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Carissa Carandas-Loaded Leciplex: Development and Optimization of a Mucoadhesive Ocular Nanovesicular System for Sustained Drug Delivery and Management of Diabetic Retinopathy

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ABSTRACT: Effective ocular drug delivery remains challenging because of the complex anatomical and physiological barriers of the eye; however, nanotechnology-based approaches offer promising strategies to overcome these limitations and enhance targeted drug delivery. In this study, a simple one-step fabrication technique was employed to develop Carissa carandas-loaded leciplex, which were then optimized using a 32 Full Factorial Design. The Carissa Carandas-LP demonstrated a uniform size distribution, impressive entrapment efficiency of $96.015 \pm 1.45\%$, and a zeta potential of 22.24 ± 1.10 mV. In addition, ex vivo corneal permeation showed consistent and sustained drug permeation, while in vitro mucoadhesive experiments with different mucin concentrations demonstrated strong electrostatic bonding, confirming the formulation's mucoadhesive qualities. Moreover, histological studies ensured that the formulation is non-toxic and non-irritating, and the Draize test confirmed its significant tolerance; the in vivo pharmacodynamic study on a zebrafish model revealed that treatment with Carissa Carandas-LP led to a significant and lasting decrease in diabetic retinopathy. Overall, Carissa carandas-LP represents a promising and versatile drug delivery system with potential for the management of diabetic retinopathy.

INTRODUCTION

Diabetic retinopathy (DR) is one of the leading causes of vision loss among working-age adults [1]. The Wisconsin Epidemiologic Study of Diabetic Retinopathy reported that DR contributes to approximately one-third of blindness among individuals with type 2 diabetes and nearly 86% of blindness among those with type 1 diabetes [2]. In addition to DR, diabetes is associated with other ocular complications, including cataracts and optic neuropathies [1]. While the rates of incidence can differ across various populations and historical contexts, research from the US and Europe indicates that DR is a major cause of blindness, particularly in individuals aged 20 to 70 years [3]. The risk of progressing to proliferative diabetic retinopathy (PDR) and experiencing significant vision loss is heightened due to inadequate screening, with many people only seeking help once symptoms become apparent [4]. The pathogenesis of DR involves multiple interrelated mechanisms, including persistent hyperglycemia, oxidative stress, inflammation, endothelial dysfunction, vascular permeability, and pathological angiogenesis [5]. These processes contribute to progressive retinal vascular damage and disruption of the blood-retinal barrier, highlighting the need for therapeutic approaches that can address multiple pathological pathways associated with DR [4, 5].

Intravitreal medication plays a crucial role in managing diabetic retinopathy, which is a progressive microvascular complication of diabetes. This is particularly true for cases involving proliferative diabetic retinopathy (PDR) and diabetic macular edema

(DME) [6]. To enhance visual acuity and reduce retinal swelling, anti-vascular endothelial growth factor (anti-VEGF) drugs like Ranibizumab, Aflibercept, and Bevacizumab are frequently injected into the eye. These treatments work by decreasing vascular permeability and preventing the growth of new blood vessels [7]. For some patients with PDR, these medications can also serve as effective alternatives to panretinal photocoagulation. Additionally, intravitreal corticosteroids, such as Dexamethasone implants, are utilized to alleviate macular edema and manage inflammation when anti-VEGF therapy isn't sufficient or suitable [8]. While these treatments can be quite effective, they do come with some notable downsides. For starters, patients often need to deal with repeated injections and continuous monitoring, not to mention the hefty price tag. There are also potential side effects to consider, like endophthalmitis, increased intraocular pressure, and the risk of developing cataracts, particularly when steroids are involved [9]. Additionally, it's important to remember that these medications primarily modify the disease rather than cure it, as responses can vary from patient to patient, and there's always a chance the disease could return once treatment is stopped. These limitations have encouraged investigation into alternative and complementary therapeutic strategies capable of providing sustained ocular exposure while reducing the frequency and invasiveness of conventional administration. In particular, localized ocular delivery of bioactive compounds may provide an opportunity to improve therapeutic exposure at the target site while minimizing unnecessary systemic exposure [10].

Herbal and plant-based compounds are gaining traction as complementary treatments alongside well-known intravitreal medications like anti-VEGF drugs, such as Ranibizumab and Aflibercept [11]. In various experimental and early clinical studies, phytochemicals like curcumin (derived from *Curcuma longa*), resveratrol (found in *Vitis vinifera*), and Ginkgo biloba extracts have demonstrated antioxidant, anti-inflammatory, and anti-angiogenic properties. This suggests they could play a role in slowing down retinal damage [12]. These substances might help reduce vascular dysfunction and retinal oxidative stress, both of which are key factors in the progression of the disease. Among medicinal plants, *Carissa carandas* has attracted interest because of its diverse phytochemical composition and reported antioxidant and other pharmacological properties. The presence of biologically active phytoconstituents in *C. carandas* suggests its potential as a source of bioactive compounds for investigating oxidative stress- and inflammation-associated disorders [13]. However, the therapeutic application of plant-derived compounds may be limited by factors such as poor aqueous solubility, instability, limited permeability, and inadequate residence time at the site of administration [13, 14]. Herbal medications offer several benefits compared to traditional intravitreal therapies like anti-VEGF injections, including the convenience of topical delivery, lower costs, systemic metabolic advantages, and a solid safety profile with fewer complications related to procedures [13, 14]. However, the potential benefits of phytoconstituents can only be translated into effective ocular therapy when adequate drug concentration and residence time can be achieved at the target ocular tissues.

Modern drug delivery systems are revolutionizing the way we manage DR by tackling those pesky ocular barriers and allowing for targeted, long-lasting drug release right to the retina. Traditional delivery methods often struggle with getting enough medication to the retina, but cutting-edge systems like nanoparticles, liposomes, hydrogels, and intravitreal implants are stepping up to the plate [15]. For topical ocular formulations, however, rapid tear turnover, blinking, nasolacrimal drainage, limited corneal permeability, and the protective epithelial barrier can substantially reduce the amount of drug reaching intraocular tissues. Consequently, strategies that increase ocular surface residence time and facilitate corneal permeation are particularly important for improving the effectiveness of topical ocular drug delivery.

One standout option is Leciplex formulations—these are cationic lipid-based nanocarriers that enhance corneal permeability, improve drug stability, and extend how long the medication stays in the eye by using electrostatic interactions with the ocular surface [16]. The positive surface charge of Leciplex is particularly relevant to ocular delivery because mucins present on the corneal and conjunctival surface contain negatively charged components. Electrostatic attraction between the positively charged nanocarrier and negatively charged mucin may enhance mucoadhesion, thereby prolonging formulation residence on the ocular surface and providing greater opportunity for drug permeation [17]. The nanosized vesicular structure may further facilitate intimate contact with the ocular surface and improve the delivery of poorly water-soluble bioactive compounds. This all leads to better therapeutic outcomes [16, 17]. In a nutshell, these innovative approaches not only make drug delivery more efficient but also less invasive, ultimately improving patient compliance in treating DR. Therefore, the development of a mucoadhesive Leciplex-based ocular delivery system may represent a promising strategy for improving the ocular delivery of plant-derived bioactive compounds intended for the management of diabetic retinopathy.

In the context of ocular delivery, this electrostatic interaction is particularly relevant because the ocular mucin layer carries a predominantly negative charge. Consequently, positively charged Leciplex may exhibit enhanced mucoadhesion at the ocular

surface, potentially increasing residence time and facilitating drug transfer across the corneal barrier. The ability of Leciplex to incorporate poorly water-soluble compounds, together with its nanoscale size and cationic surface characteristics, makes it a promising carrier for topical ocular delivery [18].

Despite these promising characteristics, limited studies have explored the application of Leciplex as a mucoadhesive ocular delivery system for plant-derived bioactive compounds in the context of diabetic retinopathy. Therefore, in the present study, *Carissa carandas*-loaded Leciplex was developed using a simple one-step fabrication approach and optimized using a 3² full factorial design. The optimized formulation was subsequently evaluated for its physicochemical characteristics, drug entrapment, zeta potential, *in vitro* release, *ex vivo* corneal permeation and retention, mucoadhesive interaction with mucin, ocular safety, and *in vivo* pharmacodynamic potential against diabetic retinopathy.

MATERIALS AND METHODS

Materials

Lipoid GmbH, a German company, gave us a gift sample of Lipoid S 100, which contains 95.40 percent phosphatidylcholine from soybeans. From Gattefosse India Ltd. (Mumbai, India), a complimentary sample of Transcutol P (diethylene glycol monoethyl ether) was acquired. Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) was the supplier of didodecyl dimethyl ammonium bromide (DDAB)

Experimental animals:

All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), Biocyte Institute of Research and Development (BiRD), Sangli, Maharashtra, India (Approval No. IAEC/BiRD/Sangli/2024-25/15; approved on 15 April 2024) and were conducted in accordance with the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India.

In the present study, adult zebrafish were sourced from a certified supplier (Wild variety from local dealer 777 Aquatics India Pvt. Ltd Madhya Pradesh) and allowed to acclimate for a week in a controlled lab environment. The fish were kept in aerated, dechlorinated water, maintaining a temperature of 26 ± 1 °C, a pH level between 7.0 and 7.5, and ensuring dissolved oxygen levels were at least 6 mg/L. The water hardness was kept between 80 and 120 mg/L, and we made sure that ammonia and nitrite levels were undetectable. The fish experienced a light cycle of 14 hours of light followed by 10 hours of darkness. During the acclimatization period, they were fed a standard pelleted diet, but we refrained from feeding them during the exposure phase, in line with OECD Guideline 203. We also made sure to regularly refresh the water using purified sources.

Methods:

Formulation of *Carissa Carandas*- leciplex (*Carissa carandas*-LP)

Lecithin-based cationic nanocarriers, *Carissa Carandas*-LP, were developed through a simple and one-step synthesis process [19]. In brief, 0.5 mL of Transcutol-P was added to 24 mM (186 mg) of soybean lecithin (Lipoid S 100) along with DDAB, 24 mM (111 mg). The excipients, primarily phospholipids and DDAB, were dissolved by heating them in a water bath set to 70°C. *Carissa Carandas* (CC) 60 mg was added to the lipid phase under heating to get a smooth and homogeneous solution. In the final step, the lipid phase was heated to 70 °C until a uniform nanodispersion (leciplex) formed. The stirring was continued at 1200 rpm, and gradually 10 mL of double-distilled water was added.

Optimization *Carissa Carandas*-LP

The *Carissa Carandas*-LP was optimised using Response Surface Methodology (RSM), employing a 3² Full Factorial Design (Design Expert®, version 13; Stat-Ease Inc., USA). This methodology helped to explore the relationship between the product's characteristics and its critical quality attributes (CQAs). The impact of independent variables like phospholipid concentration (A) and DDAB concentration (B) was studied on dependent variables like particle size (Y1), PDI (Y2), % entrapment efficiency (Y3), and zeta potential (Y4). **Table 1** summarises the dependent and independent variables with their levels.

Table 1: Details of dependent and independent variables

Independent variables	Levels		
	Low (-1)	Medium (0)	High (+1)
A = Soy lecithin	139.5	186	232.5
B = DDAB	83.25	111	138.75
Dependent Variables			
Y1 = Particle size	Y2 = Polydispersity index		
Y3 = % EE	Y4 = Zeta potential		

Evaluation of *Carissa carandas*-LP:

Appearance, pH and Refractive index (RI):

The colour, appearance, and clarity of the LP formulation were checked by visual evaluation against a dark background. The pH of the formulation was determined using a calibrated Labotronics pH meter. The RI of the LP formulation was determined using an Abbe refractometer (Unicon).

Particle size and Zeta potential

The particle size analysis for *Carissa carandas*-LP was conducted using a Sympatec NanoPhox particle size analyzer from Germany. The LP formulation was diluted with double-distilled water to get the right scattering intensity. Similarly, zeta potential was determined using the electrophoretic light scattering method [20].

Entrapment efficiency (EE):

Carissa carandas-LP dispersion was accurately measured and transferred into a centrifuge tube, and 100 mg of sodium chloride was added to facilitate the separation of the vesicular phase. This dispersion was then subjected to ultracentrifugation (Eppendorf 5430R) at 12,000 rpm for 30 min at 4°C. Following centrifugation, the supernatant containing the unentrapped/free *Carissa carandas* extract was carefully separated from the vesicular fraction.

The supernatant obtained was then collected, filtered, and analyzed with a validated HPLC method [21].

The total amount of extract incorporated into the formulation was determined from the amount initially added during formulation preparation. The EE was calculated using the following equation.

$$EE(\%) = \frac{Td - Fd}{Td} \times 100$$

Where,

Fd: Free drug found in the supernatant,

Td: Total amount of medication that has been delivered.

Transmission Electron Microscopy (TEM):

The morphological characteristics of the LP formulation were determined by using TEM. *Carissa carandas*-LP formulation was diluted in double-distilled water, and a drop of this diluted solution was then placed on a copper grid. To enhance the imaging, 2% phosphoric tungstic acid was applied to the grid as a negative stain and kept it for drying. Finally, the grid was scanned under vacuum conditions [21].

In vitro drug release study:

Cellophane membrane diffusion technique was used to study in-vitro diffusion of drug from

the prepared nanogel formulations [22]. The receptor medium used was freshly prepared phosphate buffer pH 7.4. Cellophane membrane soaked overnight in the receptor medium was on the Franz's Diffusion cell assembly 0.5 g of formulation was placed in the donor compartment and the assembly was kept on the multistation diffusion study apparatus (make Orchid Scientific) at $37^{\circ}\text{C} \pm 20^{\circ}\text{C}$ and stirred at 100 RPM. Aliquots of 0.5 ml were withdrawn at pre-determined time intervals (1, 2, 3, 4, 5, and 6 hrs.) and immediately replaced by same volume of the fresh medium. The aliquots were suitably diluted with the dissolution medium and analyzed by UV-Vis Spectrophotometer at 210 nm (λ_{max}). The data obtained from the in-vitro diffusion studies were fitted to various kinetic equations to find out the mechanism of drug release from the optimized batch.

Trans corneal permeation study:

This study was performed using isolated goat cornea along with a Franz diffusion cell to explore how *Carissa Carandas* penetrates the cornea from *Carissa Carandas*-LP [23]. The fresh eyeballs were sourced from a local slaughterhouse right after the animal was processed. The cornea was carefully extracted, making sure to leave a 2 to 4 mm ring of sclera around it. The cornea was rinsed with a cold phosphate buffer solution at pH 7.4 to get rid of any protein debris. The cornea, with a surface area of 2.2 cm^2 , was carefully placed between the donor and receptor compartments in such a way that the endothelium faced the donor side, while the epithelium was directed towards the recipient side [21].

Franz diffusion cell's temperature was kept constant 37°C by the surrounding jacket water. The magnetic stirrer was used to stir the medium at a speed of 100 rpm. The samples were collected at regular intervals i.e. 1, 2, 3, 4, 5 and 6 hr, and fresh phosphate buffer pH (7.4) in the same quantity was introduced into the receptor compartment to keep the volume constant. The spectrophotometer was used for the analysis of the drug amount in the samples at 210 nm

and phosphate buffer pH 7.4 was used as a blank.

Histopathology study:

The animal tissue sample was isolated and put in a 10% neutral buffer formalin solution. Specimens were cut into sections of 3-5- μm thickness and embedded in paraffin. Serial sections (3 μm) were cut using a microtome (Leica Biosystems, Germany) and stained with hematoxylin-eosin (H&E) stain. The prepared slides were examined under a microscope (Motic Microscopes, Tri-County Pkwy, Schertz, TX 78154, United States). The severity of the observed lesions was recorded as NAD = No Abnormality Detected, Minimal changes (+1), Mild changes (+2), Moderate changes (+3), Severe changes (+4) (Modi et al., 2022).

In vitro mucin mucoadhesion study:

An attempt was made to study the mucoadhesive potential of LP formulations by recording the variation in the zeta potential while interacting with mucin [24]. Briefly, the formulations were dissolved in double distilled water to get a final concentration of 1 mg/ml. Similarly, the bovine mucin was dispersed in double-distilled water to prepare a stock solution (10 mg/ml). A series of different concentrations of mucin (0, 0.25, 0.50, 0.75, and 1.0%) were prepared using double-distilled water and incubated with formulations (1 mg/ml) for half an hour at room temperature. After the incubation, the zeta potential was recorded, and observed the variations in the same.

Acute Eye Irritation Study

The present study was performed in albino Rabbits to evaluate the preclinical safety of ophthalmic formulations. The study was performed in one group consisting of 3 Albino Rabbit (2 male or 1 each female)). The test formulation was administered into the eye at a dose of 0.1mL (100 μL) on day one of the experiment.

Adult Wistar albino rabbits were acclimatized to laboratory conditions for a period of 5 days before dosing. On Day 1, 0.1 mL (100 μL) of the test item (for liquids) was instilled into the conjunctival sac of one eye, while the other eye served as a control. The eyelids were held gently closed for approximately 1 second after instillation. The rabbits were monitored at 1, 24, 48, and 72 hours post-application, and daily thereafter for up to 21 days to assess for signs of ocular irritation. Observations included corneal opacity, iris inflammation, conjunctival redness, chemosis, and discharge. The reactions were scored using the Draize scoring system, and reversibility of any effects was noted during the observation period [25].

Induction of hyperglycemia in Zebra Fish:

The required amount of D-glucose was weighed to prepare a 2–2.2% (w/v) solution. 20–22 g of glucose was dissolved in dechlorinated water and mixed thoroughly using a magnetic stirrer. The pH was adjusted to 7.0–7.5 to match zebrafish tank water. The temperature of the water was maintained at 25–28°C to ensure zebrafish survived. The zebrafish were divided into control and experimental groups (at least 6–10 fish per group). The experimental group animals were transferred into the prepared glucose solution and exposed for 30–60 minutes on alternate days (i.e., every other day) for 21 days. After each session, the zebrafish were transferred back to their normal tank water. The control group was maintained in regular dechlorinated water [26].

Retinal tissue and staining

1. Whole eyes were removed from the fish following hemisection of the brainstem by a rostral-caudal incision through the brain. The eyes were then incubated in a 4% paraformaldehyde solution at 4°C for at least one week. Fixed whole eyes were allowed to equilibrate in a 30% sucrose solution in phosphate-buffered saline at 4°C overnight. Whole eyes were embedded in Tissue Freezing Medium (TFM), and 20-µm cryostat sections were made and collected on silanated slides (Amine). The slides were stored at -80°C until use for staining. When labelling sections, tissue was allowed to come to room temperature. 4,6-Diamidino-2-phenylindole (DAPI) staining was used to examine the morphology of the cellular layers of the retinal tissues. DAPI (1:5000) was added to each section for 20 min, followed by washing three times with phosphate-buffered saline. Slides were mounted with a coverslip with 50% glycerol in PBS before viewing. Sections were viewed using fluorescence microscopy (Olympus BX51), and photographs were collected using a Nikon Coolpix 4500 digital camera. All pictures were greyscale and viewed at 200%. Retinal layers from sections of fish sacrificed on days 0 and 28 were quantified. “C” shaped sections from each fish were identified, and the retinal area opposite the lens was photographed at 40x magnification. Pictures were transferred to Adobe Photoshop, and looked for the ganglion layer (GL), inner plexiform layer (IPL), and INL for each section. [27].

RESULTS AND DISCUSSION:

The present study was undertaken to develop and optimize a *Carissa carandas*-loaded Leciplex (*Carissa carandas*-LP) as a mucoadhesive nanovesicular system for ocular delivery, with the aim of improving formulation stability, ocular residence, drug permeation, and sustained therapeutic performance in the management of diabetic retinopathy. A systematic formulation optimization approach was employed using a 3² full factorial design to investigate the influence of selected formulation variables on the critical quality attributes of the Leciplex system. The optimized formulation demonstrated favorable physicochemical characteristics, high entrapment efficiency, positive surface charge, controlled drug release, and enhanced corneal retention, indicating the potential of the Leciplex platform to overcome some of the limitations associated with conventional ocular delivery systems.

The levels and responses of independent variables from the experimental runs are shown through a 3² Full Factorial Design in Table 2.

Table 2: Actual values of Independent and dependent variables

Run	Independent variables		Dependent variables			
	Soy lecithin (A)	DDAB (B)	Particle size Y1(nm)	PDI (Y2)	EE Y3 (%)	Zeta Potential Y4(mV)
1	186	83.25	726.5	0.689	94.3	15.25
2	232.5	83.25	775.2	0.798	94.25	16.21
3	186	111	817.99	0.789	95.12	20.11
4	139.5	111	557.98	0.978	96.25	20.56
5	139.5	83.25	512.69	0.896	96.08	17.96
6	139.5	138.75	593.3	0.998	94.81	28.49
7	232.5	138.75	720.66	0.745	93.93	31.98
8	186	138.75	779.89	0.769	93.25	28.69
9	232.5	111	751.26	0.836	95.1	23.66

**Carissa Carandas* 60 mg

The effects of independent variables on PDI and particle size:

The particle size of the vesicles was found in the range of 512.10 ± 0.49 nm to 817.10 ± 0.89 nm (**Table 2**). It seems that the formation of larger vesicles, due to increased viscosity, could explain the significant increase in particle size with higher phospholipid levels [28]. This finding aligns with earlier research that utilized the same methods to create a leciplex loaded with Acetazolamide & Vancomycin Hydrochloride [21]. A higher concentration of DDAB would lead to the phospholipids becoming more soluble, which in turn would reduce the particle size. A 3D response surface map illustrates how the particle size evolves with various independent factors. As shown in Fig.2, the particle size decreases, as indicated by the negative coefficient of "B," in tandem with an increase in DDAB concentration in Eq. (1). A negative coefficient for "B" suggests that there's an inverse relationship between size and the individual components, while a positive coefficient (as seen in Eq. (1) points to a direct correlation between particle size and the concentration of phospholipids (A).

$$(Y1) = +791.04 + 13.2433 A + 97.1917 B - 33.7875AB - 24.37A^2 - 122.945 B^2 \dots\dots\dots(1)$$

The ANOVA statistical analysis showed a significant model ($p < 0.05$) with an F-value of 25.82. There was a strong correlation between the adjusted R^2 (0.9394) and the expected R^2 (0.8041). Additionally, Adeq Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Our ratio of 13.252 indicates an adequate signal. This model is well-suited for exploring the design space, as evidenced by a precision value of 13.252, which indicates a reliable signal.

The polydispersity index (PDI) of the *Carissa carandas*-LP formulations ranged from 0.11 ± 0.05 to 0.83 ± 0.03 . Nanoparticles with a PDI below 0.3 are considered monodisperse, while those above 0.7 are classified as polydisperse [29]. For this study, the optimal formulation was selected based on a PDI value of 0.8. The PDI was positively influenced by the phospholipid content, while the surfactant concentration had the opposite effect (Eq. (2)). With a significance level of $p < 0.05$, the model's F-value of 35.36 indicates statistical significance. The modified R^2 of 0.9555 and the expected R^2 of 0.8002 were quite close to each other. A precision of 16.063 suggests a suitable signal, confirming that the model can effectively guide the design space.

$$(Y2) = +0.783556 + 0.0215A - 0.0821667 B - 0.03875AB - 0.0518333A^2 + 0.126167B^2 \dots\dots\dots (2)$$

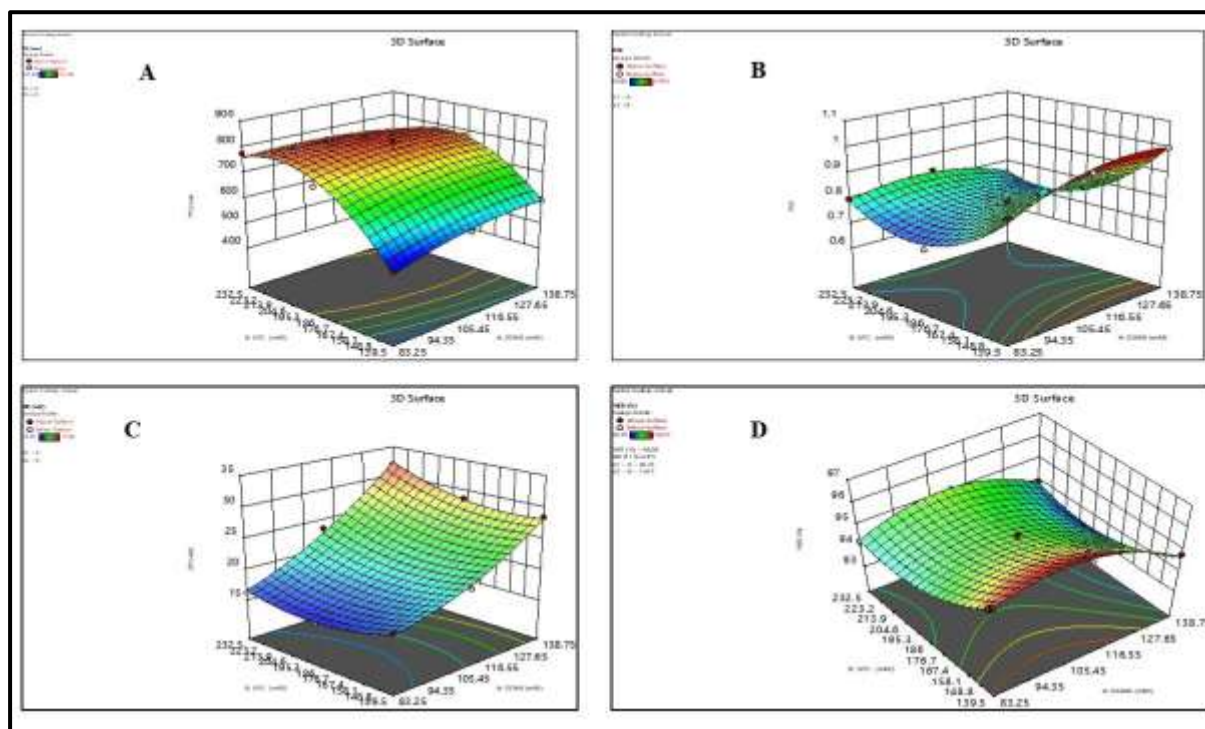


Fig. 2: 3D response graph showing impact of independent variables on dependent variables

Impact of independent variables on % entrapment efficiency:

The entrapment efficiency ranged from 94.81% to 96.25%, as shown in **Table 2**. The EE of *Carissa carandas*-loaded Leciplex was significantly influenced by the levels of phospholipid and surfactant used in the formulation. At a constant phospholipid-to-

surfactant molar ratio, an increase in the phospholipid concentration resulted in a corresponding improvement in entrapment efficiency. In contrast, formulations containing a relatively higher proportion of surfactant exhibited comparatively lower entrapment, which may be attributed to the solubilizing effect of excess surfactant on the phospholipid bilayer. Excessive surfactant incorporation may disrupt the structural integrity of the vesicular membrane, thereby increasing membrane permeability and facilitating leakage of the entrapped phytoconstituents into the surrounding aqueous phase [30].

The enhanced entrapment observed with increasing phospholipid concentration may be associated with the formation of a more organized and structurally robust vesicular bilayer [31]. At a constant molar ratio, the increased availability of phospholipid may favor the formation of additional or more compact bilayer structures, thereby providing greater capacity for incorporation and retention of *Carissa carandas* constituents within the vesicular system. Thus, an appropriate balance between phospholipid and surfactant appears to be critical for maintaining vesicular integrity and achieving high entrapment efficiency.

The similarity in the observed formulation behavior supports the role of optimized lipid–surfactant interactions in achieving efficient drug incorporation within Leciplex systems.

The combined influence of the independent formulation variables on entrapment efficiency was further illustrated by the three-dimensional response surface plot (Fig. 1c). The curvature of the response surface demonstrates the interactive effect of the investigated variables and indicates that entrapment efficiency was dependent on their relative concentrations rather than on the individual contribution of either component alone. The response-surface analysis therefore provided a useful basis for identifying the formulation region associated with higher entrapment efficiency and for selecting the optimized *Carissa carandas*-loaded Leciplex formulation.

In the polynomial (Eq. (3)), the positive coefficient for "A" indicates that as the concentration of phospholipid increases, so does the entrapment efficiency. Conversely, the negative coefficient for "B" suggests that higher concentrations of DDAB lead to a decrease in entrapment efficiency. An F value of 37.38 suggests that the model is statistically significant ($p < 0.05$) based on the ANOVA results. The modified R^2 of 0.9579 aligns well with the expected R^2 of 0.8603. This model could be quite helpful for exploring the design space, given its precision of 18.212.

$$(Y_3) = +94.9256 - 0.44 A - 0.643333B + 0.2375AB - 1.05333A^2 + 0.846667B^2 \dots\dots(3)$$

Impact of independent variables on Zeta Potential:

The zeta potential of the *Carissa carandas*-LP formulations ranged from 15.25 to 31.98 mV, indicating that variation in the composition of the vesicular system substantially influenced its surface charge characteristics.

The increase in zeta potential with increasing cationic surfactant concentration may be attributed to the greater availability of positively charged species at the vesicular surface. A higher positive surface charge can enhance electrostatic repulsion between individual vesicles, thereby reducing their tendency to aggregate and contributing to improved physical stability of the dispersion. This observation is in agreement with previous reports demonstrating the role of cationic components in modulating the surface charge and stability of Leciplex-based vesicular systems [32].

The three-dimensional response surface plot (Fig. 2) visually illustrates the combined influence of phospholipid and cationic surfactant concentrations on the zeta potential of *Carissa carandas*-LP. The progressive change in the response surface across the experimental design space supports the positive contribution of both formulation variables and their interaction toward modulation of the vesicular surface charge. The response-surface analysis therefore provided a quantitative basis for selecting the formulation composition capable of producing the desired zeta potential for the optimized *Carissa carandas*-LP system.

The effect of the independent variables, i.e., phospholipid concentration (A) and cationic surfactant concentration (B), was evaluated using the fitted polynomial equation:

$$Y_4 = 20.2478 + 6.62333A + 0.806667B + 1.31AB + 1.6533A^2 + 1.79333B^2 \quad (4)$$

where Y_4 represents the zeta potential, while A and B represent the coded levels of phospholipid and cationic surfactant, respectively. The positive regression coefficient for A (+6.62333) indicates that phospholipid concentration exerted a positive influence on zeta potential within the investigated formulation range. Similarly, the positive coefficient for B (+0.806667) suggests that increasing the concentration of cationic surfactant contributed to an increase in the surface charge of the vesicles. The comparatively larger coefficient associated with A indicates that phospholipid concentration had a greater influence on zeta

potential than the cationic surfactant concentration under the investigated conditions.

The positive interaction coefficient for AB (+1.31) further indicates that the combined effect of phospholipid and cationic surfactant concentrations was favorable for increasing the zeta potential. In addition, the positive quadratic coefficients for A^2 (+1.6533) and B^2 (+1.79333) indicate a nonlinear relationship between the formulation variables and zeta potential, demonstrating that the response was influenced not only by the individual effects of the variables but also by their combined and quadratic contributions.

The statistical significance and adequacy of the developed model were confirmed by analysis of variance (ANOVA). The model exhibited a high F-value of 99.24 with a corresponding p-value < 0.05 , indicating that the model was statistically significant. The adjusted R^2 value of 0.9840 and predicted R^2 value of 0.9274 demonstrate a high degree of agreement between the experimental observations and model-predicted values, with a relatively small difference between the two parameters. Furthermore, the adequate precision value of 27.683, which is considerably higher than the desirable value of 4, indicates an adequate signal-to-noise ratio and confirms that the model can be effectively used to navigate the investigated design space.

During the formulation process, 60 mg of *Carissa Carandas*-LP was effectively captured in 10 mL of Leciplex. To determine the best batch of *Carissa Carandas*-LP, a desirability plot (Fig 3) was used that focused on achieving the highest EE and ZP values while minimizing particle size (PS) and polydispersity index (PDI) values (Abd-Elal et al., 2020). The optimized *Carissa Carandas*-LP formulation consisted of 60 mg of *Carissa Carandas*, 136.95 mg of phospholipids, 83.2 mg of DDAB, 0.5 mL of Transcutol P, and 9.5 mL of double distilled water. Further studies were carried out on this selected optimized *Carissa carandas*-LP formulation.

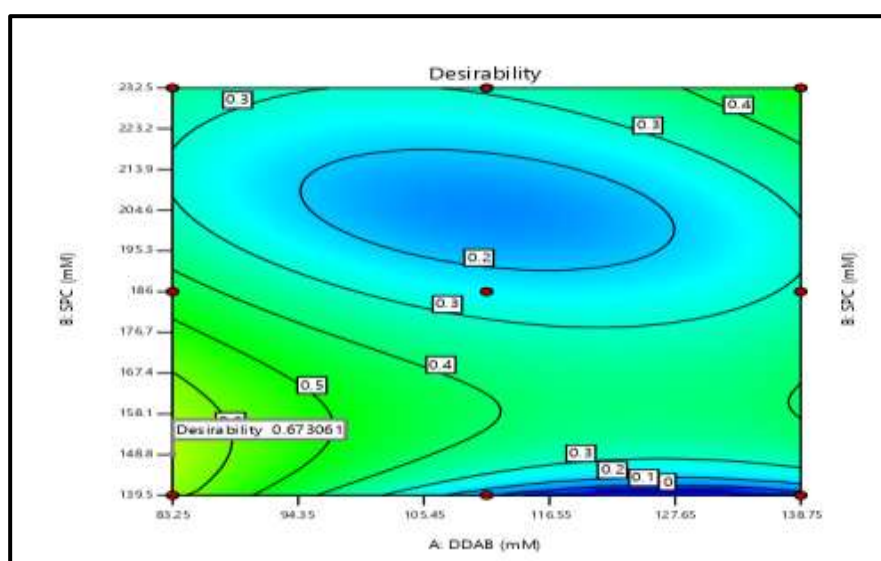


Fig 3: Desirability plot

Evaluation of optimized *Carissa Carandas*-LP

Physical Parameters:

The RI, pH, and physical characteristics of the optimised batch were evaluated. The selected batch of *Carissa Carandas*-LP was found to be uniform and had a cream, milky white colour. The constant replenishment of tear fluid, along with its buffering capacity, helped the eye's surface handle a broad pH range from 3.50 to 8.50, which is essential for maintaining physiological balance. The pH of *Carissa Carandas*-LP measured at 5.80 ± 0.20 , making it suitable and well-tolerated for ophthalmic formulations. Additionally, *Carissa Carandas*-LP has an RI of 1.35 ± 0.05 . The RI of ophthalmic formulations is an important parameter that may influence visual clarity following ocular administration. Ideally, the RI of an ophthalmic formulation should be close to that of the tear film to minimize transient visual disturbances; a range of 1.34–1.36 has been reported as suitable for ophthalmic formulations.

Viscosity Studies:

As shown in **Fig. 3**, *Carissa Carandas*-LP exhibited pseudoplastic flow characteristics, especially shear thinning. This pseudoplastic nature helps ensure that the drug is evenly dispersed and maintains consistent contact with the cornea. It is well known that high-viscosity fluids can cause eye irritation when exposed to significant force [33]. Therefore, the demonstration of shear-thinning (pseudoplastic) behavior is advantageous for ophthalmic formulations, as the reduction in viscosity under applied shear facilitates ease of administration and spreading across the ocular surface, while the viscosity increases under low-shear conditions after administration, potentially promoting prolonged ocular residence.

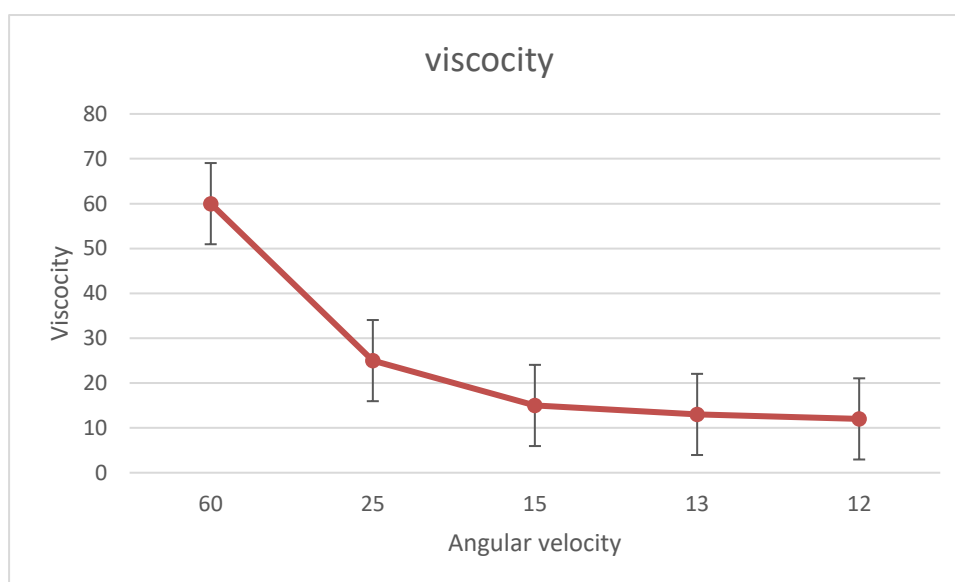


Figure 3: Viscosity graph

Particle size, PDI, entrapment efficiency, zeta potential, and *Carissa Carandas*-LP content

The PDI and average particle size play a crucial role in the stability of the *Carissa Carandas*-LP formulation, and they're also key for enhancing corneal permeability. For the selected batch, the particle size and PDI were found to be 103.17 ± 3.25 nm and 0.877 ± 0.05 , respectively. The PDI reflects the uniformity of the particles. Impressively, the *Carissa Carandas*-LP EE was found to be $96.015 \pm 1.45\%$, indicating an excellent blend of *Carissa Carandas* and lecithin. Typically, a zeta potential value between -30 and +30 mV suggests that the dispersed system is stable. The optimised batch demonstrated solid colloidal stability, boasting a zeta potential of $+22.24 \pm 1.10$ mV. The positive charge of the formulation helped *Carissa Carandas*-LP to interact with the negatively charged sialic acid present in corneal mucus through electrostatic attraction [19]. *Carissa Carandas* content in the lecithin was found to be $96.015 \pm 1.45\%$, showing that there was no significant degradation of *Carissa Carandas* during the formulation process.

TEM

The particle size measured by the particle size analyzer seemed to be larger than what was observed with the TEM. The dynamic light scattering technique looks at the hydrodynamic layers surrounding nanoparticles and their apparent size, which can sometimes result in an overestimation of their diameter. On the other hand, the TEM provided clear images of *Carissa Carandas*-LP, indicating a smooth, well-defined surface and a spherical shape. The particles were consistently sized, appeared black, and were evenly dispersed without any noticeable clumping, as shown in Figure 4.

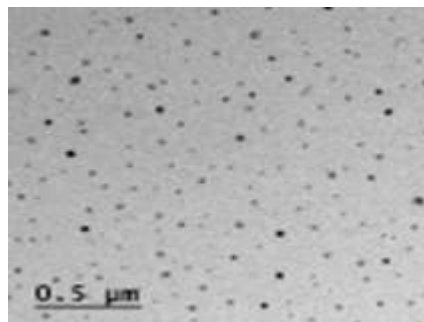


Figure 4: TEM of optimised *Carissa Carandas*-LP formulation

***In vitro* Drug release:**

The optimized *Carissa carandas*-LP exhibited a characteristic biphasic drug-release profile, comprising an initial rapid-release phase followed by a sustained-release phase (**Fig. 5**). Approximately 35.75% of the incorporated drug was released within the first hour, followed by a gradual and controlled release that reached 96.85% at the sixth hour. The initial burst release may be attributed to the rapid diffusion of drug/extract components located near or associated with the surface of the vesicles, whereas the subsequent sustained-release phase may be governed by the slower diffusion of the entrapped components through the phospholipid bilayer and gradual disruption or reorganization of the vesicular structure [34].

The biphasic release behavior may be advantageous for ocular delivery, as the initial release could provide an early availability of the active constituents, while the subsequent sustained-release phase may help maintain drug availability over an extended period and reduce the rapid loss of formulation from the ocular surface [35]. The overall release pattern therefore suggests that incorporation of *Carissa carandas* into the Leciplex system modified its release characteristics compared with a conventional drug/extract solution. The progressive increase in cumulative drug release throughout the study period further demonstrates the ability of the optimized Leciplex system to provide controlled release of the incorporated *Carissa carandas* constituents. Collectively, these findings support the potential of the optimized formulation to improve the duration of ocular drug availability following administration.

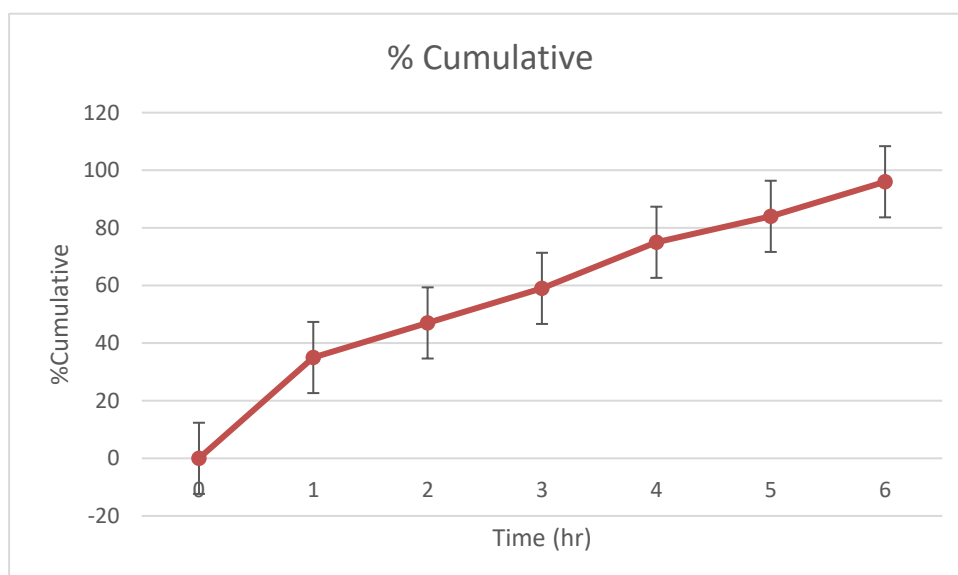


Figure 5: *In vitro* drug release profile

Release kinetics model:

The *in vitro* drug release data from the various formulations were evaluated kinetically using various mathematical models like zero order the highest coefficient ($R^2 = 0.9157$). This indicated that the release from the formulation was not concentration-dependent. After analysis, it became clear that the drug release was controlled by a diffusion mechanism, which aligns well with

the Higuchi model ($R^2 = 0.9963$). So the best-suited model for the drug in vitro kinetic study was the Higuchi model.

Ex-vivo trans corneal permeation study

Trans corneal permeation study showed a progressive increase in drug permeation through the goat cornea over time, indicating an effective permeation behavior of the formulation. A significant release was observed within the first hour (36.33%), possibly due to initial burst release or surface drug diffusion. This was followed by a consistent and sustained drug permeation pattern, reaching over 98% cumulative release by the 6th hour, as shown in Fig.6. The standard deviation values remained low throughout the experiment, suggesting high reproducibility and reliability of the results. The findings suggest that *Carissa Carandas-LP* allows for a longer release of the drug, which could be attributed to the strong mucoadhesive properties of the positively charged formulation. This interaction with the negatively charged mucus layers and the outer surface of the cornea helps more of the medication to seep in and stay in the cornea for an extended time. Additionally, the buildup of *Carissa Carandas* in the cornea was boosted by the electrostatic attraction between the opposing charges.

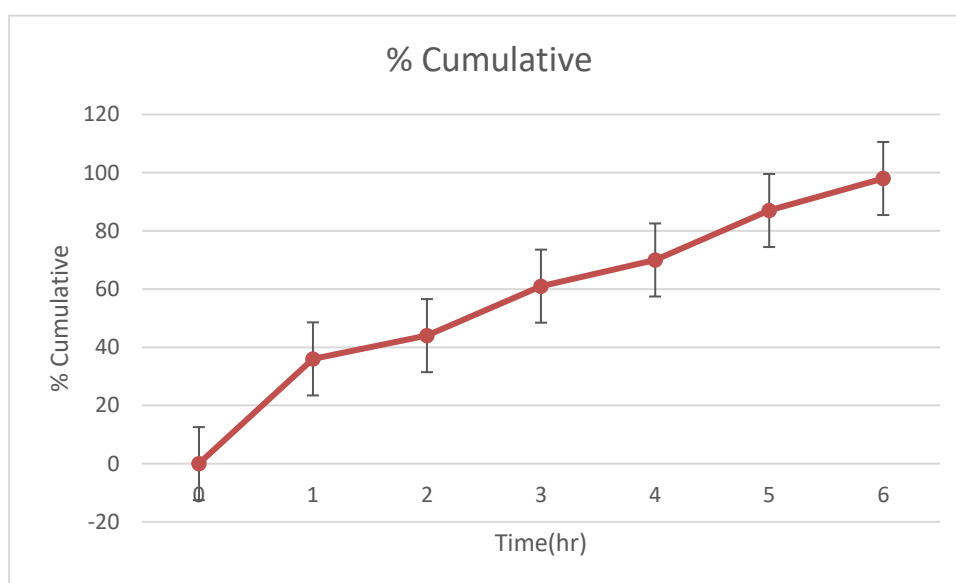


Figure 6: *Ex-vivo* corneal permeation study

Study of ex vivo histopathology:

The *ex vivo* histopathological evaluation of the retina was performed to assess the structural integrity and tissue-level compatibility of the developed formulation. Histological examination of the retinal sections revealed no apparent pathological abnormalities or significant structural alterations following treatment with the formulation. As shown in Fig. 6, the retinal tissue exhibited a well-preserved and organized laminar architecture, with the major retinal layers maintaining their normal arrangement and cellular organization. No evident signs of tissue disruption, cellular degeneration, edema, or abnormal morphological changes were observed in the examined sections.

The preservation of the retinal architecture at both 10× and 40× magnifications (Fig. 7a and 7b) indicates that exposure to the developed formulation did not produce detectable structural damage to the retinal tissue under the experimental conditions. The intact retinal morphology further supports the favorable tissue compatibility and preliminary ocular safety of the developed formulation. Thus, the histopathological findings, together with the absence of observable morphological abnormalities, suggest that the optimized formulation was well tolerated by the retinal tissue during the *ex vivo* evaluation.

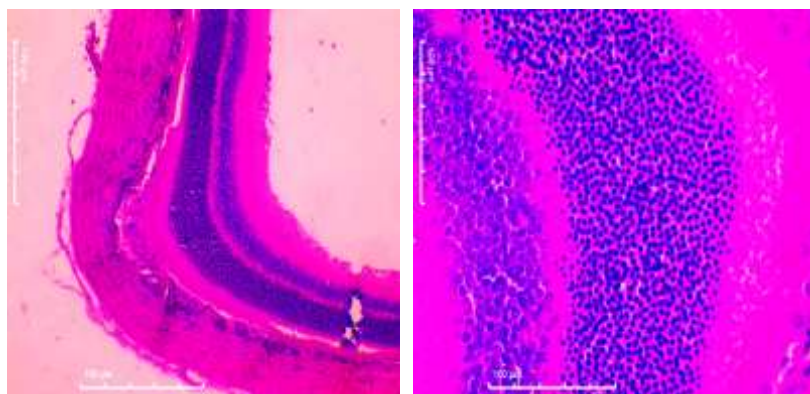


Figure 6: (a) Formulation under 10x (b) Formulation under 40x

***In vitro* mucoadhesion study:**

The Fig. 8 shows how the zeta potential of the formulation changes when it comes into contact with different concentrations of mucin. At the beginning (0% mucin), the formulation had a high positive zeta potential of about +40 mV. As the concentration of mucin increases, the zeta potential gradually decreased. At 0.25% mucin, the value dropped to around +34 mV, and further decreased to about +29 mV at 0.50%. When the mucin concentration was increased to 0.75% and 1.00%, the zeta potential reduced more noticeably to approximately +22 mV and +15 mV, respectively. This steady decrease in ZP suggested that the formulation is interacting with mucin. Since mucin carries a negative charge, it binds to the positively charged surface of the formulation, reducing its overall charge [36]. This indicates good mucoadhesive properties, which can help the formulation stay longer at the site of application and improve drug effectiveness.

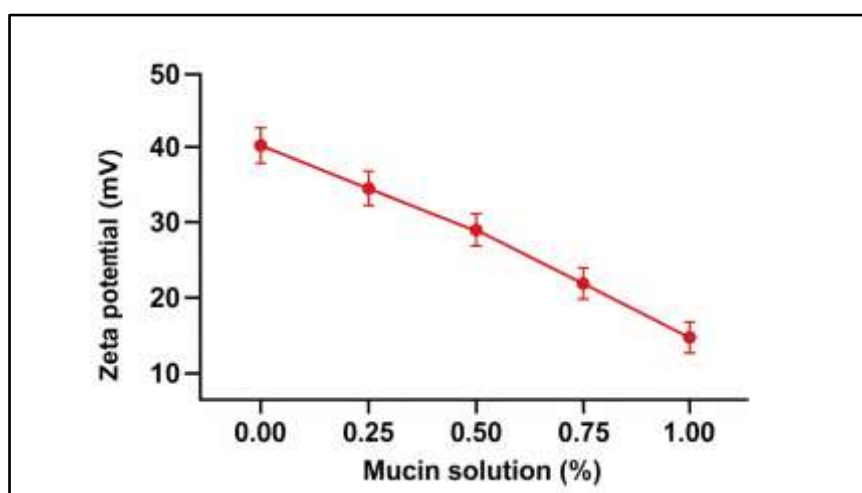


Figure 8: Mucin Mucoadhesion Graph

Acute Eye Irritation Test:

The acute ocular irritation potential of the optimized LP formulation was evaluated in an acute eye irritation test. During the observation period, no visible clinical signs of ocular irritation or toxicity were observed in the treated animals. In particular, no evidence of corneal opacity, iritis or iris inflammation, conjunctival redness, chemosis, or ocular discharge was detected following administration.

The absence of these characteristic signs of ocular irritation indicates that the developed formulation was well tolerated at the administered dose under the experimental conditions. These findings are consistent with the *ex vivo* histopathological observations, which demonstrated preservation of the retinal tissue architecture without apparent morphological abnormalities. Collectively, the results provide preliminary evidence of the favorable ocular tolerability and safety profile of the optimized formulation.

Retinal tissue and staining:

The retinal tissue of the normal control group (**Fig. 8A**) exhibited a well-organized and clearly defined laminar architecture. The retinal layers were distinctly arranged with preserved tissue morphology, indicating normal retinal organization. The disease control group (**Fig. 8B**) showed disruption and loss of the normal stratification of the retinal layers. The retinal architecture appeared irregular, with apparent thinning and degeneration of the outer nuclear layer (ONL), suggesting retinal tissue damage and photoreceptor degeneration. The formulation-treated group (**Fig. 8C**) demonstrated comparatively improved organization of the retinal layers. The major retinal layers, including the retinal pigment epithelium (RPE), outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL), appeared better preserved compared with the disease control group.

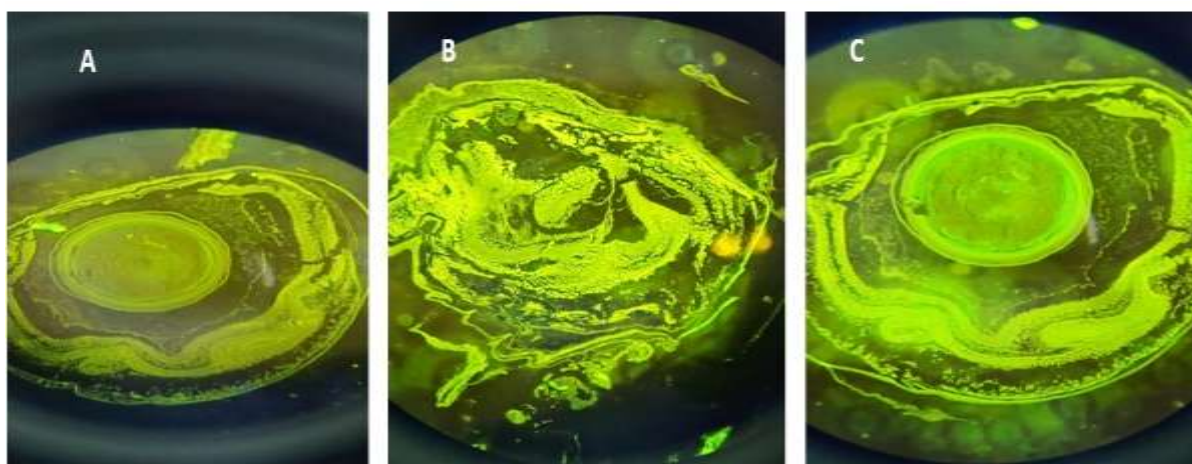


Figure 8: Retinal Tissue and Staining in A: Normal Control; B: Disease control; and C: Formulation

A relative improvement in retinal thickness and tissue organization was also observed, suggesting a protective or restorative effect of the formulation on retinal tissue. The formulation-treated group showed comparatively better preservation of retinal architecture than the disease control group. The retinal layers appeared more organized, with relatively improved tissue thickness and preservation of the major retinal layers. These findings suggest that the formulation may have a protective effect against disease-associated retinal structural alterations. However, confirmation of specific cellular and molecular mechanisms would require further immunohistochemical and molecular investigations.

CONCLUSION

In this study, we successfully synthesized and optimized the *Carissa Carandas-LP* loaded vesicular formulation, known as Leciplex, using a 3^2 Complete Factorial Design. The *Carissa Carandas-LP* exhibited a spherical shape, a uniform size distribution, a high zeta potential, and impressive entrapment efficiency. Furthermore, tests on ex vivo corneal penetration and in vitro drug release revealed a biphasic release pattern, featuring an initial burst in the first hour followed by a sustained release lasting up to 7 hours. We found that the *Carissa Carandas LP* formulation is both non-toxic and non-irritating. The overall safety and corneal tolerance of the formulation were validated through the Draize test. Additionally, the ex vivo corneal drug retention and in vitro mucin mucoadhesive tests suggest that there is an electrostatic interaction between the *Carissa Carandas-LP* and the mucin present on the ocular surface. Retinal tissue also showed the nontoxic nature of the formulation; prepared retinal layers are well organised as compared with the disease control. In this high-glucose model, we observed some significant changes, including morphological lesions and disruptions in tight junction proteins.

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Conflict of interest: No

Author Contribution: Sharvari Chavan: Research Scholar; DR.U. Bagul Sir: Supervisor; Dr. J. Disouza: guide

Ethical statement:

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