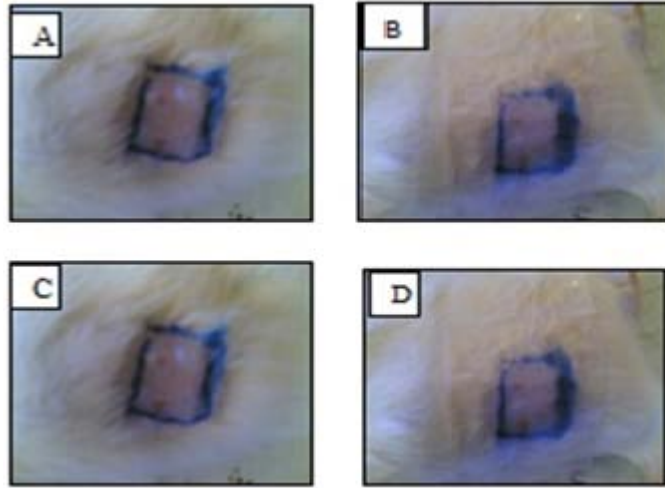


Fig. 8: Shows Photographs of rabbit skin treated with developed Niogel: (A) intact and (B) abraded after 24 hours; (C) intact and (D) abraded after 72 hours

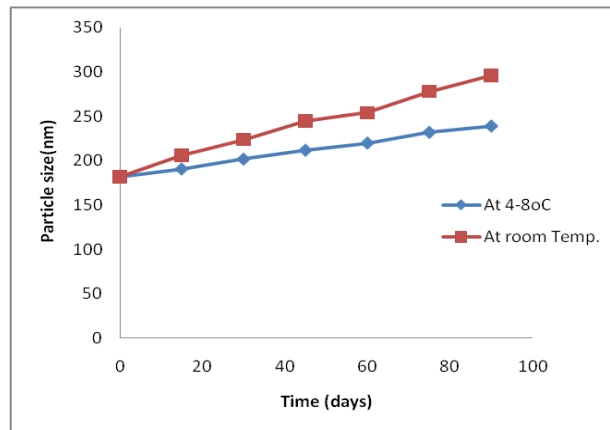


Fig. 9: Shows Effect on Particle size (nm) during stability study

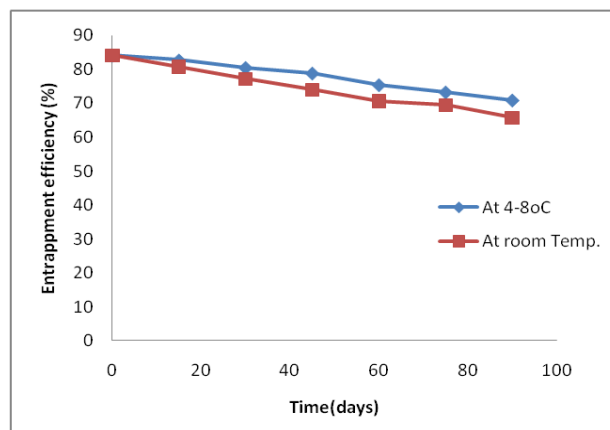


Fig. 10: Shows Effect on % Entrapment Efficiency during stability study

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