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RESEARCH ARTICLE

Design and Characterization of Fluconazole Loaded Elastic Liposome Based Gel for Treatment of Keratomycosis

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SUMMARY

Fungal corneal ulcers, also known as keratomycosis, occur due to a breach in the corneal epithelium. According to WHO, it is the leading cause of blindness. The eye consists of a variety of different structures having different physiological functions that make it highly resistant to external substances, thus resulting in low bioavailability of drugs from most of the conventional dosage forms. To improve drug effectiveness, a series of research groups have tried a variety of strategies. The majority of these modifications provide some benefit over traditional dosage forms, but they have their own set of drawbacks. To overcome the side effects of the formulations mentioned above, Fluconazole-loaded elastic liposome-based gel was prepared. The elastic liposomes were prepared by rotary evaporation method using soya lecithin and sodium deoxycholate. The elastic liposomal suspension was then incorporated into optimised gelling agent (carbopol 934) to have sufficient contact time of the drug in the eye. The elastic liposome-based gel was then characterized for pH, drug content, rheological study, homogeneity and grittiness, in vitro release study, ex vivo permeation study, toxicity study, bio adhesion study and antifungal activity. The optimized formulation had pH 7.0 ± 0.01, drug content 98.5 ± 3.9%, viscosity 7217 ± 340 mPa.s, in vitro release 80.5± 0.32%, ex vivo permeation 72.27±0.65 % and the bio adhesion time of the optimized formulation was found to be significantly higher (p≤ 0.05) as compared to marketed gel. Antifungal activity of the optimized gel was also found to be significantly higher (p≤ 0.05) as compared to the marketed gel. The Fluconazole-loaded elastic liposome gel was prepared successfully and was found to be a good choice over conventional gel formulation for the treatment of keratomycosis.

Key Words: *Fungal corneal ulcers, Fluconazole, elastic liposomal gel, antifungal activity.*

Keratomikoz Tedavisi İçin Flukonazol Yüklü Elastik Lipozom Bazlı Jelin Tasarımı ve Karakterizasyonu

ÖZ

Keratomikoz olarak bilinen mantar kaynaklı kornea ülseri, korneal epitelyumunun yırtılması sonucu ortaya çıkar. WHO'ya göre keratomikoz körlüğün önde gelen nedenidir. Göz, dış faktörlere karşı oldukça dirençli olmasını sağlayan farklı fizyolojik fonksiyonlardaki farklı yapılardan oluşur, bu durum çoğu konvansiyonel dozaj formunun düşük ilaç biyoyararlanımı göstermesine neden olur. İlaç etkinliğini artırmak amacıyla birçok araştırma grubu çeşitli stratejiler denemişlerdir. Bu modifikasyonların büyük bir kısmı geleneksel dozaj formlarına kıyasla bazı yararlar sağlamışlardır ancak kendi dezavantajları vardır. Yukarıda bahsedilen formülasyonların yan etkilerinin üstesinden gelmek amacıyla, flukonazol yüklü elastik lipozom temelli jel hazırlanmıştır. Elastik lipozomlar, soya lesitin ve sodyum deoksikolat kullanılarak döner evaporasyon metodu ile hazırlanmışlardır. Sonrasında ilacın göz ile yeterli temasını sağlamak amacıyla elastik lipozomal süspansiyonlar optimize edilmiş jelleştirme ajanı (karbopol 934) ile birleştirilmiştir. Daha sonra elastik lipozom temelli jel pH, ilaç içeriği, reolojik çalışma, homojenite ve sürülebilirlik, in vitro salım çalışması, ex vivo permeasyon çalışması, toksisite çalışması, biyoadhezyon çalışması ve antifungal aktivite açısından karakterize edilmiştir. Optimize formülasyon 7,0 ± 0,01 pH değerine, %98,5 ± 3,9 ilaç içeriğine, 7217 ± 340 mPa.s viskoziteye, %80,5± 0,32 oranında in vitro salıma, %72,27±0,65 ex vivo permasyon değerine sahiptir ve biyoadhezyon süresi piyasa jeli ile karşılaştırıldığında anlamlı derecede (p≤ 0,05) yüksek bulunmuştur. Optimize jelin antifungal aktivitesi de ticati jel ile karşılaştırıldığında anlamlı derecede (p≤ 0,05) yüksek bulunmuştur. Flukonazol yüklü elastik lipozom jel başarılı bir şekilde hazırlanmış ve keratomikozis tedavisi için konvansiyonel jel formülasyonlarına göre ümit verici bir seçenek olarak bulunmuştur.

Anahtar Kelimeler: *Fungal kornea ülserleri, Flukonazol, elastik lipozomal jel, antifungal aktivite.*

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INTRODUCTION

Fungal corneal ulcers, also known as keratomycosis, are marked by stromal infiltration produced by fungi. Since the corneal epithelium acts as a barrier to microorganisms, these fungi induce infection when there is a breach in the epithelium (Chandra *et al*., 2013). The development of problems is sometimes preceded by a lack of suspicion and a delayed diagnosis, resulting in permanent eyesight loss or, worse, enucleation (Sharma *et al*., 2014).

The tropical climate and agriculture as significant occupation are the main causes of its high prevalence. Because of their increased vulnerability to occupational trauma, Indians are at an increased risk of keratomycosis. Annually, 1.5 to 2 million new cases of keratomycosis are reported, and keratomycosis is the leading cause of blindness, according to the World Health Organization (WHO). Keratomycosis is said to affects 44-47 percent of people in India (Chandra *et al*., 2013).

Candida, Aspergillus flavus, Fusarium species, etc., are a few microorganisms responsible for the onset of keratomycosis (Chandra *et al*., 2013).

The eye is an organ of vision and its physiology and anatomy make it a unique organ. The eye consists of various structures having different physiological functions that make it highly resistant to external substances (Palani *et al*., 2010), thus resulting in the low bioavailability of drugs from most of the conventional dosage forms (Yu *et al*., 2015). Another reason for the poor bioavailability of drugs from the conventional dosage forms is the pre-corneal loss variables such as insufficient residence time in the conjunctival sac, tear dynamics and non-productive absorption (Budai *et al*., 2007).

A series of research groups have tried a variety of strategies to improve drug effectiveness, including suspensions, ointments, inserts, hydrogels and polymeric micelles. The majority of these modifications provide some benefit over traditional dosage forms. Still, their drawbacks of obscured sight and patient non-compliance are the primary cause behind their lack of widespread acceptance (Yu *et al*., 2015).

To overcome the side effects associated with the formulations mentioned above, Fluconazole-loaded elastic liposomes were prepared for the treatment of keratomycosis. As reported in various literature, elastic liposomes have multiple advantages such as patient compliance due to painless administration of the drug, systemic side effects associated with oral formulations being bypassed, the drug is delivered to the target site directly (Hussain *et al*., 2017) and their ability to squeeze through channels 1/10th of their size and enter deeper tissues due to their elasticity (Benson, 2009), ensures a sustained release of drug with improved antifungal activity (Kumar *et al*., 2012).

However, due to their low viscosity, elastic liposomes generally need to be integrated into a suitable semi-solid dosage form for topical application and ample exposure time to drug in the eye. It is crucial to ensure product quality and production efficiency by controlling the flow qualities. By loading elastic liposome suspension in gel, the rheological properties of the suspension are improved (Dar *et al*., 2020) and the retention time of the formulation within the eye is increased (Sayeh *et al*., 2014).

In the current study, Fluconazole-loaded elastic liposomes were prepared to treat keratomycosis, using a Rotary evaporator. To improve the rheological properties, the elastic liposomal suspension was loaded in a gelling agent. The gel prepared was then evaluated for various parameters.

MATERIALS AND METHODS

Chemicals

Fluconazole was provided kindly by Ramson Remedies, Amritsar. Soya lecithin, sodium deoxycholate, carbopol 934 and sabouraud dextrose agar were procured from Hi-Media, Mumbai, India; chloroform was purchased from Molychem, Mumbai, India; methanol, sodium alginate and triethanolamine were procured from Merck life sciences Pvt. Ltd. Mumbai,

India. Carbopol 940 was purchased from Qualikems Fine Chem, Pvt. Ltd., Vadodra, India; HPMC was purchased from Thermo Fischer Scientific Pvt. Ltd., India. All the other chemicals and reagents were of analytical grade.

Preparation of elastic liposomes

The Rotary evaporation method was used for the development of drug-loaded elastic liposomes. Accurately weighed amounts of soya lecithin (280 mg), fluconazole (50 mg) and sodium deoxycholate (120 mg) were taken in a clean and dry round bottom flask and then the mixture was dissolved in an optimized quantity of organic solvent (Chloroform, 10 ml). The organic solvent was then evaporated under reduced pressure and high temperature (40° C) using a rotary evaporator. The film obtained at the base of the flask was then rehydrated with an aqueous phase (10 ml) (Kumar *et al*., 2012).

Preparation of elastic liposome-based gel

Selection of gelling agent

For the preparation of elastic liposome-based gel, different gelling agents (such as carbopol 934, carbopol 940, sodium alginate, and hydroxypropyl methylcellulose (HPMC)) were utilized at a concentration of 1%. In elastic liposomal suspension, the gelling agent was dispersed with constant stirring. In the case of carbopol 934 and carbopol 940, triethanolamine was utilized to alter the pH of the gel to 7 and was stirred continuously till a clear gel was obtained. The gels prepared were then characterized for physical appearance, homogeneity, grittiness and consistency (Kaur *et al*., 2018).

Formulation of elastic liposome-based gel

Four different concentrations (0.5%, 1%, 1.5%, and 2%) of the optimized gelling agent were taken and dispersed in elastic liposomal suspension. The mixture was stirred continuously. Triethanolamine was added under continuous stirring to adjust the pH of the mixture to 7 and was added until clear gel was obtained (Dar *et al*., 2020).

Evaluation of elastic liposome-based gel

Physical evaluation: Under normal daylight, the different gel formulations prepared were analyzed for physical changes (Abou *et al*., 2014). The experiment was done in triplicate.

***pH*:** The pH of different gel formulations was determined using a digital pH meter (Tawfeek *et al*., 2020). The experiment was done in triplicate.

Homogeneity and grittiness: By pressing a few milligrams of gel between the thumb and index finger, the homogeneity and grittiness of the prepared gel formulations were assessed (Abdellatif *et al*., 2016). The experiment was done in triplicate.

Drug content: By dissolving 1 g of properly weighed gel in methanol, the drug content of the gel was calculated. A UV spectrophotometer was used to measure absorbance at 261nm after appropriate dilution. The slope of the standard curve was used to determine the drug content (Phaldesai *et al*., 2014). The experiment was done in triplicate.

Rheological studies: Rheometer (Anton- Paar) was used for to analyze the rheological properties of the different gel formulations prepared. At 25° C, the viscosity of elastic liposome-based gel was measured using a spindle 25 (PP 25). The number of data points was set to 25, and the behaviour was set to ramp linear (Varges *et al*., 2019). The experiment was done in triplicate.

In vitro drug release study: Franz diffusion cell and treated dialysis membrane were used to determine the *in vitro* drug release of Fluconazole from different elastic liposome-based gels and drug solution gel (plain gel). Simulated tear fluid pH 7.4 (Sodium chloride = 0.670g, sodium bicarbonate = 0.2g, calcium chloride dehydrate = 0.008g, purified water = 100 ml) was used as a medium in the receptor chamber. The dialysis membrane was positioned amid the receptor chamber and the donor chamber. 1 g of accurately weighed gel was deposited on the dialysis membrane in the donor chamber. The whole assembly was

kept on the magnetic stirrer under constant stirring and the temperature of the assembly was maintained at 37°± 2°C. Samples were taken at preset time intervals and the amount withdrawn was restored with an equivalent amount of fresh media. The collected samples were filtered using a 0.45µm membrane filter prior to analysis.

UV spectrophotometer was used to determine the drug content in the samples withdrawn at a wavelength of 261 nm. A graph was plotted between the cumulative percent of drug released and time (El-Gizawy *et al*., 2020). The experiment was done in triplicate.

Ex vivo trans-corneal permeation study: Using Franz diffusion cell and excised goat corneal membrane, the *ex vivo* permeation study was carried out. The elastic liposomal gel (1 g) was evenly applied to the corneal membrane that separated the donor and receptor compartments. Simulated tear fluid was taken in the receptor region. The assembly was kept on the magnetic stirrer under constant stirring. The temperature was maintained at 37 °C. At pre-planned time intervals, the samples were taken. The amount of sample withdrawn was replenished with an equal amount of fresh media. UV spectrophotometer was used to evaluate the samples withdrawn at 261 nm. The percent cumulative drug permeated was determined using a calibration plot (Tiwari *et al*., 2020). The experiment was done in triplicate.

Data analysis (determination of flux and permeability coefficient): The amount of Fluconazole that permeated through the goat corneal membrane from the elastic liposome-based gel (Q, g/cm^2) was reported as a function of time (hr). The slope and intercept of the straight line generated by plotting the amount of fluconazole permeated against time under steady-state conditions were used to quantify the drug flux (permeation rate) at a steady state (Jss, g/cm^2/hr). The flux was divided by the initial drug concentration (Co) in the donor compartment of the cell to obtain the permeability coefficient (kp) (Tawfeek *et al*., 2020). The experiment was done in triplicate.

Determination of drug retention in the corneal membrane: Cotton soaked in a 0.05% sodium lauryl sulphate was used to remove the formulation that remained on the excised goat corneal membrane. Then the membrane was washed with distilled water. To extract fluconazole, the ocular membrane was weighed, chopped into small pieces, and sonicated for 15 minutes with methanol. After centrifuging and filtering the resultant solution, the drug concentration (g/cm^2) of the corneal membrane was measured using a UV spectrophotometer (Pathak *et al*., 2020). The experiment was done in triplicate.

Toxicity study: Toxicity studies were used to look into the formulation's safety. The following investigations were performed to see if the formulation had any unfavorable effects on the cornea of the eyes.

Corneal Hydration Test: The corneal hydration of goat corneas was determined using the same goat corneas used in the permeation study. Each cornea was weighed at the end of the experiment, then dipped in 1ml methanol, dried night long at 90°C, and was weighed again. The difference in weights was used to calculate corneal hydration (Zubairu *et al*., 2015). The experiment was done in triplicate.

Histopathological study: Each goat cornea was detached from the Franz diffusion cell and was kept in 10% formalin solution in distilled water after the *ex vivo* permeation study. Two untreated goat corneas were kept in potassium chloride solution and normal saline solution, respectively, before being fixed in 10% formalin solution. Following that, the slices were cut and stained with eosin and hematoxylin before being examined under a microscope (Zubairu *et al*., 2015).

Effect of formulation on corneocytes: To gain a better understanding of the tissue injury induced by formulation on corneocytes, a comparative toxicity study was performed. The assembly for the toxicity study was set using the same approach as the *ex vivo* permeation study assembly. In separate diffusion

cells, the drug-loaded formulation, normal saline, and a mixture of span 80 and soya lecithin were introduced into the donor compartment. Tyrode solution was poured into the receptor chamber. After 60 minutes, samples were obtained from the receptor chamber and analyzed utilizing the LDH assay kit from Coral clinical systems (Zubairu *et al*., 2015). The experiment was done in triplicate.

Bio adhesion testing: The experiment was carried out per the procedure reported in the literature by Zubairu et al. (21) with slight modifications. An agar plate was formulated. In the center of the plate, the test sample was placed. The prepared agar plate was attached to an IP disintegration test apparatus after 5 minutes after placing the test sample on it and was pushed up and down in simulated tear fluid at 37° C ± 1° C. At the lowest point, the sample on the plate was immersed in the solution, and at the highest point, it was out of the solution. The visual appearance of the formulation over the plate indicated the residence time of the elastic liposome-based gel on the plate (Zubairu *et al*., 2015). The experiment was done in triplicate.

Antifungal activity of the prepared gel by cup and plate method: The suspension of *Candida albicans* was prepared by taking a small quantity of the lyophilized powder of *Candida albicans* in a test tube containing 100 ml of nutrient broth and was incubated at 28° C for 24- 48 hours. After 24-48 hours, 50 µl of the suspension was added into 900 µl of sterile water. 50 µl of the suspension, which was prepared in sterile water, was grabbed and spread aseptically on Sabouraud dextrose agar plates using a sterile cotton swab, rotating the plates through a 60° C angle after each application. Finally, the swab was pushed along the agar surface's margins. With the lid closed, the plates were left to rest at room temperature. Then, using a sterile cork borer, three wells were bored into the agar medium and filled with elastic liposome-based gel (1g containing 5mg fluconazole), marketed gel (1g containing 5 mg fluconazole) and placebo gel (1g), respectively. To ensure equal drug distribution, the plates were placed in the refrigerator for two hours. The plates were incubated for 24-48 hours at 28° C. Around the wells; assessments were done for zones of inhibition. The inhibitory zones produced from all of the formulations evaluated were compared and results were reported (Kumar *et al*., 2012). The experiment was done in triplicate.

Stability studies: The gel formulation was stored at two different temperatures, at 4° C and room temperature for three months. The formulation's physical stability was then determined by visual inspection for phase separation. Additionally, pH, viscosity and drug content of the gel formulations were also evaluated (Shakeel *et al*., 2008).

Statistical analysis: Results were expressed as mean ± standard deviation (SD). The data obtained from various groups were statistically analysed using Graph Pad Instat 3, using two- tailed unpaired t-tests. Values at $p \leq 0.05$ were considered significant.

RESULTS

Preparation of elastic liposomes

Elastic liposomes were formulated using the rotary evaporation method. The optimised elastic liposomes exhibited particle size of 173.6 ± 5.9 nm, polydispersity index of 0.303 ± 0.03 and zeta potential of -10.0 ± (-0.311).

Preparation of elastic liposome-based gel

Selection of a gelling agent

To optimize the gelling agent, different gels were prepared using a 1% concentration of different gelling agents. Then the gels were characterized for various parameters. The results of the characterization of gels are given in Table 1 and based on the results of characterization carbopol 934 was selected for the development of elastic liposome- based gel.

Table 1: List of different gelling agents used for gel formulations

S. No.	Gelling agent	Concentration (w/v)	Observation	Homogeneity
1	Carbopol 934	1%	Very clear, stable, good consistency	Homogeneous
2	Carbopol 940	1%	Clear, hard	Non-homogeneous
3	Sodium alginate	1%	Phase separation	Non-homogeneous
4	Hydroxyl propyl methyl cellulose	1%	Grittiness and phase separation	Non-homogeneous

From the results, it was concluded that gels formulated using carbopol 934 were clear, stable and exhibited good consistency and therefore carbopol 934 was selected as the gelling agent. Further gels with different concentrations of carbopol 934 (from 0.5% to 2%w/v) were formulated.

Evaluation of elastic liposome-based gel

Physical evaluation: The different gels prepared were evaluated for physical appearance under normal daylight and the results are given in Table 2.

Table 2: Physical evaluation of different gel formulations

Property	E2 containing 0.5% carbopol 934	E2 containing 1% carbopol 934	E2 containing 1.5% carbopol 934	E2 containing 2% carbopol 934
Colour	Slightly yellowish	Slightly yellowish	Slightly yellowish	Slightly yellowish
Appearance	Opaque	Opaque	Opaque	Opaque
Odor	Characteristic	Characteristic	Characteristic	Characteristic
Washability	Washable	Washable	Washable	Washable
Consistency	+	++	+++	+++
Type of smear	Non greasy	Non greasy	Non greasy	Non greasy

(+) = low, (++) = medium, (+++) = good

pH: The pH of the different gel formulations prepared was determined and was found to be ranging from 7.0 to 7.3, indicating that the formulations prepared were safe for ocular administration. The results are given in Table 3.

Table 3: pH, drug content and viscosity of different gel formulations

S. No.	Gel formulations	pH± SD	Drug content % ± SD	Viscosity (mPa.s) ± SD
1	E2 (0.5% w/v)	7.1±0.01	88.3± 2.5	4361± 240
2	E2 (1% w/v)	7.3±0.02	95.1± 1.8	4984±250
3	E2 (1.5% w/v)	7.0±0.01	98.5±3.9	7217±340
4	E2 (2% w/v)	7.3±0.03	92.7±2.7	9969±410

Homogeneity and grittiness: Homogeneity and grittiness of different gel formulations prepared were determined and all the gel formulations were found to be homogeneous and free from gritty particles, indicating that the gel, when applied, would not cause any discomfort.

Drug content: The Drug content of the different gel formulations prepared was evaluated and was found to be ranging from 88.3 % to 98.5%. The results of the drug content determined are given in Table 3. Elastic liposome-based gel containing 1.5% w/v carbopol 934 exhibited a maximum drug content of 98.5%.

Rheological studies: The viscosity of the different gel formulations was determined using a rheometer (Anton- Paar). It was noticed that with the increase in the concentration of the gelling agent in a gel formulation, there was an increase in viscosity of the gel. The viscosity of the different gel formulations was found to

be ranging from 4361 to 9969 mPa.s. The results are given in Table 3.

In vitro release study: The *in vitro* release of the different gel formulations prepared was determined utilizing Franz diffusion cell and treated dialysis membrane. The percent cumulative drug release from the plain gel formulation was found to be significantly ($p \leq 0.05$) more (91±1.42 %) when compared with release from the elastic liposomal suspension gel over a period of six hours. The percent cumulative drug release from the different elastic liposomal gel formulations containing different concentration (0.5% w/v, 1% w/v, 1.5% w/v and 2% w/v) of gelling agent was found to be 86.45±0.107%, 85.4±0.18%, 80.5±0.32% and 77.7±0.21% respectively. From the results, it was noticed that with the rise in the concentration of the gelling agent, there was a significant decrease ($p \leq 0.05$) in the release of the drug from gel formulations. The elastic liposome-based gels showed continuous and sustained release of Fluconazole. *In vitro* release profile of different gel formulations and drug solution gel (plain gel) is given in Figure 1.

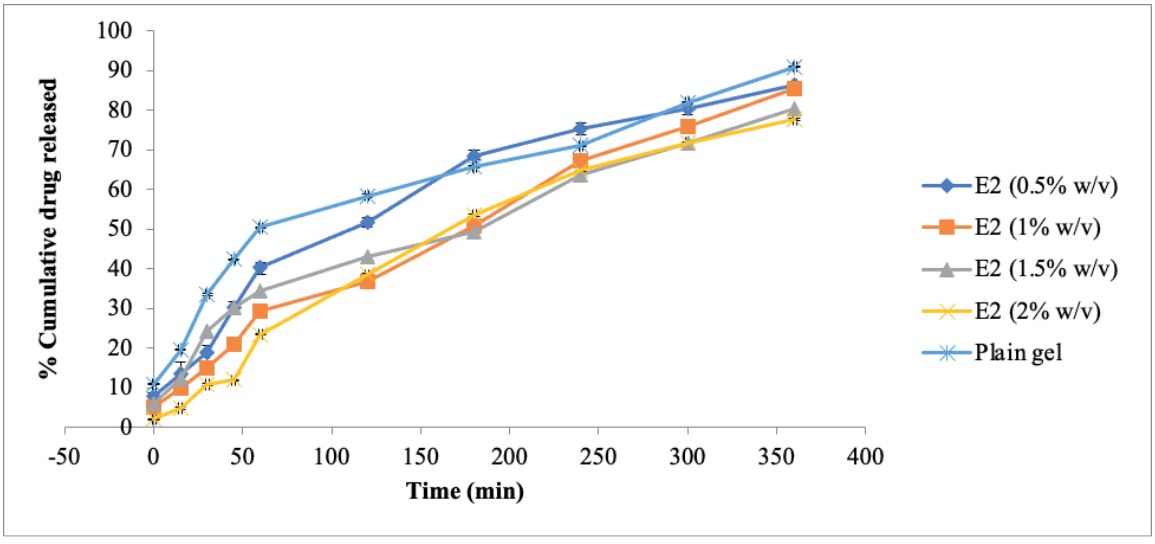

Figure 1: *In vitro* release profile of various gel formulations and drug solution gel (plain gel) (n=3)

For further studies, elastic liposome-based gel E2 $_{(1.5\%\ w/v)}$ was chosen instead of elastic liposome-based gel E2 $_{(2\%\ w/v)}$ because phase separation was observed in the case of elastic liposome-based gel E2 $_{(2\%\ w/v)}$ after 2 to 3 days.

Ex vivo permeation study: The *ex vivo* permeation study of the optimized gel formulation E2 $_{(1.5\%\ w/v)}$ was performed utilizing Franz diffusion cell and excised goat cornea for a period of six hours. The permeation of the optimized gel E2 $_{(1.5\%\ w/v)}$ was found to be significantly ($p \leq 0.05$) more (72.27±0.65 %) as compared to the plain gel (44.1±0.33 %), which could be attributed to the flexible and deformable nature of the elastic liposomes. Percentage cumulative amount of drug permeated versus time plot for optimized gel formulation E2 $_{(1.5\%\ w/v)}$ and plain gel formulation is given in Figure 2.

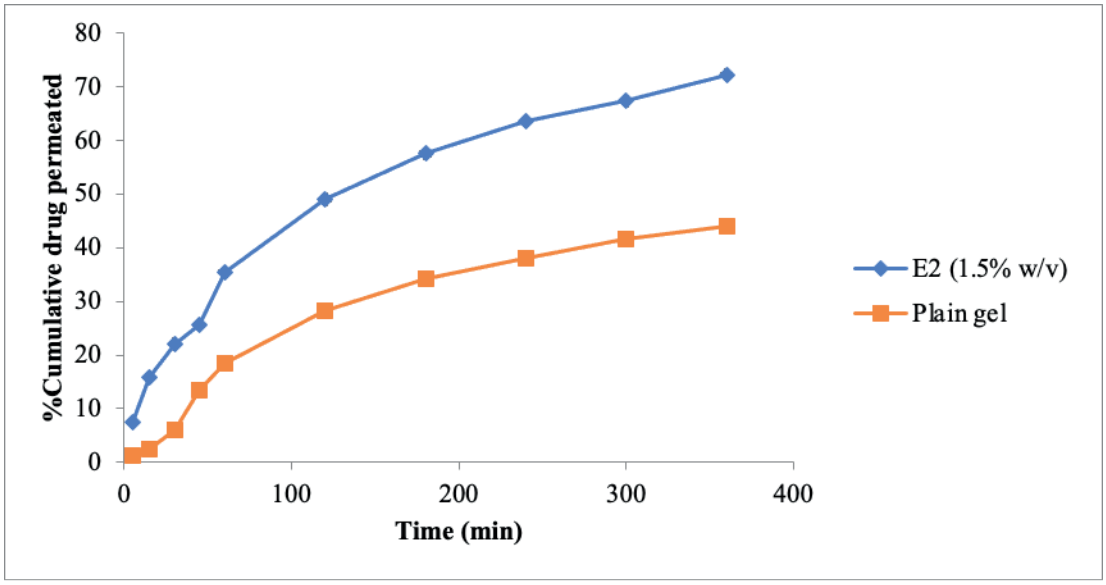

Figure 2: Percentage cumulative amount of drug permeated versus time plot for optimized gel formulation E2 $_{(1.5\%\ w/v)}$ and plain gel formulation (n=3)

Data analysis (determination of flux and permeability coefficient): Flux and permeability coefficient of the optimized gel formulation E2 $_{(1.5\%\ w/v)}$ were determined. The flux and permeability coefficient of E2 $_{(1.5\%\ w/v)}$ elastic liposomal based gel was significantly higher($p \leq 0.05$) as compared to plain gel formulation, which exhibited low flux and permeability coefficient. The results are shown in Table 4.

Table 4: Flux (J_{ss}) and permeability coefficient (k_p) of optimized gel formulation (E2 $_{(1.5\%\ w/v)}$) and plain gel formulation

S. No.	Formulations	Flux (µg/cm²/h) ± S.D (n=3)	Permeability coefficient (k_p)± S.D (n=3)
1	Optimized formulation (E2 $_{(1.5\%\ w/v)}$)	519.5± 0.110	0.1039 ± 0.002
2	Plain gel	367.8± 0.125	0.07356 ± 0.012

Determination of drug retention in the corneal membrane: Determination of drug retention (mg) and % drug retained in the corneal membrane was determined and significantly higher ($p \leq 0.05$) drug retention was observed in the case of optimized gel formulation E2 $_{(1.5\%\ w/v)}$. Drug retention (mg) and % drug retained in the case of optimized gel formulation E2 $_{(1.5\%\ w/v)}$ was found to be 1.05± 0.23 mg and 21±0.547 %, respectively. Drug retention and % drug retained in the case of plain gel were found to be 0.77± 0.11 mg and 15.4± 0.236 %, respectively.

Toxicity study

Corneal hydration test: Corneal hydration test was carried out and hydration was found to be 76.78%± 1.8%, which was within the range of (75- 80%) (Zubairu *et al.*) (Zubairu *et al.*, 2015), indicating that the formulation did not damage the cornea.

Histopathological study: Histopathological study was carried out and the examination revealed that the exposure of goat cornea to potassium chloride solution has shown considerable damage to the corneal tissues

(Figure 3), whereas no remarkable change was noticed in the corneal tissues treated with normal saline (Figure 4) and elastic liposomal based gel (Figure 5). The results indicated that elastic liposomal based gel did not cause any alteration in the structure of cornea thus conserving the histological structure of all corneal layers: the epithelium, the stroma and the endothelium. It was concluded that elastic liposome-based gel could be safely applied to the eye (Zubairu *et al*., 2015).

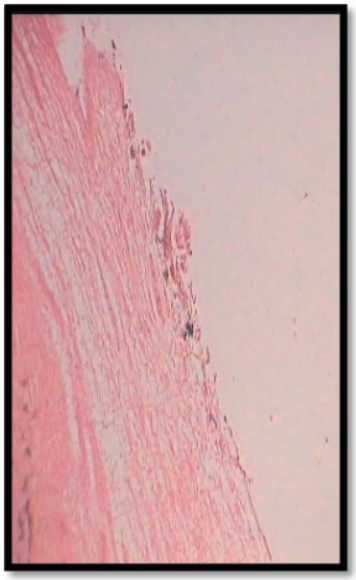

Figure 3: Goat cornea in saturated potassium chloride

Figure 4: Goat cornea in normal saline

Figure 5: Goat cornea treated optimized gel formulation

Effect of formulation on corneocytes: Biochemical estimation of LDH was carried out using a Coral clinical systems kit for LDH. LDH release in case of saline was found to be 11.9 ± 1.8 units/ cm^2, and LDH release in the case of elastic liposome-based gel was found to be 12.1 ± 1.3 units/ cm^2. In the case of span 80- soya lecithin mix, high LDH release was observed (18.62± 1.45 units/ cm^2) due to tissue destruction. Thus, a complete toxicological examination disclosed that the prepared elastic liposomal gel formulation had lower toxic potential.

After testing the levels of LDH in the Tyrode solution present in the receptor compartment, the goat corneas were homogenized using a tissue homogenizer. The homogenate obtained after homogenization was centrifuged. After centrifugation, the supernatant obtained was used for testing the LDH level. However, no significant difference was found in LDH levels in the case of normal saline (332.2± 1.2 units/ cm^2) and optimized elastic liposomal gel formulation (339.2 units/ cm^2), indicating that the optimized formulation was safe. On the contrary, span 80- soya lecithin mixture had a significantly higher (427.52 units/ cm^2) LDH level as compared to normal saline, further confirming that the elastic liposomal gel formulation ($E2_{1.5\%\ w/v}$) had lower toxic potential.

Bio adhesion testing: Bio adhesion testing of the optimized gel formulation was carried out, figure 6 shows the image of bio adhesion assembly. The bioadhesive potential of optimized elastic liposomal gel was compared to the marketed formulation (Flucos gel). From the results, it was observed that the optimized gel formulation ($E2_{(1.5\ \%\ w/v)}$) had a significantly ($p \leq 0.05$) higher (205± 5.5 min) bio adhesion time on the agar plate as compared to the marketed formulation (Flucos gel) (17± 0.5 min). The results indicated that the optimized gel formulation had better bio adhesion property than the marketed formulation.

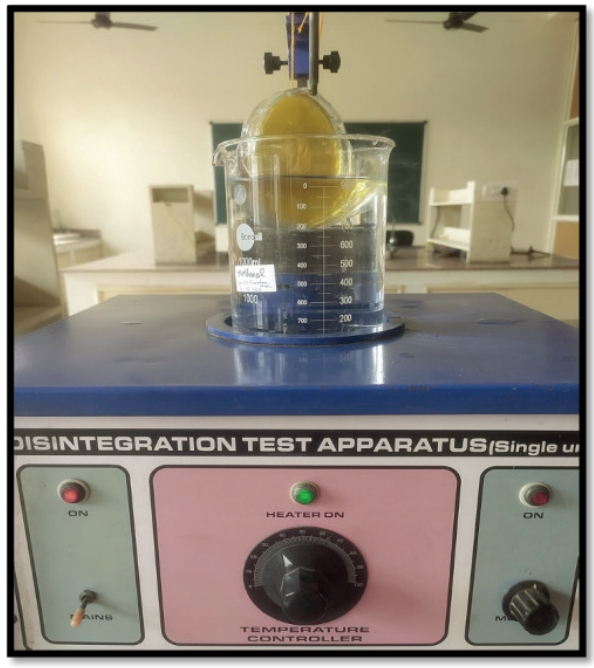

Figure 6: Bioadhesion testing assembly

Antifungal activity of the prepared gel by cup and plate method: Zone of inhibition for different formulations was determined. A significantly larger ($p \leq 0.05$) zone of inhibition was observed for elastic liposome-based gel E2 $_{(1.5\%\ w/v)}$ compared to the marketed formulation (Flucos gel) and placebo formulation, after 48 hours of the study period. The results are revealed in Table 5, Figure 7, and Figure 8.

Table 5: Zones of inhibition for Fluconazole loaded different formulations against *Candida albicans*

Formulations	Zone of inhibition (mm)	
	24 hrs± S.D (n=3)	**48 hrs± S.D (n=3)**
Elastic liposomal gel (El.G)	28 ± 0.9	34±1.4
Marketed gel (Flucos gel) (M.F)	18±1.1	20±1.0
Placebo gel (P.G)	0	0

Figure 7: Comparison of the zone of inhibition for various formulations evaluated against *Candida albicans* after incubation of 24 hours

(Here, El.G= Elastic liposome-based gel, P.G= Placebo gel and M.F= Marketed Formulation)

Figure 8: Comparison of the Zone of inhibition for various formulations evaluated against *Candida albicans* after incubation of 48 hours

Stability study: The stability study of elastic liposome-based gel E2 $_{(1.5\% w/v)}$ was carried out. The elastic liposome-based gel E2 $_{(1.5\% w/v)}$ (1g) was withdrawn at the end of 30, 60 and 90 days and was evaluated for pH, viscosity and drug content. The results are revealed in Table 6, Table 7, Figure 9 and Figure 10. No significant difference was observed in pH, viscosity, drug content and phase separation were not noticed in the elastic liposomal gel formulation, kept for stability study at 4° C.

Table 6: Stability study of optimized elastic liposome-based gel E2 $_{(1.5\% w/v)}$ when it was kept at 4° C

Time (Days)	pH ± S.D	Viscosity ± S.D	Drug content (mg) ± S.D	% Drug remaining	Log % drug remaining	Phase separation
0	7.0±0.036	7217±0.21	4.925± 0.023	100	2	No
30	7.01±0.041	7214±0.24	4.923± 0.025	99.96	1.9998	No
60	7.07±0.043	7213±0.29	4.919±0.029	99.87	1.9994	No
90	7.09±0.038	7211±0.25	4.915±0.031	99.8	1.9991	No

Figure 9: Gel formulation at the end of 90 days when it was kept at 4° C

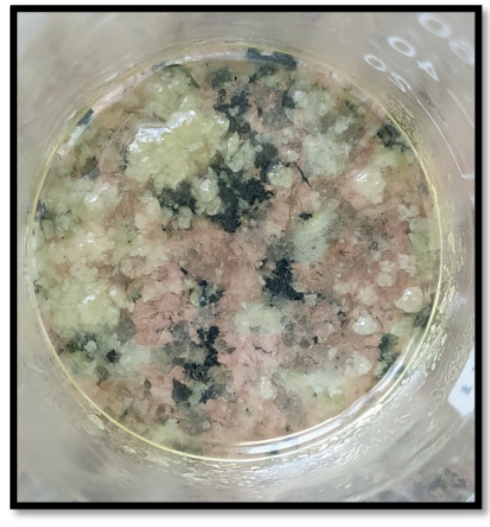

Figure 10: Gel formulation at the end of 30 days when it was kept at room temperature

Table 7: Stability study of optimized elastic liposome-based gel E2 $_{(1.5\%\ w/v)}$ when it was kept at room temperature

Time (month)	pH ± S.D	Viscosity ± S.D	Drug content (mg) ± S.D	% Drug remaining	Log % drug remaining	Phase separation
0	7.0±0.025	7217±0.19	4.925± 0.022	100	2	No

Phase separation was observed in elastic liposomal gel formulation after 30 days, when kept at room temperature.

So from the above results, it was concluded that the elastic liposomal gel formulation was more stable when kept at 4° C.

DISCUSSION

Preparation of elastic liposome-based gel

The optimized elastic liposomal suspension was incorporated into the gel to increase the retention time of the formulation in the eye. Enhanced retention would improve the therapeutic action by decreasing the lacrimal discharge of elastic liposomal suspension (Sayeh *et al*., 2014).

Evaluation of elastic liposome-based gel

In vitro release study: From the results obtained, it was observed that the elastic liposome-based gels showed continuous and sustained release of Fluconazole which may be because of the reservoir effect of elastic liposomes due to a combination of release from elastic liposomes first followed by diffusion through the gelling agent. The entrapped fluconazole molecules leaked out slowly from the vesicles into the enclosing gel and then into the release media, thereby providing a sustained effect. The advantage of sustained-release could be reduced application frequency and increased patient compliance (El-Gizawey *et al*., 2020).

Ex vivo permeation study: From the above *ex vivo* trans-corneal permeation studies, maximum permeation of Fluconazole was detected for the formulation E2 $_{(1.5\%\ w/v),}$ which was significantly higher ($p \leq 0.05$) than the plain gel formulation after 6-hour study. The higher permeation of Fluconazole from the elastic liposomal formulation E2 $_{(1.5\%\ w/v)}$ could be attributed to the presence of edge activators which render the formulation more flexible and permeable for transcorneal transport. The flux (519± 0.110 μg/cm^2/h) and permeability coefficient (0.1039) of E2 $_{(1.5\%\ w/v)}$ elastic liposomal based gel were significantly higher ($p \leq 0.05$) as compared to plain gel formulation which exhibited low flux (367.8± 0.125 μg/cm^2/h) and low permeability coefficient (0.07356).

The significantly higher ($p \leq 0.05$) permeation of Fluconazole from formulation E2 $_{(1.5\%\ w/v)}$ could be attributed to the smaller size of particles, the ability of elastic liposomes to squeeze through channels 1/10th of their diameter and their ability to penetrate deeper tissues (Benson, 2009). Another reason could be the presence of an edge activator, which worked as a permeation enhancer and played a significant role in enhancing the permeation (Tiwari *et al*., 2020).

Determination of drug retention in the corneal membrane: From the results obtained, a significantly higher amount of Fluconazole was localized into the cornea after application of elastic liposomal gel E2 $_{(1.5\%\ w/v)}$ as compared to plain gel formulation. More corneal retention in the case of elastic liposomal gel E2 $_{(1.5\%\ w/v)}$ was due to greater penetration of the drug from elastic liposomal gel due to nanosized elastic liposomal vesicles and their ability to penetrate through smaller pores and to penetrate deeper tissues (Benson, 2009). Besides this, another reason could be the establishment of an interaction with the outer structures of the eye, which results in the accumulation of elastic liposomes in the corneocytes. This interaction could allow systems to be released for more than 24 hours (Cristiano *et al*., 2019).

Toxicity study

Effect of formulation on corneocytes: In the case of span 80- soya lecithin mix, LDH release was observed to be significantly higher ($p \leq 0.05$) (18.62± 1.45 units/

cm^2), which could be attributed to tissue destruction. On the contrary, elastic liposomal gel formulation had a significantly lower (12.1± 1.3 units/ cm^2) LDH levels. As reported in the literature by Negi *et al.* (Negi *et al*., 2013), it may be due to the fact that surfactants bound to the vesicular system had a lower potential to cause cellular damage than free molecules.

Bioadhesion testing: The results indicated that the optimized gel formulation had better bioadhesion property than the marketed formulation. Due to better bioadhesion, the formulated elastic liposomal gel formulation would have better residence time on the eye, thereby improving the therapeutic efficacy with a reduction in dosing frequency.

Antifungal activity of the prepared gel by cup and plate method: A significantly larger zone of inhibition was observed for elastic liposome-based gel $E2_{(1.5\%\ w/v)}$ compared to marketed formulation (Flucos gel) and placebo formulation, after 48 hours of the study period. The larger zone of inhibition for elastic liposome-based gel $E2_{(1.5\%\ w/v)}$ could be attributed to nanosized particles of Fluconazole contained in elastic liposomes and due to the flexibility of elastic liposomes and ability to penetrate through small pores, which resulted in greater penetration through fungal cell walls, to inhibit ergosterol synthesis (Basha *et al*., 2013).

CONCLUSION

The elastic liposome gel was prepared successfully and was satisfactory in terms of release, bioadhesion and viscosity, with significantly higher penetration and better corneal drug retention. The present study endorsed that the elastic liposomal-based topical ophthalmic gel formulation was a good choice over conventional gel formulation for the treating keratomycosis.

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CONFLICT OF INTEREST

All the authors of this research declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Experimenting and the study text (RN), Developing hypothesis, Statistics, analysis and interpretation of the data (JKN), Literature Research (M), Histopathological experiments and interpretation of the data (RSN)

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