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PREPARATION AND CHARACTERIZATION STUDIES OF LEVOFLOXACIN HEMIHYDRATE (LVF) LOADED NLCS FOR OCULAR DELIVERY AND COMPARISON WITH VARIOUS MARKETED FORMULATIONS

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ABSTRACT

In recent studies, there has been an extensive increase in fluoroquinolone-resistant conjunctivitis. The current study's objective was to prepare and optimize Levofloxacin Hemihydrate (LVF)-loaded NLCs for conjunctivitis treatment. It aims to provide sustained release, which can improve corneal permeation and tend to decrease the adverse side effects as observed in other fluoroquinolones. Levofloxacin Hemihydrate (LVF)-loaded NLCs with particle size **239.7nm,** polydispersity index **0.182** and entrapment efficiency % (EE) **86.21%** were achieved. The Franz diffusion cells were used for the *ex-vivo* tests of the NLCs formulation. The NLCs formulation provided nearly **65.77%** *in-vitro* release and **55.23%** *ex-vivo* release in 8 hours of time span. The *in-vitro* corneal permeation was discovered to be comparable with the novel formulation, demonstrating the practical use of the optimised NLCs. The antimicrobial studies of the NLCs formulation were performed against *E. coli* and *S. aureus* strains. Against *E. coli* and *S. aureus*, the improved NLCs formulation was found to be more effective compared to the marketed eye drops of other fluoroquinolones. This effectiveness was confirmed using antimicrobial tests mainly consisting of zones of inhibition testing and minimum inhibitory concentration testing. The results obtained from the above studies concluded that NLCs are an efficient form of ocular drug delivery system. Levofloxacin Hemihydrate (LVF) is compatible with NLCs as a drug delivery system and is efficiently and sustainably delivered at the ocular target sites. Ocular drug permeability is observed in Levofloxacin Hemihydrate (LVF)-loaded NLCs used in the form of eye drops.

KEYWORDS: Levofloxacin Hemihydrate (LVF), Fluoroquinolones, NLCs, Nanoformulation, Ocular Drug Delivery.

1. INTRODUCTION

Keratitis and conjunctivitis are ocular diseases that can be effectively treated with antibiotic eye drops. Eye drops rapidly deliver a large amount of antibiotics at the site of infection, in contrast to systemic antibiotics.[1] Fluoroquinolones, aminoglycosides, cephalosporins, and oxytetracycline are some of the antibiotics that are commonly found in eye drop compositions. Fluoroquinolone eye drops are now commonly used by general practitioners in various countries to treat bacterial keratitis and conjunctivitis because of their high stability, broad spectrum, and efficacy.[2] To extend the residence time and increase bioavailability of fluoroquinolone eye drops, a variety of novel formulations have been developed for ocular applications, including ocular inserts, collagen shields, or particulate systems such as SLN, NLCs, niosomes, and liposomes.[3]

Due to their advantages over alternative drug delivery methods, nanoparticle formulations have attracted significant interest during the past two decades. Benefits of these systems include targeted drug delivery to a particular location in order to reduce toxicity, enhanced bioavailability by lowering drug level fluctuations, improved drug stability towards degradation by enzymes, and a sustained gradual release effect that decreases dosing frequency with increased patient compliance.[4]

Third-generation Levofloxacin Hemihydrate (LVF) works similarly to various fluoroquinolones by suppressing the activity of two bacterial enzymes that regulate the topological state of DNA. For the growth of bacteria, both type II DNA topoisomerase enzymes are necessary.[5] In gram-negative bacteria like *E. coli* and *N. gonorrhoeae*, the enzyme DNA gyrase is the main target of Levofloxacin Hemihydrate (LVF), whereas in gram-positive bacteria like *S. aureus* and *S. pneumoniae*, topoisomerase IV is the main target. Levofloxacin

Hemihydrate (LVF), like other fluoroquinolones, has the ability to cause a post-antibiotic effect against a variety of microorganisms and has concentration-related antibacterial properties. In comparison to other fluoroquinolones, including fourth-generation fluoroquinolones such as moxifloxacin, gatifloxacin, and ofloxacin, Levofloxacin Hemihydrate (LVF) was found to have less toxic effects on human corneal keratocytes and epithelial cells.[6]

In order to provide a sustained ocular system for drug delivery and decrease the frequency of dosage, Levofloxacin Hemihydrate (LVF), a hydrophobic medication, is an ideal option for the formulation of NLCs.[7]

2. MATERIALS AND METHODS

2.1 Materials

The sample gift of Levofloxacin Hemihydrate (LVF) Hemihydrate was provided by Aarti Drugs Ltd., Mumbai, India. The marketed formulation of moxifloxacin was purchased from Cipla Ltd., gatifloxacin from Sun Pharmaceutical Industries Ltd., and Levofloxacin Hemihydrate (LVF) from Entod Pharmaceuticals Ltd. Tween 80, compritol, oleic acid, and poloxamer 188 were procured from SDFCL (SD Fine Chem Ltd., Mumbai, India). All the extra chemicals were of the analytical class.[8]

2.2 Methods

2.2.1 Screening of excipients

Nanostructured lipid carriers (NLCs) are made up of biodegradable lipids such as solid and liquid lipids which are blended to form a structurally imperfect matrix. This kind of imperfect structure leads to high drug loading and sustained drug delivery. To obtain most of the compatible excipients for formulation, various excipients were screened in the following pre-formulation study.[9,10]

2.2.2 A Screening of solid lipid

The solubility of Levofloxacin Hemihydrate (LVF) was determined in solid lipid by the test tube method. Levofloxacin Hemihydrate (LVF) 10 mg was transferred to a test tube maintained at a water bath. The solid lipids such as stearic acid, glyceryl monostearate, Compritol ATO 888, Precirol ATO 5 and Apifil were added in increments until Levofloxacin Hemihydrate (LVF) was completely dissolved.[11]

2.2.3 B Screening of liquid lipid

The saturation solubility of Levofloxacin Hemihydrate (LVF) was determined in liquid lipids by the test tube method. Liquid lipids such as oleic acid, castor oil, capryol 90, miglyol 812, olive oil (1 mL) were transferred to a test tube maintained at vortex mixture. The drug was added in increments until the mixture was completely hazy. The amount of liquid lipid required to solubilize Levofloxacin Hemihydrate (LVF) was determined.[12]

2.2.4 C Screening of surfactants:

1% surfactant solution in distilled water was prepared for the screening of surfactant. For surfactant screening excess of drug was added individually to known amounts of surfactants such as Tween 80, Tween 20, Cremophore EL, Kolliphore RH 40, Lutrol F68. Mixtures were shaken for 72 hrs in a water bath maintained at 37±2°C. After completion of 72 hours cycle, mixture of drug and excipient was centrifuged at 18000 rpm for 20 mins to separate the undissolved drug from the excipient. Then, from supernatant 0.5 mL of liquid withdrawn and diluted with Methanol (AR) suitably and analysed for the amount of drug dissolved by UV spectrophotometry at 298 nm wavelength against Methanol (AR) as blank.

2.3 Formulation of Levofloxacin Hemihydrate (LVF)-loaded NLCs

Levofloxacin Hemihydrate (LVF)-NLCs were developed by Solvent Injection-Evaporation method. According to this method, lipids and drugs are dissolved in a water-miscible solvent (e.g., Methanol (AR), Ethanol, Isopropanol, or Acetone) or a water-miscible solvent mixture, which constitutes the organic phase. The aqueous phase is usually prepared by adding surfactant to water. The organic phase is then quickly injected into the aqueous phase under continuous mechanical stirring using a needle. The organic solvent is then evaporated either in a rotary evaporator or by mechanical stirring. After the solvent has evaporated, lipid precipitation causes NLCs to develop.[13] The various batches were prepared as per the given composition in Table 4.1. Levofloxacin Hemihydrate (LVF) was briefly dissolved in a sufficient amount of Oleic Acid, Compritol 888 ATO and Isopropyl Alcohol in order to produce the organic phase of the formulation. The solution was then heated for a sufficient amount of time until it became transparent. Additionally, Poloxamer 188 and Tween 80 (surfactants) were dissolved in distilled water in separate beaker to create an aqueous phase.[14]

The mixture was then kept on a magnetic stirrer for 45 minutes at a speed of 120 rpm at 37°C to ensure proper homogenization. With the help of a syringe, the organic phase was gradually added in a dropwise manner.[15] The formulation was then subjected to a probe sonicator for 5 minutes to reduce the particle size and ensure a uniform distribution of nanoparticles.[16]

2.4 Particle size and Polydispersity index

The particle size measurement of the Levofloxacin Hemihydrate (LVF) loaded NLCs batches were determined by laser light scattering on a Beckman Coulter Particle Size Analyzer.[17] Photon Correlation Spectroscopy (PCS) is used by the N5 Submicron Particle Size Analyzer to determine particle size by monitoring the rate of fluctuations in laser light intensity scattered by particles as they diffuse through a fluid. PCS is a non-destructive technique for evaluating particle size to a few nanometers.[18] To determine particle size, a disposable sizing cuvette constructed of polystyrene and

particularly built for the device was rinsed with MilliQ water. Sample intensity is adjusted between 5e+4 and 1e+6 by dilution with MilliQ water passing through a 0.44 µm filter membrane. At 25 °C, the mean particle size was estimated at a 90° angle using a light source of 25 mW helium-neon laser (632.8 nm). Following dilution, the sample cuvette was placed in the instrument for analysis.

2.5 Entrapment Efficiency (%EE)

The entrapment efficiency of Levofloxacin Hemihydrate-loaded NLCs was determined indirectly. The formulated Levofloxacin Hemihydrate-loaded NLCs were separated from non-entrapped LVF by centrifugation at 18,000 rpm for 10 minutes at 25°C.[19]

To determine the amount of drug present, the supernatant was collected and diluted (10-fold) with Methanol (AR), and then subjected to an analysis using a UV spectrophotometer at 298 nm.[20] The following formula was used to calculate the % entrapment efficiency:

$$Entrapment\ efficiency\ \% = \frac{Initial\ Drug - Free\ Drug}{Initial\ Drug} \times 100$$ [21]

2.6 *In-vitro* drug release of formulation

The comparative release study of Levofloxacin Hemihydrate-loaded NLCs and three marketed formulations, such as Gatiflox HS 0.5%, Moxicip 0.5% and LV Flox 0.5% was performed using a dialysis bag method. Simulated Tear Fluid (STF) hydrated the dialysis membrane. Using release media as the Simulated Tear Fluid (STF), the release study was conducted in a 100 mL beaker. The dialysis membrane was filled with 1 mL of the formulation, and the threads were used to secure the membrane's ends. It was then added to a beaker with Simulated Tear Fluid (STF) and stirred continuously for 100 rpm.[21] At a particular time point, the aliquots were removed. When the aliquot was removed, the release medium also received the same volume of freshly prepared Simulated Tear Fluid (STF). Following that, the sample was examined using Simulated Tear Fluid (STF) as a blank and UV spectroscopy at 288 nm.[22]

Table no. 1: Protocol for *In-vitro* release study of Levofloxacin hemihydrate NLCs.

Apparatus	100ml Beaker
Release medium	Simulated Tear Fluid (pH 7.4)
Membrane	Dialysis membrane (cut-off molecular weight 12,000-14,000 Daltons)
Temperature	36-38 °C
Stirring Speed	100 rpm
Quantity of formulation	1ml
Duration of Study	8 hours
Volume of aliquot	1ml
Time interval of aliquot withdrawn	0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 Hr

2.7 *Ex vivo* corneal permeation study

To evaluate the drug's permeability into ocular tissue, *ex-vivo* studies are important. The cornea of a goat was used in the study. Goat corneas are identical to human corneas, they are frequently used to investigate ocular permeability. From a nearby butcher shop, fresh goat eyeballs were bought. The eyeballs were kept in a sterile saline solution (0.9% NaCl solution) and kept at 4°C. Using forceps and scissors, the cornea was separated. With 5 to 6 mm of surrounding sclera removed, the cornea was gently removed. The Franz diffusion apparatus was used in the study to examine permeability. The Franz diffusion cell includes an acceptor compartment with releasing medium and a donor compartment with formulation.[23]

A water jacket surrounds the donor chamber to keep the release medium's temperature at the right condition. The acceptor compartment's 7.0 ml volume is appropriate for studies of ocular permeability. 7.0 ml of simulated tear fluid are placed in the acceptor compartment as a release medium. There is a side expansion on the acceptor compartment that can hold aliquots. Using clamps, the 1.84 cm^2 goat cornea was fixed in the space between the donor and acceptor compartments. The donor compartment was then filled with 0.5 mL of Levofloxacin Hemihydrate (LVF) loaded NLCs. A water jacket was used to keep the release medium's temperature at 34 °C while stirring it continuously at 25 rpm. A fixed 1 mL aliquot was taken out of the system every 8 hours, and an equal amount of fresh Simulated tear fluid was put in to maintain the sink condition. Following that, the aliquots were examined using Simulated Tear Fluid (STF) as a blank in UV spectroscopy at 288 nm wavelength.[24,25]

$$Permeation\ \% = \frac{Amount\ of\ drug\ permeated\ in\ receptor}{Initial\ amount\ of\ drug\ in\ donor} \times 100$$

Table no. 2: Protocol for *Ex-vivo* release study of Levofloxacin Hemihydrate (LVF)-loaded NLCs

Apparatus	Franz Diffusion Cell
Release medium	Simulated Tear Fluid (pH 7.4)
Membrane	Goat Cornea
Temperature	36-38 °C
Stirring Speed	25 rpm
Quantity of formulation	0.5 ml
Duration of Study	8 hours
Volume of aliquot	1ml
Time interval of aliquot withdrawn	0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 Hr

2.8 Antimicrobial assay

2.8.1 Zone of Inhibition (ZOI)

The ability of formulation to inhibit the growth and colonisation of bacteria is assessed by antimicrobial testing. The agar-cup plate method (IP 2018) was used to evaluate the antimicrobial activity of the formulation against *S. aureus* and *E. coli* in comparison with marketed formulations. To compare the results, marketed levofloxacin hemihydrate eye drops were used along with test formulation.[27]

The zone of inhibition has been found to be directly related to drug concentration. Cultures of microorganisms were transferred to solidified agar after a layer of Mueller–Hinton agar has solidifies in a Petri plate and the culture was evenly dispersed. Levofloxacin Hemihydrate (LVF) loaded NLCs and marketed formulations were poured into separate cups. All of these steps were carried out aseptically, and all of the Petri plates were incubated for 24 hours at 37°C after being kept at room temperature for 2 hours in order to allow the drug to diffuse into the medium. The petri plates were examined, and the zone of inhibition was calculated using a zone reader.[28]

2.8.2 Minimum Inhibitory Concentration (MIC)

The MIC of Levofloxacin Hemihydrate (LVF) was determined in two different strains, i.e *S.aureus* (gram-positive bacteria) and *E.coli* (gram-negative bacteria), with the help of nutrient broth. The nutrient medium was made by adding 3.25 g of powdered nutrient broth to 250 ml of a conical flask. All the instruments, along with the nutrient medium, were autoclave to maintain sterility.[29]

The volume was made up with the help of Milli Q water, up to 250 ml. Different drug concentrations were taken in a volume of 1 ml in a sterilised test tube. Then 1 ml of nutrient broth was added, followed by the subsequent addition of cultures of *S. aureus* and *E. coli* in different test tubes. All the test tubes were covered with sterilised cotton to avoid the incorporation of external microorganisms. All of these steps were carried out aseptically, and all of the Petri plates were incubated for 24 hours at 37°C.[30]

2.9 Stability study

The Stability of Levofloxacin Hemihydrate (LVF) loaded NLCs were checked to assess the long-term usability of formulation with the help of ICH Q1A(R2) guideline. The stability study of formulation gives us ideas about potential excipient reactions, long term drug stability, possible drug expulsion from formulation. Stability study also assesses any possibility of breaking during storage of formulation. It also assesses the stability of formulation at different environmental and storage conditions. Briefly, 5 mL of Levofloxacin Hemihydrate (LVF) loaded NLCs was poured in a transparent glass vial and sealed properly. Then these vials were stored at room temperature (25±2°C/60%±5% RH) and in the refrigerator (5±3°C) for the duration of 1 month. The formulation was investigated every month for change in appearance, PDI, particle size and entrapment efficiency.[31]

3. RESULTS

3.1 Screening of components

Levofloxacin Hemihydrate (LVF) showed maximum solubility in isopropyl alcohol. Screening of lipids was performed to find out the lipid which could give maximum drug loading. Compritol 888 ATO and oleic acid were the only lipids in which the drug was found to be highly soluble; hence compritol 888 ATO and oleic acid were selected for preparation of NLCs. Also solubility of compritol and oleic acid with isopropyl alcohol was determined; compritol and oleic acid found to be completely soluble in 1 ml of isopropyl alcohol at room temperature. In addition, Levofloxacin Hemihydrate (LVF) also presented maximum solubility in isopropyl alcohol hence stearic acid as lipid and isopropyl alcohol as solvent were selected for the preparation of Levofloxacin Hemihydrate (LVF) loaded NLCs formulations.

3.2 Entrapment efficiency, Particle size and pDI

All of the formulations' entrapment efficiencies were shown to be more than 83% (Table 3). We can clearly state that the ratio of solid to liquid lipids had an impact on the percentage entrapment efficiency. Except for F6, which may be related to agitation speed, the results

showed that the entrapment effectiveness of all formulations was boosted by reducing concentration of the total lipid content in the formulations.

Table 3: Physical characterization of formulated Levofloxacin Hemihydrate (LVF) nanoformulation batches.

Formulation code	Total Lipid Content	EE (%)	PS (nm)	PDI
F1	2%	89.38±0.52	552.2±20.1	0.986±0.17
F2	1.5%	92.97±0.51	349.1±10.2	0.746±0.14
F3	1.5%	83.01±0.57	266.7±19.7	0.388±0.07
F4	1%	86.25±0.51	239.7±12.9	0.182±0.03
F5	1.5%	88.18±0.53	224.2±10.8	1.014±0.41
F6	1%	86.60±0.52	309.2±12.7	0.201±0.02

3.3 *In-vitro* drug release of formulation

In-vitro release study is the measure of release of active pharmaceutical reagent from the formulation. The release profile of Levofloxacin Hemihydrate (LVF) loaded NLCs was obtained by plotting % cumulative release versus time.

In comparison to other commercially available formulations, formulation F4 had the highest cumulative release (%), or 65.77% ± 0.22 at the completion of 8 hours. The regression coefficient (R^2) of various kinetic models was obtained by plotting the data in graphs. The goodness of fit of any model is decided by how close the value of regression coefficient is to the 1. The R^2 values of all models are tabulated in table no. 4. The R^2 value of the Korsmeyer peppas model for Levofloxacin Hemihydrate (LVF) loaded NLCs is 0.9956 and it is closest to 1 compared to the other three models. This indicates that the Korsmeyer peppas model graph has better linearity than other models. According to the *in-vitro* release studies, the F4 formulation of NLCs shows a greater drug release profile than the other formulation. This might be because the particle size is smaller and the polydispersity index is lower, which could improve the dissolving and penetration profile.

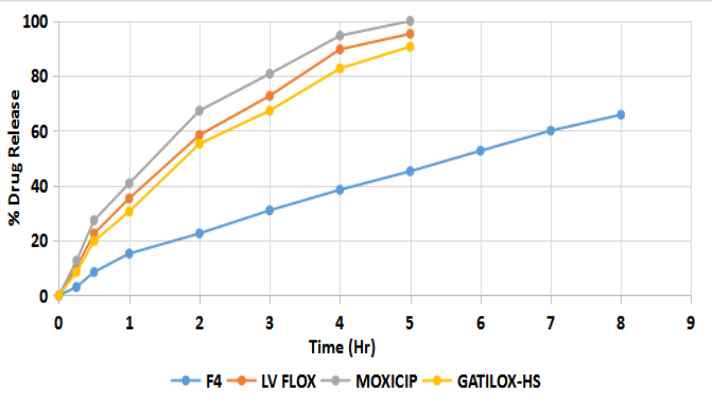


Figure 1: Comparative *in vitro* release profile of Novel and Marketed formulation.

Table no. 4: Data for various release kinetics of Levofloxacin Hemihydrate (LVF) loaded NLCs.

Kinetic Model	Zero Order	First Order	Higuchi Model	Korsmeyer Peppas Model
Regression coefficient (R^2)	0.9884	0.7144	0.9748	0.9956
Model followed	Korsmeyer Peppas Model			

3.4 *Ex vivo* transcorneal permeation study

Drug permeability across ocular barriers varies depending on a number of variables, including the chemical composition of the drug, its size and structure, the lipid/water partition coefficient, and others. The primary barrier is made up of lipidic epithelium. *ex-vivo* permeation indicated that medication release is slower than that observed in *in-vitro* release studies. Using Franz diffusion cell equipment, *ex-vivo* permeation studies were conducted. The *ex-vivo* release investigation demonstrates that the Levofloxacin Hemihydrate (LVF) loaded NLCs released 55.23% of the drug in 8 hours. The main problem in drug permeation in *ex-vivo* study is the epithelial barrier. The structure of epithelium of ocular tissue contains lipid bilayer.The lipids present in NLCs provide better permeation of drug through the epithelium of ocular membrane. The smaller size of NLCs enhances the drug release and permeation through the membrane.

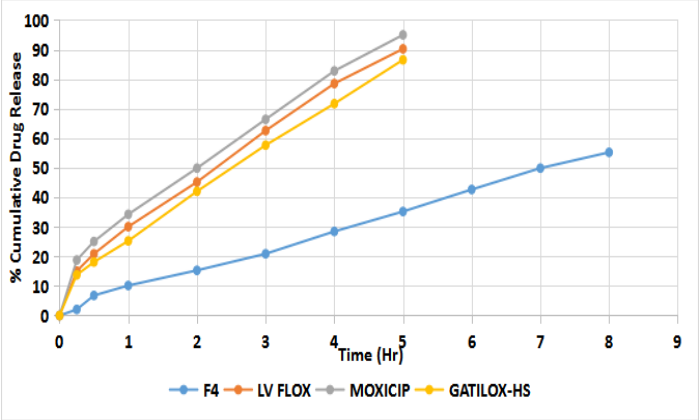


Figure 2: Comparative *ex vivo* release profile of Novel and Marketed formulations.

3.5 Antimicrobial assay

3.5.1 Zone of Inhibition (ZOI)

Zone of Inhibition was carried in a cup plate method. The activity was assessed by measuring the zone of inhibition. The activity of Levofloxacin Hemihydrate (LVF) loaded NLCs was compared with the marketed formulations. It shows that Levofloxacin Hemihydrate (LVF) loaded NLCs have antimicrobial activity against *S.aureus* and *E.coli.* The zone of inhibition was measured by the zone reader. From Fig 3 and 4, it is observed that Levofloxacin Hemihydrate (LVF) loaded NLCs have a better zone of inhibition than the marketed eye drops. Levofloxacin Hemihydrate (LVF) loaded NLCs and marketed eye drops have statistical differences in antimicrobial activity. This concludes that the developed formulation has equivalent activity as other marketed formulations.

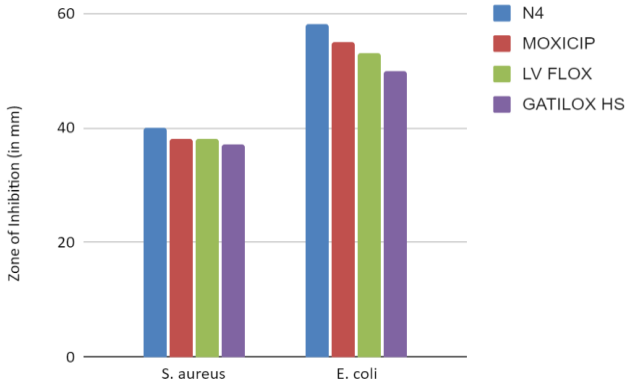
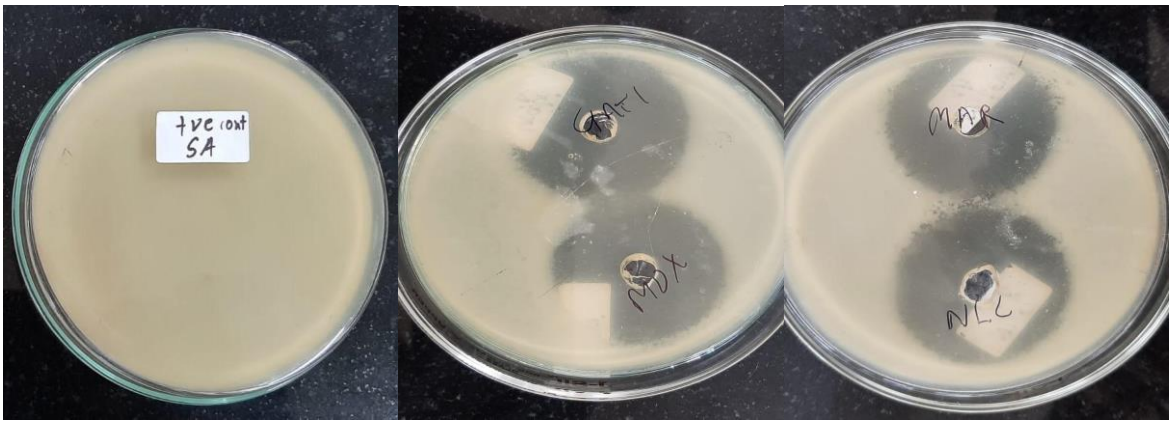
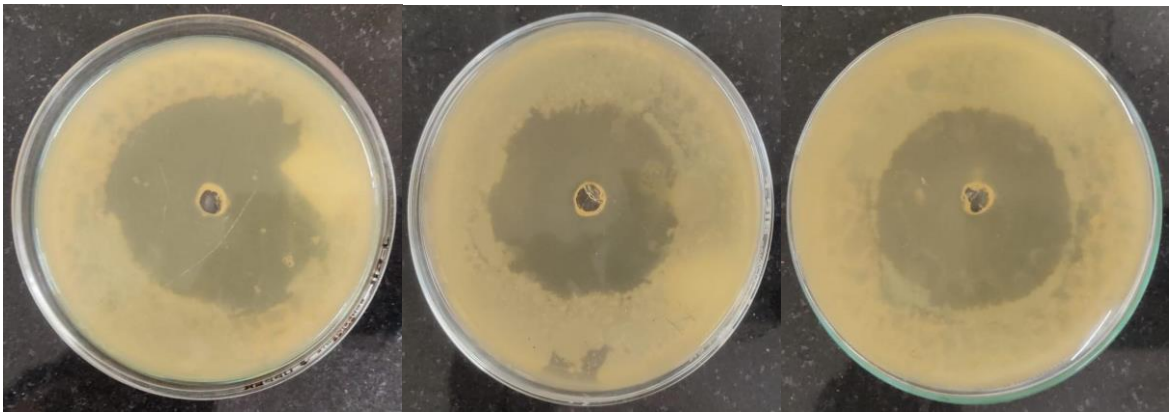


Figure 3: Diameter of zones of inhibition for marketed eye drops and the optimized nanoformulation (F4) using *S. aureus* and *E. coli.*



a) Positive control **b) Gatilox HS (*S. aureus)*** **c) LV Flox and F4 (*S. aureus)***

d) Positive control **e) Negative control (*E. coli*)** **f) F4 (*E. coli*)**



g) Moxicip (*E. coli*) **h) LV Flox (*E. coli*)** **i) Gatilox HS (*E. coli*)**

Figure 4: Antimicrobial Study of Levofloxacin Hemihydrate (LVF) loaded NLCs and Marketed eye drops in *S. aureus* and *E. coli*

3.5.2 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the nanoformulation was tested in *S. aureus* and *E. coli*. It was found to have MIC of 2 ppm and 1 ppm in *E. coli* and *S. aureus* as mentioned in Fig 5. The results obtained were superior when compared with the all marketed drops, showing the efficiency of the formulations.

MIC (ppm)	MIC (ppm)						
	0.1	0.2	0.5	1	2	3	4
E. coli	+	+	+	+	-	-	-
S. aureus	+	+	+	-	-	-	-

+: Growth, -: No Growth

Figure 5: Minimum Inhibitory Concentration (MIC) of nanoformulation in *S. aureus* and *E. coli.*

3.6 Stability study

The stability of formulation was checked for one month at room temperature (25±2°C/ 60±5% RH), accelerated condition (40±2°C/ 75±5% RH) and at refrigerator temperature (5 ± 3°C) as mentioned in ICH Q1A(R2). The results show an increase in particle size at 5 ± 3°C from 239.7 nm to 247.6 nm over the period of 3 months. The entrapment efficiency decreases from 86.25% to 80.54%. The change in particle size at room temperature is more than that of refrigerator temperature. The particle size increases from 239.7 nm to 258.0 nm over the period of 3 months at room temperature. The decrease in entrapment efficiency at room temperature from 86.25% to 78.24% over the period of 3 months. The particle size increase at accelerated condition was from 239.7 nm to 264.3nm over the period of 3 months. The entrapment efficiency decreases from 86.25% to 76.73% over the period of 3 months. The increase in particle size during storage conditions is due to the formation of more stable NLCs in formulation. The decrease in entrapment efficiency of formulation is due to the leakage of drug from lipid core to the medium. Leakage of drug from formulation is slower when the formulation is stored in the refrigerator.

4. CONCLUSION

From the experiments done so far, the outcomes obtained were as follows:-

- Successful optimization of Levofloxacin Hemihydrate (LVF) loaded NLCs for ocular drug delivery.
- Characterization of Levofloxacin Hemihydrate (LVF) loaded NLCs concluded desired formulation attributes.
- *In-vitro* and *ex-vivo* studies revealed that the proposed formulation had good efficacy, sustained release, and greater penetration through the ocular membrane.
- The newly developed formulation demonstrated improved antibacterial activity
- Levofloxacin Hemihydrate (LVF) loaded NLCs can be considered as an effective alternative to the conjunctivitis treatments currently on the market.
- Additional studies, such as those on long-term stability and comprehensive, in-depth animal studies, must be carried out to realize the clinical potential of the formulation.

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