Original Article

Development of Natamycin Loaded Glycerosomes–A Novel Approach to Defend Ophthalmic Keratitis

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ABSTRACT

Objectives: Glycerosomes represent novel drug delivery systems and are characterised by addition of different amounts of glycerol to liposomal preparations. The present research work aims to formulate eyedrops of natamycin loaded glycerosomes and thereby improve its entrapment efficiency in the vesicles and enhance its corneal penetration. **Methods:** Liposomes and glycerosomes loaded with antifungal drug, natamycin, were prepared by thin film hydration technique and the prepared glyerosomes were optimized by 3^2 factorial design. **Results:** The best results were displayed by optimized glycerosome formulation. Results indicated higher efficacy of natamycin glycerosomes in terms of entrapment, *in vitro* penetration, *ex vivo* percentage penetration and also stability, as compared to that of pure drug and drug loaded liposomes. Natamycin glycerosomes exhibited entrapment efficiency of 80.8471%, *in vitro* percentage penetration of 93.422%, particle size of 394.5 nm and zeta potential of - 27.6, on other hand liposomes exhibited entrapment efficiency of 59.5%, in vitro percentage penetration of 57.6%, particle size of 231.4 nm and zeta potential of -16.5. **Conclusion:** Optimized glycerosome formulation exhibited maximum entrapment and when compared with pure natamycin solution and liposomes, it exhibited increased *ex vivo* corneal penetration. Thus, glycerosomes and liposomes of natamycin were successfully prepared and characterized.

Key words: Glycerosomes, Thin Film Hydration, Optimization, Factorial Design, Ocular, *Ex vivo*.

INTRODUCTION

Vesicular systems provide a useful mean for ocular drug delivery. These not only help in the localization of the drug to the desired site but also ease the administration of ocular drugs to the eye. Liposomes represent one such system by which one can obtain desired therapeutic effect without causing any harm to the ocular tissues. Although, liposomes manifest such potent ocular drug delivery still they are not the preferred drug delivery systems. Their stability issues, low entrapment efficiency limit their usage in ophthalmics.[1] To overcome these problems a novel drug delivery system referred to as glycerosomes can be used. Composed of phospholipids, different amount of glycerol and water, these new drug delivery systems result in improved vesicular entrapment and penetration of the drug through cornea than the conventional liposomes. These drug delivery systems increase the viscosity of formulations and also act as penetration enhancers.[2]

Eye is divided into two parts namely anterior and posterior parts. Cornea, conjunctiva, sclera and anterior uvea form anterior portion of the eye whereas posterior portion of eye consists of retina and vitreous choroid.[3] Drug delivery through eye is cumbersome due to its complex structure that does not allow the entry of foreign agents in eye.[4] Cornea which is the outermost layer of the eye poses as an obstacle during drug delivery due to its different polarity. It consists of epithelium, stroma and endothelium. Hydrophilic stroma is present between hydrophobic epithelium and endothelium. Polar stroma poses as a barrier for the deliv-

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ery of lipophilic agents whereas nonpolar epithelium and endothelium poses as a barrier in the delivery of hydrophilic agents.[5,6]

Natamycin is an antifungal drug that belongs to polyene class of antifungals. When natamycin is administered in eye, it does not acquire sufficient concentration in cornea and results in poor bio distribution in eye due to its high molecular weight and long molecular structure. Due to this reason, it has to be administered every hour for effective treatment that results in poor patient compliance.[7-9] Incorporating it in vesicular structure can result in its improved characteristics, *i.e.*, increase in entrapment and increase in corneal penetration.

Although vast research have been performed out in the field of vesicular systems for delivering drug to eye, there remain many challenges those liposomes results in. Therefore, it is the need of the hour to develop a system of drug delivery that will be proficient of surpassing the limitations of conventional liposomes and will result in effective delivery of drugs to eye.

The current research work focussed on increasing the vesicular entrapment efficiency and corneal penetration of natamycin by encapsulating it in novel glycerosomes, thereby providing a controlled and retention effect in eye.

MATERIALS

Natamycin was supplied from Himedia Laboratories, Mumbai. Soy lecithin, chloroform and cholesterol were supplied from CDH, Daryaganj, New Delhi and glycerol was procured from Rankem, Okhla Industrial Area, New Delhi. Himedia Laboratories of Mumbai supplied dialysis membranes. Analytical grade chemicals were used. *Ex vivo* study was carried out using goat's cornea for which the protocol was passed from Animals Ethics Committee (Protocol No. IAEC/NIET/2018/01/14).

METHODS

The following procedures and methods were employed in the present research work:

Preformulation studies

The following preformulation studies were carried out on natamycin and other excipients used in the study.

Identification of Drug

Various techniques were utilised for the identification of drug. The techniques included Fourier Transform Infrared Spectroscopy (FTIR), Ultraviolet - visible spectroscopy (UV) and determination of melting point.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectrum of natamycin was performed using KBR pellets. It gave the results regarding the functional groups present in natamycin. The spectra was recorded by scanning the drug in the range of 4000 to 400 cm^{-1}.[10,11]

Ultraviolet - visible spectroscopy (UV)

Absorbance spectra of natamycin in simulated tear fluid

Simulated tear fluid of pH adjusted to 7.4 was used as medium for dissolution for plotting calibration curve of pure natamycin.[10,12] The readings were taken at 304 nm. The wavelength was decided by running the sample in spectrum mode.

Calibration curve of natamycin

10mg natamycin crude drug was dissolved in 10ml simulated fluid. This was sonicated for 30 min and then filtered. From filtered drug solution, 1ml was taken out and 10 ml simulated fluid was added to it. This resulted in the formulation of Stock solution.5 µg/ml solution was prepared from the stock solution. 0.5ml solution was taken out and diluted upto 10 ml. It was then scanned in UV spectrophotometer at spectrum mode for determining the wavelength. 304nm was selected as the wavelength of natamycin drug. From the stock solution dilutions were then prepared to prepare 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml and 10 µg/ml, scanned at 304 nm. Absorbances were obtained.

Determination of melting point

The M.P. of pure natamycin was performed by capillary melting point method. According to USP capillaries must possess the following specifications for their use in capillary method of melting point determination. The length of the capillary must be 10 cm and it should possess an internal diameter of 0.11 cm. One end of capillary was sealed using the flame. After this small amount of crude drug was filled in the capillary and placed in the melting point apparatus. When the drug started melting or started decomposing the melting point of natamycin was recorded.[13]

Drug Excipient Compatibility studies[14]

The drug excipient compatibility studies were performed by the following procedures.

FTIR of drug and excipients

This was carried out by mixing them and scanning in the range of 400 to 4000 cm^{-1}.

Physical Compatibility studies

Drug and excipients individually and mixture of drug and excipients were kept at accelerated conditions. The

samples were kept at 60°C in sealed glass vials and 40°C/75% relative humidity (RH) in open glass vials. After 30 days the samples were analyzed against control samples that were kept at 4°C.

Procedure

Formulation of liposomes and glycerosomes

Liposomes and nine batches of glycerosomes were formed. 3^2 factorial design of experiment was followed for the optimization of nine batches of glycerosomes (unpublished observations). Glycerol concentration and hydration volume were taken as independent variables, whereas, entrapment efficiency and *in vitro* percentage penetration were taken as dependent variables.

Preparation method utilised for liposomes and glycerosomes

Thin film lipid hydration method was used for the preparation of liposomes and glycerosomes.[15] In this procedure, phospholipid namely, soy lecithin and cholesterol were dissolved in chloroform. This mixture was then added to a RBF which was connected to a rotary evaporator. The flask was rotated at 100 rpm for half hour at 45°C. Thin film was visible on the walls of the flask in half hour. Dry lipid film was then hydrated using phosphate buffered saline and drug for liposomes and with phosphate buffered saline, glycerol and drug solution (for glycerosomes). The flask was rotated for 5 min manually. It was then again rotated at the same temperature and rpm for one hour. The flask was removed after one hour and the solution was transferred to the beaker.

Evaluation of prepared glycerosomes and liposomes

Natamycin liposomes and glycerosomes were evaluated on the basis of the following parameters.

Entrapment efficiency

Various methods are used for finding the entrapment efficiency. The preset study utilised the method of ultracentrifuge. The formulations were placed in centrifuge and were rotated for 50 mins at 45000 rpm. The supernatant was collected after 50 mins. It was analyzed at 304nm using UV spectroscopy.[16,17]

$$\textbf{Formula} = \frac{\text{Total Drug Content} - \text{Free drug Content} * 100}{\text{Total Drug Content}}$$

In vitro penetration studies

Franz diffusion cell and dialysis membrane were used for determining the *in vitro* drug penetration studies. Dialysis membrane (weight cut off 12000 to 14000 Da) was placed on donor side. Simulated tear fluid was added in the receptor chamber. It was maintained at 37°C and was stirred by magnetic stirrer at 100 rpm. The required formulation (glycerosome or liposome) were placed on dialysis membrane and samples were taken out at definite time intervals. Fresh simulated fluid was added each time after sample withdrawal. Analysis of samples were carried out by UV spectroscopy.[18]

Particle size and zeta potential analysis

It was carried out by Dynamic light scattering analyzer (Malvern Zetasizer Version 6). It gave the results regarding polydispersity index and average particle size along with charge and stability of liposomes and glycerosomes.[17,18]

Morphological Analysis

The vesicular structures of liposomes and glycerosomes were confirmed by observing under trinocular microscope at 45x. Sample was set on slide (drop form) and was covered with coverslip. Excess sample was wiped off and the samples were then observed.[19]

Formulation of Eye Drops with Pure Natamycin and Natamycin Loaded Liposomes and Glycerosomes

Pure natamycin and natamycin loaded liposomes and glycerosomes were formulated into their specific eye drops and these processes were carried out under aseptic conditions. The prepared eye drops were evaluated for the following parameters:

Evaluation of Eye Drops[20]

Clarity

For determining clarity of eyedrop, the formulations were observed under white and black coloured backgrounds.

pH

pH of the formulations were checked using pH meter.

Viscosity

Eye drop formulations were analysed for viscosity using Ostwald viscometer.

In vitro percentage penetration

In vitro percentage penetration of the prepared eyedrops of pure drug and natamycin loaded liposomes and glycerosomes were determined using the same technique as described under the evaluation section of liposomes and glycerosomes.

Release kinetics of eyedrop of optimized glycerosome formulation

Various release models were applied for determining the release kinetics of the eye drops of the optimized glyc-

erosome formulation. The kinetics was decided based on the R^2 value obtained from various models.[21]

First order equation

$\log C = \log C_0 - Kt / 2.303$

where C_0 and Kt denotes the initial concentration of drug and first order rate constant respectively.

Zero order equation

$Q_t = Q_0 + K_0 t$

where Qt, Q_0 and $K_0 t$ stands for amount of drug present in solution at time t, initial amount of drug and zero order release constant respectively.

Higuchi model equation

$f_t = Q = K_H \times t^{1/2}$

where Q, K_H and $t^{1/2}$ stands for amount of drug released at time $t^{1/2}$ per unit area A and Higuchi dissolution constant respectively.

Korsemeyer Peppas equation

$Mt / M_\infty = Kt^n$

where Mt, M_∞ and Kt^n stands for drug released in fractions at time t, release rate constant and release exponent respectively.

Figure 1: Goat Eye.

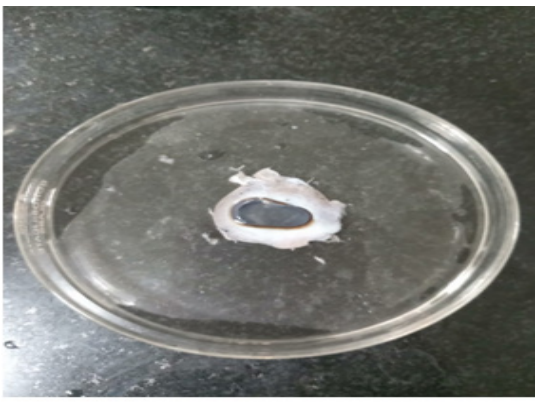

Figure 2: Isolated goat cornea from goat eye.

Ex vivo corneal penetration studies

For this study, excised goat corneas were used. Goat eyes were procured from local slaughter house (Protocol No. IAEC/NIET/2018/01/14). These were transported in cold normal saline. The cornea was separated from the whole goat eye by cutting along the edges. After that, it was washed and placed between the donor and receiver compartments. Simulated tear fluid about 20 ml in quantity was added in receiver chamber, so that it remained in contact with cornea. The individual formulations were added to the donor compartment. After definite time interval, samples were withdrawn and analyzed by UV spectroscopy.[22] Figure 1-3 shows the isolated goat cornea and mounted cornea on Franz diffusion cell.

RESULTS AND DISCUSSION

Identification of drug

Fourier transform infrared spectroscopy (FTIR)

The following FTIR spectrum was obtained for natamycin and is shown in Figure 4. Functional groups obtained for natamycin are shown in Table 1.

Determination of melting point

It was estimated to be 280°C.

Estimation of λ_{max} by UV spectroscopy

When natamycin was scanned in UV, 304 nm was chosen as it wavelength. Table 2 depicts the calibration curve readings obtained for natamycin when its different concentrations were scanned in UV spectrophotometer at 304 nm. Figure 5 depicts the calibration curve obtained for natamycin.

Table 1: Functional Groups of Natamycin.

Assignment	IR Bands (cm^{-1})
H-Bonded N-H	3070-3350
C-O-C Vibrations in Esters	1010-1040
C-O	1070-1150
Aromatic C=C	1400-1600
C=O	1670-1820

Table 2: Calibration Curve of Natamycin Drug.

Concentration (µg/ml)	Absorbance
2	0.205
4	0.397
6	0.554
8	0.731
10	0.921

Table 3: Physical Compatibility Studies of Natamycin Liposome.

Parameter	Ratio	Initial	Control	40 °C/75 % RH -open	40 °C/75 % RH – closed	60°C (close)	60°C (open)
Natamycin	Control	Yellow	No change	No notable change	No notable change	No notable change	No notable change
Soylecithin	Control	Yellow	No change	No notable change	No notable change	No notable change	No notable change
Cholesterol	Control	White	No change	No notable change	No notable change	No notable change	No notable change
Natamycin+ soylecithin	1:1	Yellow	No change	No notable change	No notable change	No notable change	No notable change
Natamycin + cholesterol	1:1	Yellow and white powder	No change	No notable change	No notable change	No notable change	No notable change
Natamycin+ soylecithin+ cholesterol	1:1:1	Yellow and white powder	No change	No notable change	No notable change	No notable change	No notable change

Table 4: Physical Compatibility Studies of Natamycin Glycerosome.

Parameter	Ratio	Initial	Control	40 °C/75 % RH -open	40 °C/75 % RH -closed	60°C (close)	60°C (open)
Natamycin	Control	Yellow	No notable change	No notable change	No notable change	No notable change	No notable change
Soylecithin	Control	Yellow	No notable change	No notable change	No notable change	No notable change	No notable change
Cholesterol	Control	White	No notable change	No notable change	No notable change	No notable change	No notable change
Glycerol	Control	Transparent	No notable change	No notable change	No notable change	No notable change	No notable change
Natamycin + soylecithin	1:1	Yellow	No notable change	No notable change	No notable change	No notable change	No notable change
Natamycin + cholesterol	1:1	Yellow and white powder	No notable change	No notable change	No notable change	No notable change	No notable change
Natamycin + glycerol	1:1	Yellow powder with a drop of glycerol on it	No notable change	No notable change	No notable change	No notable change	No notable change
Natamycin+ soylecithin+ cholesterol+ glycerol	1:1:1:1	Yellow and white powder with a drop of glycerol on it	No notable change	No notable change	No notable change	No notable change	No notable change

Drug Excipient Compatibility Studies

FTIR of drug and excipients

FTIR spectra of drug and excipients as obtained have been shown in Figure 6 and Figure 7.

Physical Compatibility studies

Physical compatibility studies are shown in Table 3 and Table 4.

Natamycin Liposome

Natamycin liposome exhibited the following characteristics

Entrapment efficiency of 59.5%

In vitro % penetration of 57.6%

P.S. of 231.4 nm and Z.P. of -16.5

Results of DLS studies and zeta potential are shown in Figure 8 and Figure 9.

Natamycin Glycerosome

The optimized formulation of glycerosomes exhibited (unpublished observations)

Entrapment efficiency of 80.8471%

In vitro percentage penetration of 93.422%

Figure 3: Goat cornea mounted on franz diffusion cell.

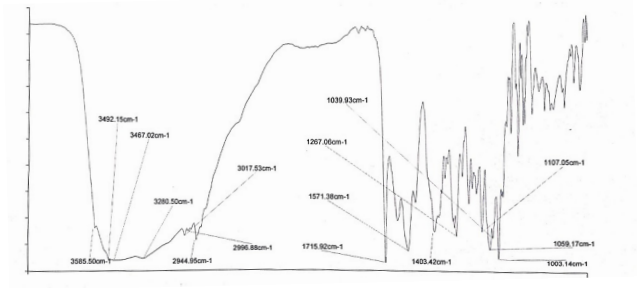

Figure 4: FTIR spectrum of natamycin.

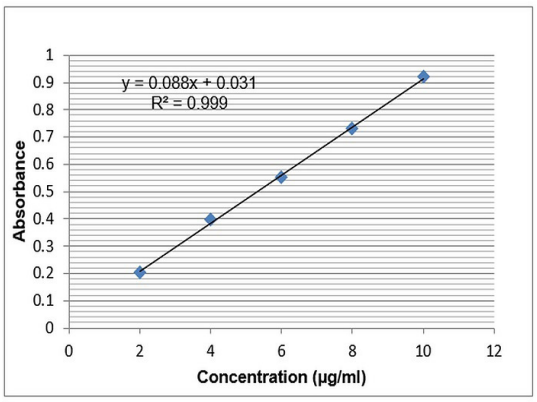

Figure 5: Calibration curve of natamycin.

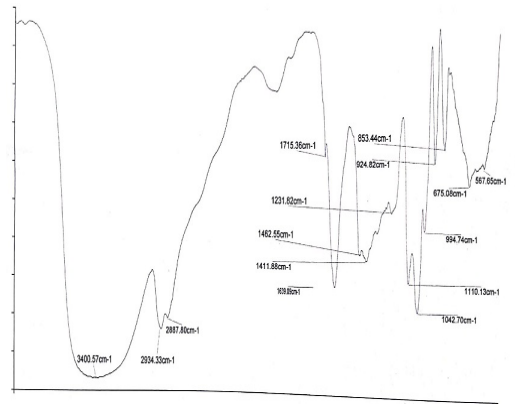

Figure 6: FTIR spectrum of drug and excipients for glycerosomes.

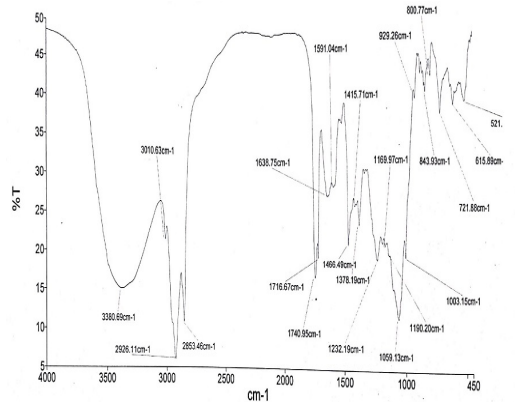

Figure 7: FTIR spectrum of drug and excipients for liposomes.

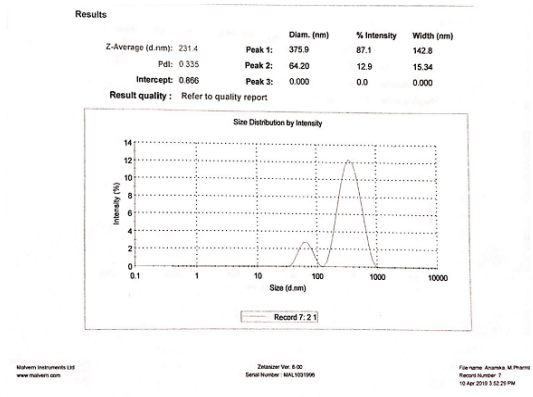

Figure 8: DLS studies (particle size and polydispersity index) of natamycin liposomes.

P.S. of 394.5 nm and Z.P. of -27.6

Results of DLS studies and zeta potential are shown in Figure 10 and Figure 11.

Trinocular Images: The microscopic images obtained for natamycin loaded liposomes and natamycin loaded glycerosomes are shown in Figure 12 and Figure 13.

Evaluation of Eye Drops

Clarity: All the eye drops were clear in appearance.

pH: Eye drop of natamycin, natamycin loaded liposome and natamycin loaded glycerosome exhibited pH of 6.78, 7.2 and 7.4 respectively.

Viscosity: Eye drop of natamycin and natamycin loaded liposome showed viscosity around 0.073 poise and natamycin loaded glycerosome exhibited viscosity of 1.94 poise.

***In vitro* percentage penetration:** Table 5 and Figure 14 show the *in vitro* percentage penetration obtained for

Table 5: *In vitro* Penetration of Eye Drops (%).

Time (hrs)	Cumulative *in vitro* penetration of Natamycin (%)	Cumulative *in vitro* penetration of Liposomes (%)	Cumulative *in vitro* penetration of Glycerosomes (%)
1 hr	3	13.25	16.04
2 hr	7.4	24.56	35.01
3 hr	12.8	30.09	55.32
4 hr	18.4	47.8	77.25
5 hr	24.3	51.04	86.21

Table 6: Release Kinetic Profiles.

Name of Formulation	Zero Order	Higuchi	First Order	Korsemeyer Peppas Model	
Eye drop of glycerosomes	R^2 = 0.985	R^2= 0.980	R^2= 0.917	R^2 = 1	n = 1.127

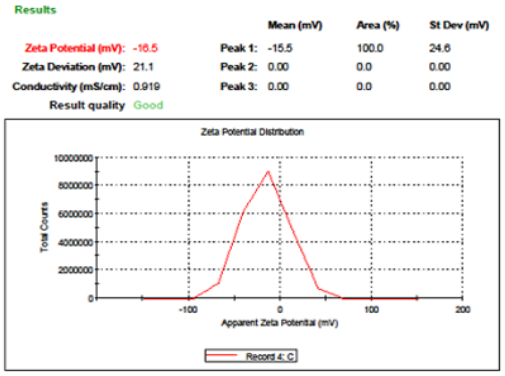

Figure 9: Zeta potential of natamycin liposomes.

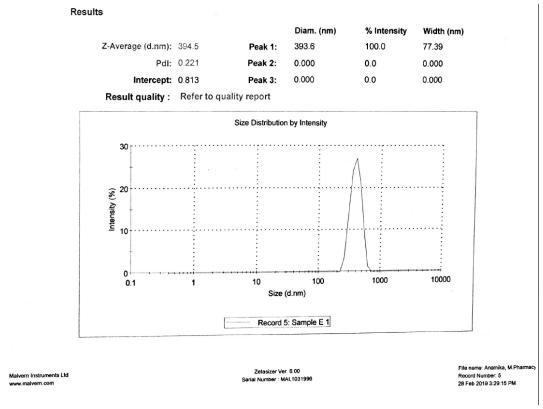

Figure 10: DLS studies (particle size and polydispersity index) of natamycin glycerosomes.

eye drop of natamycin, natamycin loaded liposome and natamycin loaded glycerosomes.

Release Kinetics

R^2 Values Obtained for optimized glycerosomes formulation after applying various release models is as shown in Table 6.

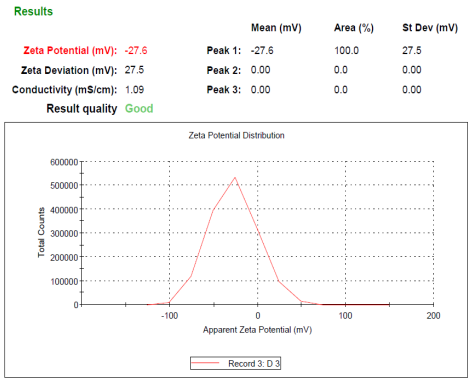

Figure 11: Zeta potential of natamycin glycerosomes.

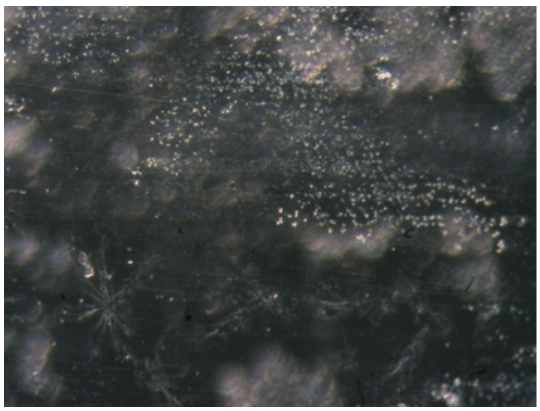

Figure 12: Trinocular image of natamycin liposomes.

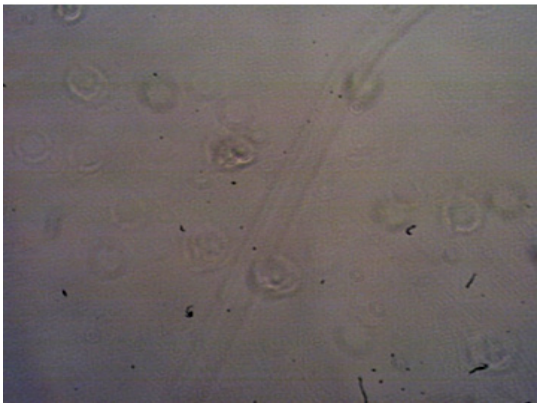

Figure 13: Trinocular image of natamycin glycerosome.

The graphs of release kinetics of optimized natamycin loaded glycerosomes eye drop are shown in Figure 15-18.

The optimized eye drops of natamycin loaded glycerosomes depicted zero order release kinetics with R^2 value of 0.985 and exhibited supercase II transport based on the n value obtained from Korsemeyer Peppas model.

Ex vivo Studies

The results gave the observation that eye drops of glycerosome penetrated the cornea to relatively more extent

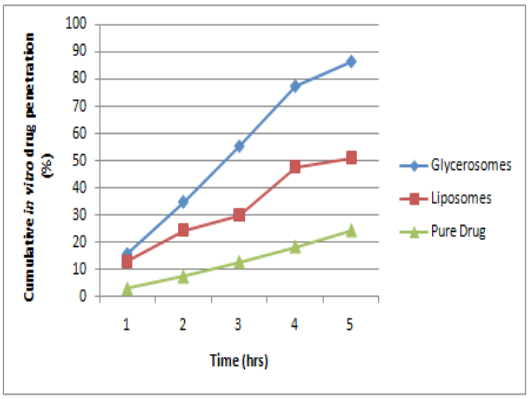

Figure 14: In vitro penetration of eye drops (%).

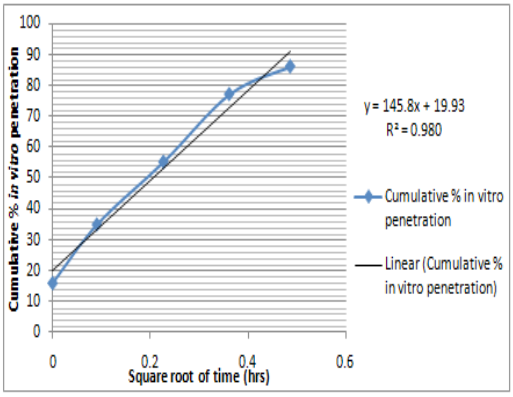

Figure 17: Release kinetics of optimized natamycin loaded glycerosomes eye drop (higuchi model).

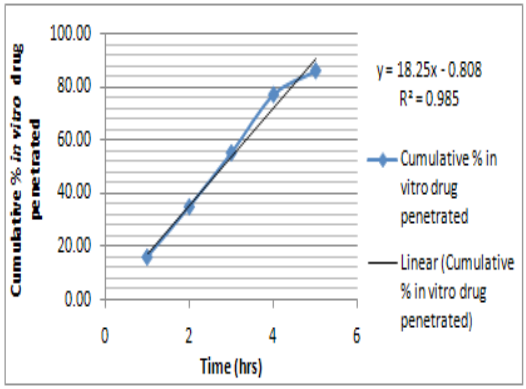

Figure 15: Release kinetics of optimized natamycin loaded glycerosomes eye drop (zero order).

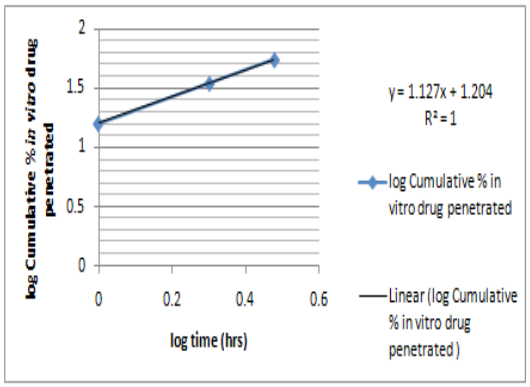

Figure 18: Release kinetics of optimized natamycin loaded glycerosomes eye drop (korsemeyer peppas).

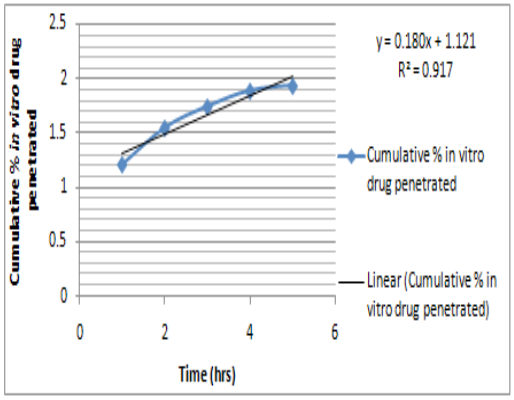

Figure 16: Release kinetics of optimized natamycin loaded glycerosomes eye drop (first order).

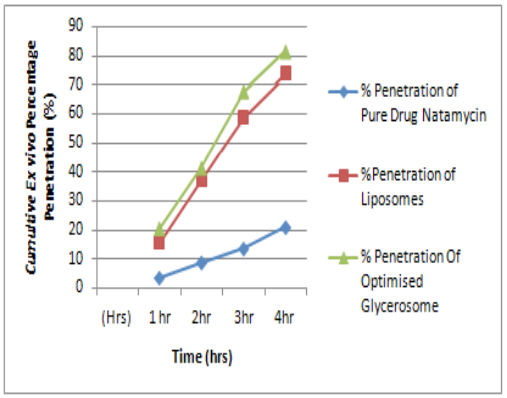

Figure 19: *Ex vivo* corneal penetration studies (%).

than the eye drops of pure natamycin and eye drops of natamycin liposomes. Figure 19 depicts the *ex vivo* studies of the three formulations.

CONCLUSION

The current study evaluated the potential of "glycerosomes" for delivering drug through eye. Natamycin glycerosomes and liposomes were successfully prepared employing lipid thin film hydration technique. The glycerosomes exhibited increased entrapment efficiency and *in vitro* percentage penetration than the old generation liposomes. This was due to the addition of glycerol that lead to the formation of big vesicles that can entrap more drug in themselves. On conversion to eye drop in aseptic conditions the natamycin loaded glycerosomes eye drop formulation depicted increased corneal penetration than the eye drop of pure natamycin and eye

drop of natamycin loaded liposomes. Thus, glycerosomes can be used as an effective drug delivery system for delivering drug to eye.

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CONFLICT OF INTEREST

There are no conflicts of interest.

ABBREVIATIONS

RBF: Round Bottom Flask; **UV:** Ultraviolet; **R^2:** Correlation coefficient; **DLS:** Dynamic light scattering; **P.S:** Particle size; **Z.P:** Zeta potential; **M.P:** Melting point.

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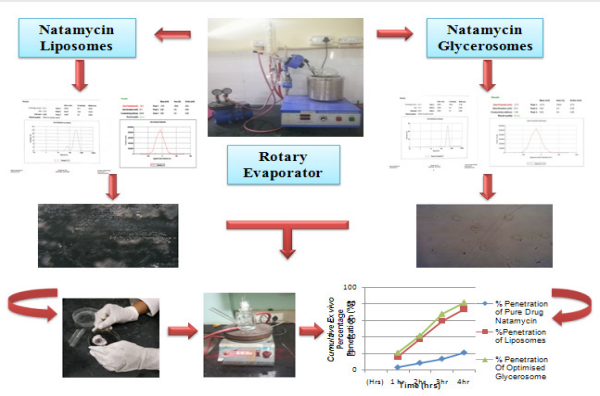
SUMMARY

In the present research work, natamycin loaded glycerosomes were formulated, optimized and evaluated for the first time for ocular drug delivery. Thin film hydration method was employed for the preparation of glycerosomes and liposomes. For optimization, glycerol concentration and hydration volume were chosen as independent factors whereas encapsulation efficiency and in vitro percentage penetration were chosen as dependent factors. After successful optimization of glycerosomes, the optimized batch of glycerosomes was fully characterized and was converted to eye drop form. Various evaluation tests were carried out on eye drops. It was further compared with the characterized natamycin liposomes and plain drug solution of natamycin on the basis of *ex vivo* corneal penetration studies and *in vitro* drug release studies in eye drop form. The results obtained depicted the increased corneal penetration as well as in vitro drug release of natamycin loaded glycerosomes than the liposomal form and solution of drug. Thus, the present research concluded that glycerosomes can be regarded as an efficient drug delivery system for the management of fungal keratitis and can function as a proficient carrier for drugs possessing low solubility and penetration.

PICTORIAL ABSTRACT



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