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Design and Evaluation of Natamycin Nanocrystals Loaded *In Situ* Gel for Ophthalmic Administration

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Natamycin belongs to a large group of naturally occurring polyene antifungal antibiotics derived from *Streptomyces natalensis.* Natamycin has a restrictive pharmaceutical role because of its extremely low aqueous solubility, which severely reduces the bioavailability of the drug. To improve the absorption of the drug, nanocrystals of natamycin were prepared and incorporated into *in situ* gel.
Aim: To improve the solubility and absorption of natamycin nanocrystals by preparing nanocrystal in situ gel of natamycin for ophthalmic delivery
Methodology: Natamycin nanocrystal was prepared using Sono-Precipitation method. Box-Behnken approach was employed to assess the influence of independent variables, namely concentration of stabilizer, sonication time and amplitude on particle size and zeta potential of the prepared nanocrystal.
Optimized natamycin nanocrystal *in situ* gel formulations was characterized for various parameters like pH, viscosity, drug content, in vitro drug release and ex vivo permeation studies.
Results: The optimized formulation of natamycin nanocrystal with a particle size of 293.9nm and

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zeta potential -14.6mV was incorporated into *in situ* gels. The pH triggered *in situ* gel was prepared using Carbopol and Hydroxypropyl methylcellulose (HPMC)., which showed clear preparation, pH of the formulation was closed to the pH of tear fluid, i.e., 7.4, viscosity showed pseudoplastic behaviour with immediate gelation remained for an extended period, and the drug content was around 99.70%. From the characterizations given above, PF-4 was optimized and evaluated for *In vitro* drug release showing slow and sustained release when compared to the marketed formulation and followed first-order kinetics with the diffusion-controlled mechanism. *Ex vivo* permeation through goat's cornea of PF-4 showed better permeation than marketed formulation. The stability studies of PF-4 showed that formulation was stable at the appropriate condition.

Conclusion: Nanocrystals formulations of natamycin was successfully formulated and incorporated into *in situ* gels. Further in vivo studies need to be carried out for confirmation of pharmacological activity

Keywords: Natamycin; nanocrystal; box-behnken design; In situ gel; pH triggered.

1. INTRODUCTION

Nanocrystals are carrier-free colloidal particles having a stable solid crystalline drug core and an outer layer of stabilizer. They are pure drug crystals with mean particle, typically between 10–800 nm. A minimum of surface active agents are required for their stabilization. When prepared in aqueous or non-aqueous medium, they form nanosuspensions, which are colloidal dispersion of drugs stabilized by polymer or surfactants delivery systems [1]. Due to the advantages of high drug loading, platform stability, and ease of scaling-up, nanocrystals have been widely used to deliver poorly water-soluble drugs [2]. As the particle size of crystals decreases to around 100 nm, the properties of the material, such as solubility are drastically changed [3].

One of the major advantages of drug nanocrystals is the possibility of improving the bioavailabilty of poorly soluble drugs by increasing their dissolution. Enhanced surface area of drug nanocrystals brought about by size reduction or nanonization helps in improving the saturation solubility and dissolution rate of the drug. For this reason, the most important application of these nanoparticles is in the oral and parenteral administration of poorly soluble drugs. However other routes of administration such as the nasal and ocular routes have also been explored [2].

The poor ocular bioavailability of slowly soluble drugs is further compounded by the availability of a very small volume of tear fluid in the *cul de sac* for dissolution. This problem could be overcome by using nanocrystal form of the drug to increase its saturatation solubility. Nanocrystal-based formulations explored for ocular drug delivery have been found successful in achieving increase in retention time, bioavailability, and permeability of drugs across the corneal and conjunctival epithelium. They show increased adhesiveness to the cornea which can increase the residence time of formulation in ocular sac [1]. The increased ocular retention can significantly increase the corneal permeation of the drug due to increased local concentrations.The retention of the particles in the conjunctival sac will also serve as a drug depot for continuous drug release leading to sustained drug levels and prolonged drug effects. Other advantages of nanocrystals include ease of large scale production and scalability due to fewer processing steps compared to polymeric nanoparticles. It involves less physical and chemical stability concerns as compared to other nanocarriers like liposomes, niosomes or solid lipid nanoparticles (SLN) [4].

A few investigations have been reported in the area of nanocrystals for ophthalmic delivery. Romero G *et al.,* prepared a cationic nanocrystal formulation containing dexamethasone acetate nanocrystals (0.05%) and polymyxin B (0.10%) for ophthalmic application. The formulation developed offered the advantage of increased saturation solubility of the drug (due to the nano-size of the crystals) and increased residence time in the eye [5]. Orasugh *et al*., reported the use of cellulose nanocrystals (CNC) biopolymern in ophthalmic delivery for sustained release [6].

Natamycin also known as pimaricin, belongs to a large group of naturally occurring polyene antifungal antibiotics derived from *Streptomyces natalensis* [7]. They treat various fungal infections around the eye, including infections around the eyelids, cornea and conjunctiva. Fungal eye infections are sporadic but can be very serious, even leading to vision loss [8].

Natamycin has been regarded as the most important agent in managing fungal keratitis (FK) and is safe and effective at low concentrations. This antibiotic is the first-line treatment for FK and acts by binding with ergosterol, which is an essential component in the fungal cell wall and blocks fungal growth [9]. This drug is currently available as a 5% w/v ophthalmic suspension meant to be instilled as drops. However the poor solubility of this drug hinders its penetration into the anterior chamber of the eye through the cornea and could reduce its effectiveness in controlling FK. Nanocrystals of Natamycin could improve its aqueous solubility and dissolution, consequently increasing its ocular bioavailability.

The use of a vehicle with *in situ* gelling effects can be utilized to further sustain the release of the drug for ocular delivery. *In situ* gelling systems are polymeric formulations in sol forms before entering the body, but they change into gel forms under physiological conditions. The transition from sol to gel depends on one or a combination of different stimuli, such as pH change, temperature modulation, exchange of solvents, ultraviolet irradiation and the presence of specific ions or molecules. In ophthalmic delivery, when these preparations are instilled into the eye as drops, they undergo a sol-gel transition in the *cul-de-sac*, thus providing a drug depot for continuous release[10]. Thus, they combine the advantages of ease of administration and prolonged drug release obviating the need for frequent administration. These systems make use of polymers such as pectin, xyloglucan, gellanic gum, chitosan and alginic acid. Several investigations have been carried out in the area of *in situ* gelling systems for ophthalmic application, for therapeutic as well as diagnostic applications [11,12,13].

In this study we have developed and investigated natamycin nanocrystals for the purpose of increasing its ocular bioavailability by virtue of enhancing its dissolution. These nanoparticles were incorporated in an in situ gelling base for providing sustained release of the drug on the corneal surface and thereby providing the necessary antibiotic levels in the precorneal film for better control of FK.

2. MATERIALS AND METHODS

2.1 Materials

Natamycin was received as a gift sample from Shandong Freda Biotechnology co., Cabopol 940P and HPMC EL 50V were obtained from Lobachemie, Pvt. Ltd, Mumbai and all other chemical were procured from local sources and were of analytical grades.

2.2 Compatibility Studies Using Fourier Transform Infrared Spectroscopy (FTIR)

FTIR can be used to find out the physicochemical interaction between the components in the formulation and therefore used to select suitable chemicals and excipients. The IR spectra of Natamycin and polyvinyl pyrrolidone were obtained individually and of the final formulation using Alpha Bruker IR spectrometer [14].

2.3 Preparation of Natamycin Nanocrystals by Sono-Precipitation Method

Nanocrystals were prepared by the Sono-Precipitation method using the ingredients shown in Table 2. Initially, Natamycin was dissolved in 2 ml of Dimethylsulfoxide (DMSO) at ambient temperature. Then it was poured into 20 ml of distilled water containing different concentrations of polyvinyl pyrrolidone (PVP K30) and stirred on a magnetic stirrer to allow for precipitation of the drug. For reducing the size of the particle, it was subjected to sonication to obtain nanocrystals. Natamycin nanocrystals were obtained by centrifugation of dispersion at 10,000 rpm for 60 mins. Crystals were separated, washed with ethanol to remove DMSO and dried [15].

2.4 Experimental Design

In the present work, Box–Behnken design and response surface curve were chosen to optimise natamycin nanocrystals using DesignExpert® software (Version 11.0.3.0 64-bit, Stat-Ease, Inc. Minneapolis, MN, and USA). A Box-Behnken design was used in the optimization operated at 3 levels and 3 factors, generating 17 runs with 5 centre points.

As shown in Table 1, the independent variables are concentration of stabilizer (mg), time of sonication (mins), and amplitude taken as major factors. The dependent variables are particle size (nm) and zeta potential (mV). The concentration of stabilizer, Time of sonication and Amplitude of sonication were within the range of 50-70mg, 15-30 mins and 50-80%, respectively, based on the preliminary screening [16].

Table 1. List of variables in Box-Beheken design

Variable	Name	Units	Levels		
Independent variables			**Low**	**Middle**	**High**
A	Conc. of stabilizer	%	50	60	70
B	Time of sonication	Min	15	22.5	30
C	Amplitude	%	50	65	80
Variable	**Name**	**Units**		**Goal**	
R1	Particle size	nm		Minimize	
R2	Zeta potential	mV		Minimize	

Abbreviation: Conc,Concentration;

2.5 Characterization of Nanocrystal

2.5.1 Particle size and Zeta potential

Particle size and polydispersity index of Natamycin naonocrystal were measured using Zeta sizer (Nano ZS Malvern, UK), which function based on laser light scattering principle. The zeta potential of natamycin naonocrystal were measured using Zeta sizer (Nano ZS Malvern, UK), which function based on electrophoretic mobility principle under an electric field. Each sample was measured at 25°C using glass cuvette in triplicate and average of each parameter was expressed [17].

2.5.2 Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The shape and surface morphology of the natamycin nanocrystal was investigated by scanning electron microscopy (ZEISS SIGMA VP Scanning Electron Microscopy). Powdered samples were used for this study. The shape and size distribution of optimized natamycin nanocrystal were observed by Transmission electron microscopy (High Resolution Transmission Electron Microscope (HRTEM) 200kV). Liquid samples were used for this study.

2.5.3 Powder X-Ray Diffraction (PXRD) studies

PXRD was imaged by an X-ray diffractometer (JEOL). The powders were placed in the sample slide and pressed smoothly. Then, samples were put into the instrument with scan speed at 2°/min, and patterns were recorded at 2θ, with angle ranging from 5° to 40° [18].

2.5.4 *In vitro* drug release studies

The *in vitro* drug release of pure natamycin and optimized nanocrystals of natamycin was carried out in a USP dissolution test apparatus type I (mode: Electrolab, Serve well instruments pvt ltd. Bangalore). The experiment was carried out using 900 ml of Simulated tear fluid (STF) pH 7.4 as dissolution medium at 37±0.5°C with 75rpm for 120 mins. Sample of 10 mg each of natamycin powder and natamycin nanocrystals subjected to dissolution studies. At predetermined time intervals, 5 ml of samples were withdrawn from the dissolution medium and replaced with freshly prepared tear buffer in order to preserve the constant volume. The samples were diluted appropriately and filtered through a 0.2 µm membrane filter. After appropriate dilutions, the clear samples were then analysed in the UV spectrophotometer at 304 nm. The release data were fitted in various kinetic models like zero order, first order, Higuchi model and Korsmeyer-Peppas model [19].

2.6 Formulation of *in situ* Gelling Solutions for pH Triggered System

Initially, HPMC E50LV and Carbopol 940 were dissolved in the appropriate quantity of phosphate buffer pH 6.8 and allowed to hydrate overnight. After 24 hours, the polymer solution was mixed together and stirred continuously for 1 hour. Optimized natamycin nanocrystal was dispersed in phosphate buffer and added to the above solution, mixed with continuous stirring. Benzalkonium chloride and Tween 80 were added with constant stirring until a uniform solution obtained. pH was adjusted with 0.5 M sodium hydroxide (NaOH). The developed formulations were filled in amber glass bottles and were subjected to sterilization by autoclaving at 121°C for 20 minutes [20].

2.7 Evaluation of pH Triggered *in situ* Gel

2.7.1 Clarity

Clarity is the most important evaluation method. All the formulation was subjected for visual inspection against a black and white background.

Table 2: Composition of polymer used in the formulation of *in situ* gelling system

Formulation Ingredient	Formulation Code				
	Formulation (F1)	Formulation (F2)	Formulation (F3)	Formulation (F4)	Formulation (F5)
Natamycin (%w/v)	5	5	5	5	5
Carbapol 940(%w/v)	0.3	0.3	0.3	0.3	0.3
HPMC 50LV (%w/v)	0.2	0.4	0.6	0.8	1.0
Tween 80	0.02	0.02	0.02	0.02	0.02
Benzalkonium chloride (%w/v)	0.01	0.01	0.01	0.01	0.01
Sodium hydroxide 0.5M (%w/v)	q.s	q.s	q.s	q.s	q.s
Phosphate buffer pH 6.8	100	100	100	100	100

2.7.2 pH

All the formulation was checked for pH using digital pH meter. pH meter was calibrated using standard buffer tablets. The tip of the pH pen (i.e electrode), brought in contact with the surface of the formulation.

2.7.3 Gelling capacity

The gelling capacity of the formulation was determined by visual inspection. A vial containing 2ml of freshly was prepared standard tear fluid and to this one drop of the formulation was added and equilibrated at 37±2°C. Time was noted for gelation and time taken for the formed gel to dissolve.

2.7.4 Drug content

The drug content of the formulation was determined by dissolving an accurately weighed quantity in tear fluid of pH 7.4. 1ml of the formulation was transferred into a 10ml volumetric flask and the volume was made up with the STF pH 7.4 and analyzed by UV spectrophotometer at 304 nm [21].

2.7.5 Rheological studies

The viscosity of the prepared formulation was measured before and after gelation using Brookfield viscometer (Brookfield DV-II +Pro) with T- bar spindle using spindle number 61 and the angular velocity increased gradually from 5 to 100 rpm. The prepared formulations were measured for sols at room temperature and gels at physiological temperature. Average of two readings was used to calculate the viscosity [14].

2.7.6 *In vitro* drug release studies

In vitro release study for *in situ* gel solution and the marketed formulation was carried out using Franz diffusion cell. Standard tear fluid of pH 7.4 as medium and cellophane membrane as diffusion membrane. The cellophane membrane previously soaked overnight in the STF was sandwiched between the donor compartment and receptor compartment. The whole assembly was thermostatically controlled with a magnetic stirrer. The temperature was maintained to 37°C. 1ml (0.05mg) of the *in situ* gel was placed in the donor compartment, and freshly prepared tear fluid of 12ml was placed in the receptor compartment.1ml of the sample was withdrawn at a predetermined time interval and the same volume of freshly prepared tear buffer was replaced. The withdrawn samples were filtered, diluted with respective solvent and analyzed using UV spectrophotometer at 304 nm [19].

2.7.7 *Ex vivo* permeation studies

Goat's cornea was used for this study to investigate the permeation across the corneal membrane. The goat eyeball was procured from a slaughterhouse and carried to the laboratory in normal saline maintained at 4°C. The cornea was separated from the eyeball and washed with cold normal saline and placed in freshly prepared tear buffer (pH 7.4). This study was carried out using a Franz diffusion cell. The cornea was kept in continuous contact with the formulation in the donor compartment. 12ml of Standard tear fluid is placed in the receptor compartment and was stirred on a magnetic stirrer. 1ml (0.05mg) of the formulation was placed in the donor compartment. The temperature was maintained at 37°C. The samples were withdrawn at

different time intervals and replaced with freshly prepared tear buffer to maintain the sink condition. 1ml of the sample was withdrawn, diluted and analyzed for drug content using UV spectrophotometer at 304nm [22].

2.7.8 Stability studies

Short term stability study of *in situ* gelling formulation was carried out for 60 days. The selected formulations were filled in amber glass containers and well stoppered with a cap. Stability studies were carried out at 4°C/100% (Refrigerated condition) and 25°C/60% RH (room temperature) according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and World Health Organization guidelines for appearance, pH, drug content and viscosity.

3. RESULTS AND DISCUSSION

3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was carried out to study drug-polymer interaction. There are some peaks of pure drug like 1109 cm^{-1} (Secondary alcohol C-O stretching), 3232 cm^{-1} (Carboxylic acid O-H stretching), 1713 cm^{-1} (Carboxylic acid C=O stretching), 3232cm^{-1} (Alcohol O-H stretching), 1642.52 cm^{-1} (Conjugated alkenes C=C stretching). The IR spectra of natamycin, polymer and optimized formulation are given in Fig 1, 2, 3. When compared with the pure drug, there was no considerable change in the IR peaks of the drug in the optimized formulation, which indicates that there is no incompatibility between drug and excipients.

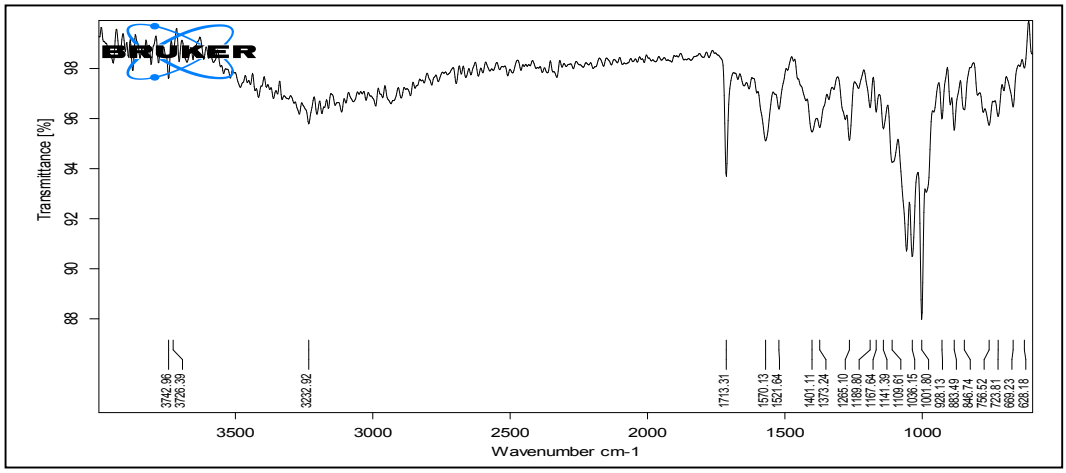

Fig. 1. FTIR spectra of Natamycin

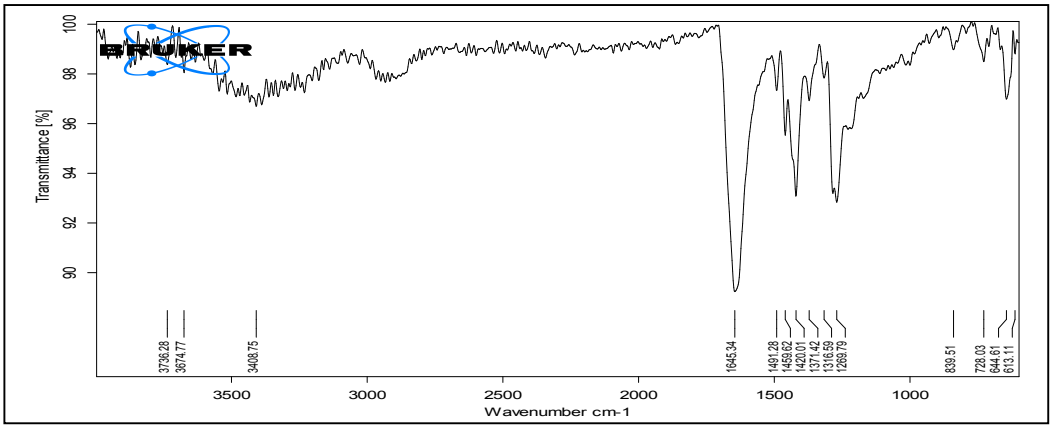

Fig. 2. FTIR spectrum of PVP

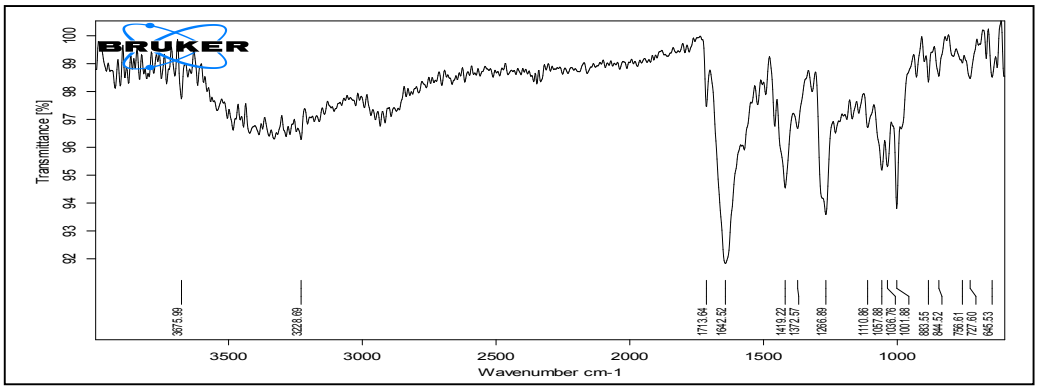

Fig. 3. FTIR spectrum of Optimized formulation

Fig. 4. Nanocrystal powder of natamycin

3.2 Preparation of Natamycin Nanocrystals by Sono-Precipitation Method

Natamycin nanocrystal was successfully prepared using the Sono-Precipitation method. Prepared nanocrystal powder was yellowish white in color as shown in Fig 4.

3.3 Experimental Design

Natamycin nanocrystal was successfully formulated by employing Box Behnken design along with response surface design and constituent's effects on its attributes were analyzed. Independent variables such as time of sonication, concentration of stabilizer and amplitude at 3 levels (low, medium and high) were evaluated for their dependent variable, i.e. particle size and zeta potential. When the constraints were selected, the software gave 17 runs. Data obtained were fed into the table and further analyzed. The results obtained are shown in Table 3. The design expert performed series of statistical analyses on the given data and chose the best model for each variable based on ANOVA. The design model selected for particle size was quadratic, and for zeta potential linear model was selected.

3.4 Particle Size

The results obtained in ANOVA indicate that all the factors (A- Concentration of stabilizer, B- Sonication time, C- Amplitude) were found to have a significant effect on particle size. The average particle size of nanocrystal is shown in Table 3. The average particle size of the natamycin nanocrystal was found to be in the range of 275-465 nm. All the nanocrystal formulations showed particle size in the nano range (<1 µm). The effect of stabilizer, sonication time and amplitude on particle size was obtained from Box-Behnken experimental design. The software selected quadratic model to be the best fitting model. The effect of the independent variable on particle size can simultaneously studied by the application of regression analysis. Quadratic model implied significance with F value of 130.20 and Predicted R^2 of 0.9260 is in reasonable agreement with Adjusted R^2 of 0.9864**.** The difference is less than 0.2. The results are given in Table 4. The equation computed the analysis is given as follows:

Particle size= $362.80-3.00A-54.50B-40.50C-6.25AB+6.75AC-10.25BC+11.373A^2-3.27B^2+20.72C^2$ (1)

Table 3. Box- Behnken experimental design

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
		A:conc of stabilizer (mg)	B:Sonication time (mins)	C:Amplitude (%)	Particle size (nm)	Zeta potential (mv)
10	1	60	22.5	65	366	-17.5
8	2	60	22.5	65	360	-16
16	3	70	22.5	80	354	-18
15	4	60	22.5	65	363	-17.7
7	5	50	22.5	80	356	-12
4	6	70	30	65	312	-19
13	7	50	30	65	321	-14
12	8	60	15	80	404	-17
9	9	70	22.5	50	421	-18.9
2	10	50	15	65	418	-15.5
11	11	60	22.5	65	367	-17.09
3	12	60	30	80	275	-17
17	13	70	15	65	434	-19
6	14	60	15	50	465	-17
5	15	50	22.5	50	450	-16.8
14	16	60	30	50	377	-17
1	17	60	22.5	65	358	-17.9

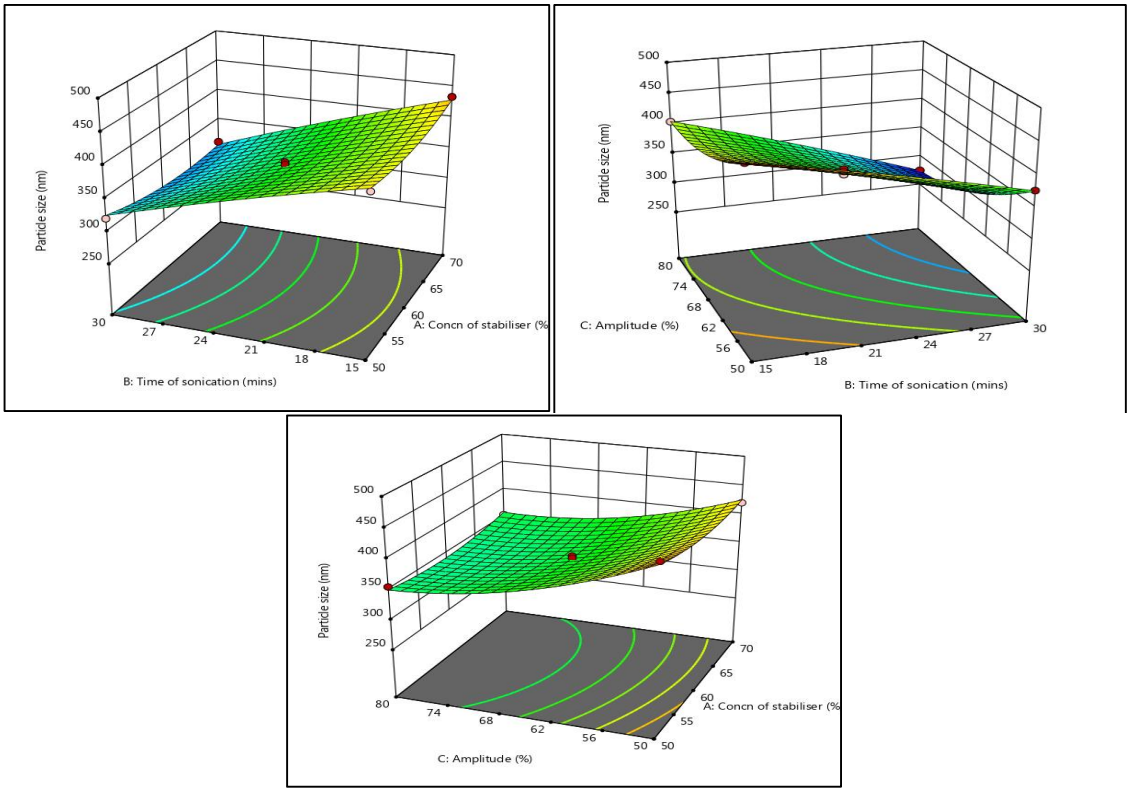

Fig. 5. 3D surface plot of particle size (nm)

Where A,B and C are the ratio of Concentration of stabilizer, Sonication time and Amplitude respectively, and AB, AC and BC are interaction effect between Concentration of stabilizer and Sonication time, Concentration of stabilizer and Amplitude, Sonication time and Amplitude. A^2, B^2

and C^2 are the quadratic effect of particle size. From the above equation, the variables A, B and C indicate negative effect on particle size. Results show that when there is an increase in the stabilizer concentration, sonication time and amplitude there is significant decrease in the particle size. The 3D response surface plot of the effects of stabilizer concentration, sonication time and amplitude on the particle size is shown in Fig.5, and it clearly shows the variables effects and interaction effect of variables on particle size.

3.5 Zeta Potential

The zeta potential values indicate the charge of the particles thereby indicates the stability of the formulation. The obtained zeta potential values of the formulations are given the Table 3. The results obtained in ANOVA indicate that all the factors (A-Concentration of stabilizer, B-Sonication time, C- Amplitude) were found to have a significant effect on zeta potential. Interaction between the variable showed no significant influence on the zeta potential. The zeta potential for the prepared natamycin nanocrystal was found within the range -12 to -19. Software selected linear model to be the best fitting model. The effect of independent variable on zeta potential can simultaneously studied by the application of regression analysis. Linear model implied significant with F value of 13.43 and Predicted R^2 of 0.5424 is in reasonable agreement with Adjusted R^2 of 0.6997**.** The difference is less than 0.2. The results are given in Table 4. The equation computed the analysis is given as follows:

$$\text{Zeta potential} = -16.91 - 2.07A + 0.1875B + 0.7125C \quad (2)$$

Where A, B and C are the ratio of Concentration of stabilizer, Sonication time and Amplitude respectively. From the above equation, the variable A indicates negative effect on zeta potential. This means that as concentration of stabilizer (A) increases, zeta potential decreases. Whereas variable B and C shows positive effect on zeta potential, this indicates as sonication time and amplitude increases, zeta potential increases. The 3D response surface plot of the effects of stabilizer concentration, sonication time and amplitude on the zeta potential is shown in the Fig. 6 and it clearly shows the variables effects and interaction effect of variables on zeta potential.

To optimize the formulation that will give minimum particle size and maximum zeta potential, numerical optimization of the design expert software was used. The software generated 43 solutions; the one that possessed desirability values (0.833) was selected. The given solution was formulated in the laboratory and the result was found to agree with the prediction made by the software with percentage error of 1.62% and 4.80% of particle size and zeta potential respectively. The optimized formulation showed size of 293.9 nm and Polydispersity index of 0.3. Zeta potential helps measure the surface charge on the particles, as surface charge directly controls the aggregation behaviour of the crystals. The optimized formulation showed a zeta potential of -14.6. The negative charge indicates that the particles have no charge and, as a whole system is stable. The optimized formulation was further analyzed for various characterizations as described in the methodology.

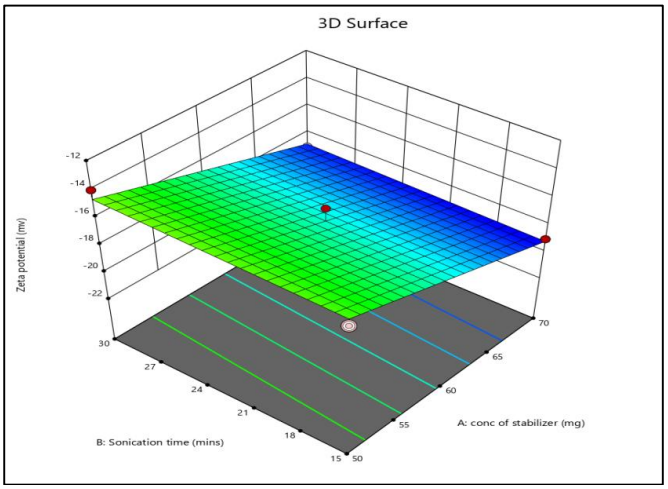

Fig. 6. 3D Surface plot of Zeta potential (mv)

Table 4. Statistical analyses of dependent variables

Response	Model	Standard deviation	Adjusted r^2	Predicted r^2	F value	P value	Predicted value	Observed value
Particle size(nm)	Quadratic	5.86	0.9864	0.9260	130.20	<0.0001	289.2	-13.93
Zeta potential (mV)	Linear	0.9813	0.6997	0.5424	13.43	0.0003	293.9	-14.6

3.4 Characterisation of the Optimized Formulation

3.4.1 Scanning Electron Microscopy (SEM)

The SEM image of optimized nanocrystal showed irregular in shape with a rough surface. The sem image of optimized nanocrystal are given in Fig 7.

3.4.2 Transmission Electron Microscopy (TEM)

The TEM was used to provide information on the morphology and size of natamycin nanocrystal. The nanocrystal as shown in Fig 8 is irregular in shape with an average size of 174nm.

3.4.2 Powder X-Ray Diffraction studies

Natamycin showed diffraction peaks at 2θ values of 16.9, 18.86, 19.88, 20.26, 20.90, 21.56, 23.29, and 24.57 as shown in Fig 9. Physical mixture and nanocrystal formulation showed peaks similar to pure drugs, as shown in Fig 10 and 11. Nanocrystal formulation peaks at 16.96, 18.86, 19.88, 20.26, 20.90, 21.58, 23.28, and 24.58. However, the peak intensity of nanocrystal was relatively low compared to pure drug. This effect is due to nanoionization. Furthermore, reduction in size and presence of stabilizer may cause the reduction in peaks of the nanocrystal. X-ray diffraction of the physical mixture showed dominant peaks of natamycin particles.

3.4.3 *In vitro* drug release studies of pure drug and nanocrystal

The *in vitro* drug release profile of pure drug (F1) and natamycin nanocrystal (F2) are shown in Fig 12. Drug release from the F2 was faster than F1. The Cumulative % drug release after 120 mins was 87.56% for the F2, and the drug release for F1 was found to be 22.75%.

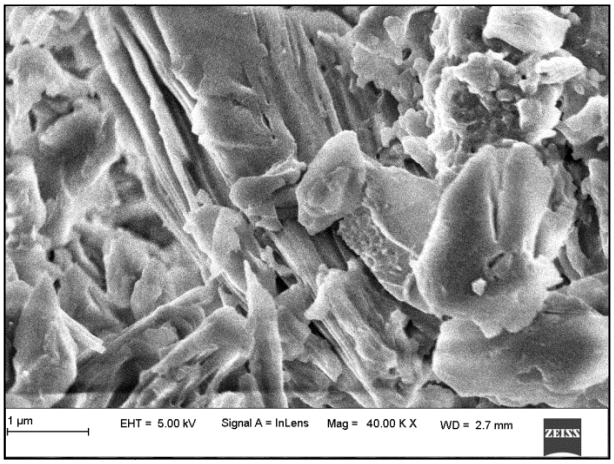

Fig. 7. SEM image Natamycin nanocrystal

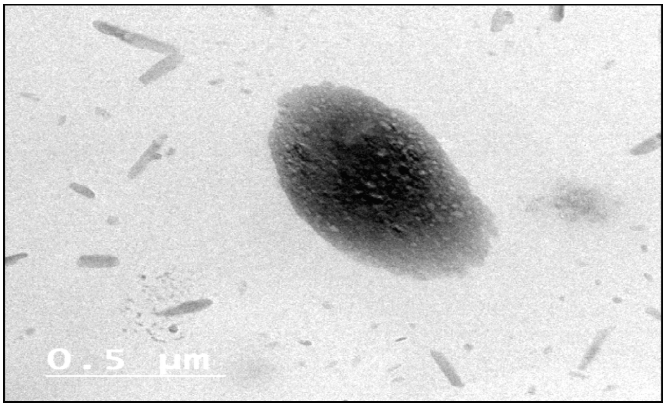

Fig. 8. TEM image Natamycin nanocrystal

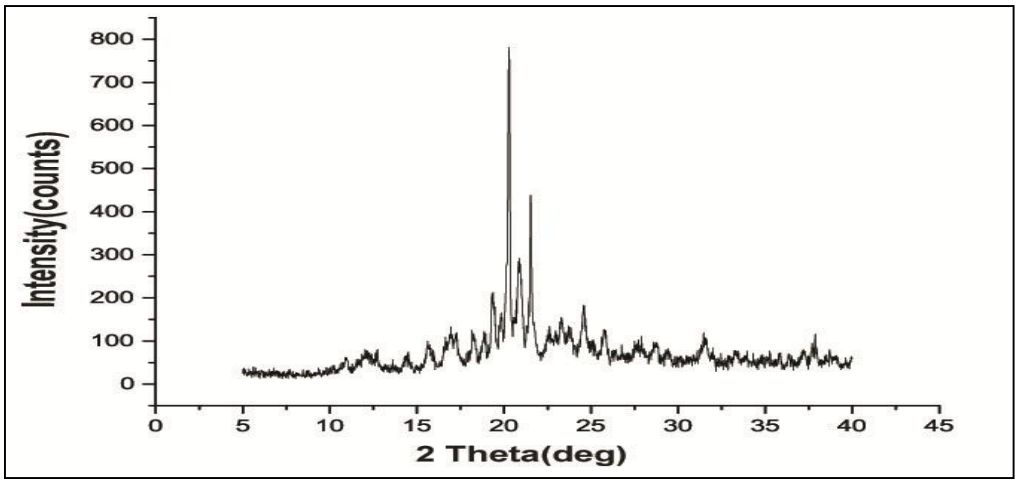

Fig. 9. PXRD image of Natamycin

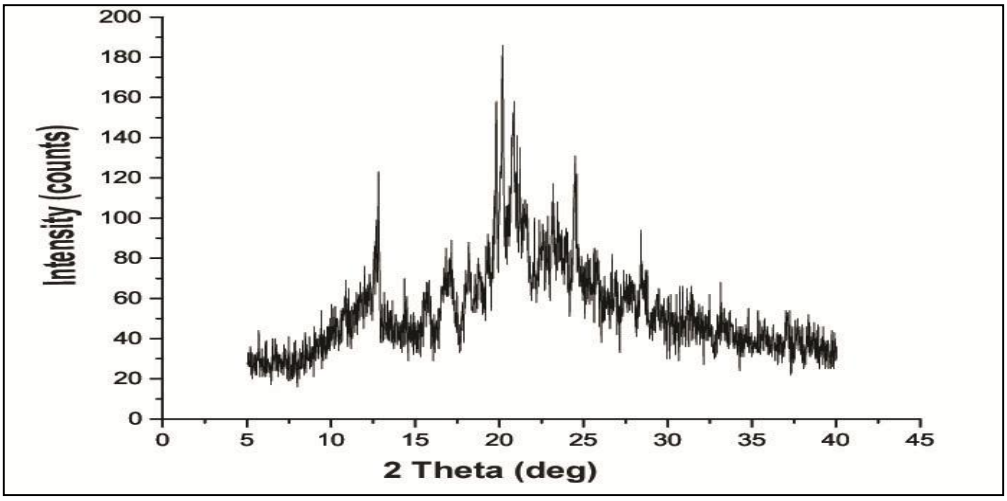

Fig. 10. PXRD image of physical mixture

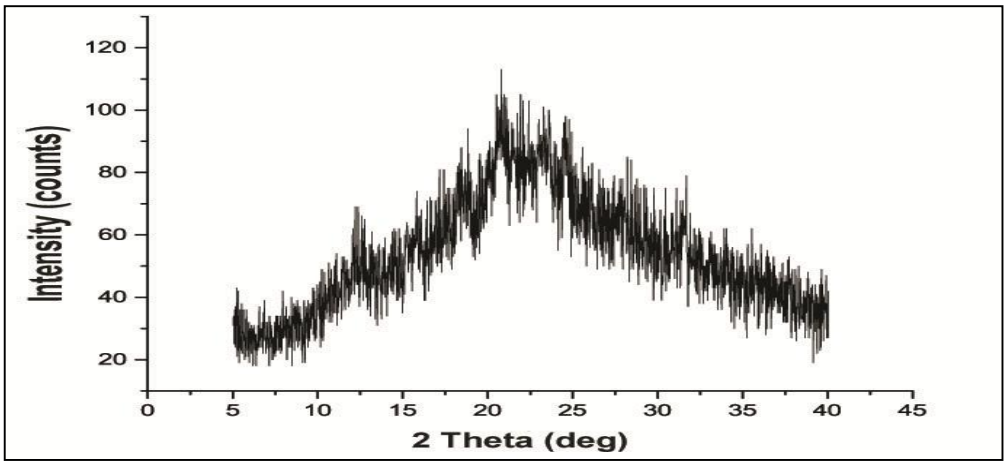

Fig. 11. PXRD image of optimized formulation

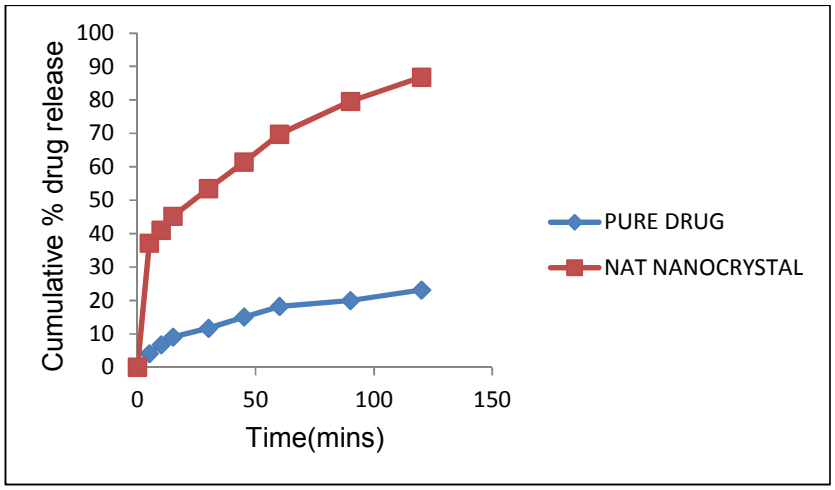

Fig. 12. Drug release profile of the pure drug and natamycin nanocrystal in Simulated Tear Fluid

3.5 Formulation and Evaluation of *in situ* Gelling Solutions for pH Triggered System

3.5.1 Clarity and pH

All the formulation was clear in appearance when observed in black and white background and found free from particular matter. The pH of the formulation was closed to the pH of tear fluid, i.e., 7.4 as per literature. All the results are shown in Table 5.

3.5.2 Drug content

The drug content of F1, F2, F3, F4 and F5 was found to be 98.15%, 97.24%, 98.23%, 99.70%, and 99.20% respectively.

3.5.3 Gelling capacity

All the formulation, when it comes in contact with STF, it shows instantaneous gelation. F5 and F4 showed immediate gelation and remained for an extended period when compared to other formulations. The gelling capacity of all the formulations depicted in Table 5.

3.5.4 Rheological studies

The angular viscosity of five formulations was determined by Brookfield viscometer at room temperature from 5 to 100 rpm. From the table 6 and 7, it can be observed that the formulations were less viscous in solution form compared to the gel state. All the formulations showed pseudoplastic flow. Increasing the concentration of polymer significantly increased the viscosity of both the gels and the solution. The formulation F4 and F5 showed higher viscosity for both gels and solution when compared to other formulations.

From the above-given results of Clarity, pH, Gelling capacity and viscosity of all the formulations (F1- F5) showed that formulation F4 showed optimum gelling capacity and the highest drug content. The formulation was clear and free from particulate matter and, upon administration, would not cause any irritation. The viscosity of the formulation was around 200 cps, which was within the range and showed pseudoplastic behaviour. Hence the F4 was selected as the optimized formulation.

Table 5. Clarity, pH, Drug content, Gelling capacity of *in situ* formulation

Formulation code	Clarity	pH	Drug Content	Gelling Capacity
PF1	Clear	7.4±0.1	98.15±0.68	-
PF2	Clear	7.5±0.25	97.24±0.52	+
PF3	Clear	7.4±0.05	98.23±0.19	++
PF4	Clear	7.4±0.1	99.70±0.39	+++
PF5	Clear	7.4±0.15	99.20±0.34	+++

All values are reported as an average of three determination± SD

Table 6. Viscosity of formulation before gelling at 25±1°C (sol form)

RPM	Viscosity in (cps)				
	F1	F2	F3	F4	F5
100	5.7±0.23	8.76±1.77	13.7±1.23	17.6±0.56	19.4±0.32
50	8.48±1.78	11.6±0.45	15±0.52	28.3±1.54	29.6±0.21
20	10.4±0.28	17.8±2.22	28.4±0.39	48.3±0.95	53.8±0.74
10	12.4±0.36	23.4±1.36	41.2±1.22	56.4±2.27	76.4±1.39
5	21.6±1.29	31.2±1.25	50.6±0.67	66.6±0.90	112.3

Table 7. Viscosity of formulation after gelling at 37±1°C (gel form)

RPM	Viscosity in (CPS)				
	F1	F2	F3	F4	F5
100	20.27±0.55	30.45±1.35	60.4±0.82	80.20±1.98	99.01±0.78
50	32.01±1.39	48.7±1.68	82.02±1.25	110.2±1.95	130.21±1.29
20	38.4±1.47	59.48±0.72	109.5±2.22	150.40±1.16	170.35±1.35
10	42.86±1.56	66.7±0.53	119.01±2.69	170.4±1.36	200.45±2.66
5	50.85±1.44	89.9±0.69	129.2±2.80	200.1±1.55	230.03±2.11

3.5.5 *In vitro* drug release study of *in situ* gel and marketed formulation

The *in vitro* diffusion study was carried out in the Franz diffusion cell. This study was done for the optimized *in situ* formulation and marketed formulation for 8 hours. The optimized in situ gel (PF-4) drug release showed 78.68%, whereas the marketed formulation drug release was found to be 96.38%. Drug release is faster in marketed formulation when compared to *in situ* formulations. The purpose of preparing *in situ* gel is to ensure that the residence time is increased so that the drug release is sustained due to the gel's retardant effect, thereby enhancing the ocular availability of the drug. This comparison of the release profile of optimized formulation and marketed formulation was depicted in Fig. 13.

3.5.6 Kinetic analysis of drug release

In this present study, the data obtained from *in vitro* drug release studies were employed to the four different mathematical models, i.e., zero-order kinetics, first-order kinetics, Higuchi square root of time equation and Korsmeyer-Peppas diffusion model, to predict the kinetics and release mechanism of the drug. The results of the *in vitro* drug release kinetics of optimized nanocrystal are given in Table 8. Drug release of nanocrystal was found to be first-order kinetics with the regression coefficient (R^2) of 0.998, based on the greater regression value (R^2) compared to that of zero-order kinetics. Whereas *in situ* formulations (F4) follows first-order kinetics. The drug release mechanism of F4 was studied by comparing the Higuchi model and Korsemeyer-Peppas model. F4 showed good linearity for the Higuchi model, suggesting diffusion controlled drug release. The value of release exponent 'n' obtained by applying the Korsemeyer-Peppas equation for F4 was 0.594. Hence the mechanism of drug release from the formulation follow non-fickian model, which is the combination of diffusion and erosion mechanism.

3.5.7 *Ex vivo* permeation studies

The *ex vivo* permeation through the goat's cornea was carried out using Franz diffusion cell and the study profile is given in Fig 14. The cumulative drug release from optimized formulation (F4) was found to be 69.32% in 8 hours and marketed formulation was found to be 93.15%. The release profile is similar to the *in vitro* release profile with *in situ* gel showing greater permeation because of a greater extend of dissolution than marketed formulation, which showed a poor permeation.

3.5.8 Stability studies

The optimized formulations (F4) were subjected to stability studies for a period of two months as given in the methodology. The formulations were evaluated for Appearance, pH, Drug content, Viscosity. Fig. 15 and 16 compare the parameters of the formulation under different conditions at initial, 15, 30 and 60 days. Results clearly indicate that, there was no significant change in these parameters. The drug content of the formulation did not deviate by more than 3%, indicating that the drug was stable in the formulation. The optimized formulation showed

good stability at 4□C/100% RH (Refrigerator temperature) and 25□C/60% RH (Room temperature) in terms of various physicochemical properties.

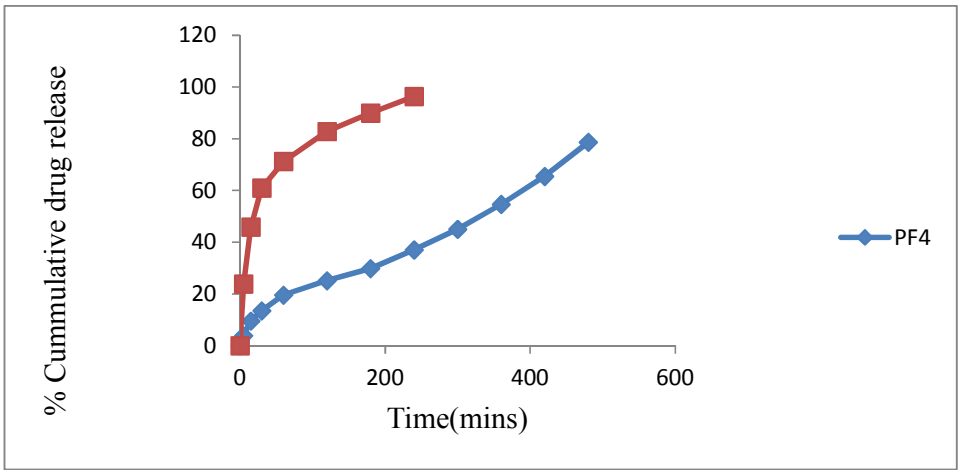

Fig. 13. *In vitro* drug release profile

Table 8. Data for drug release kinetics of the formulation

Release Model		Nanocrystal	PF-4
Zero Order	R^2	0.968	0.983
	K	-0.439	-0.139
First order	R^2	0.998	0.909
	K	-0.005	-0.001
Higuchi	R^2	0.996	0.983
	K	5.907	2.496
Korsmeyer-peppas	R^2	0.978	0.978
	n	0.277	0.594

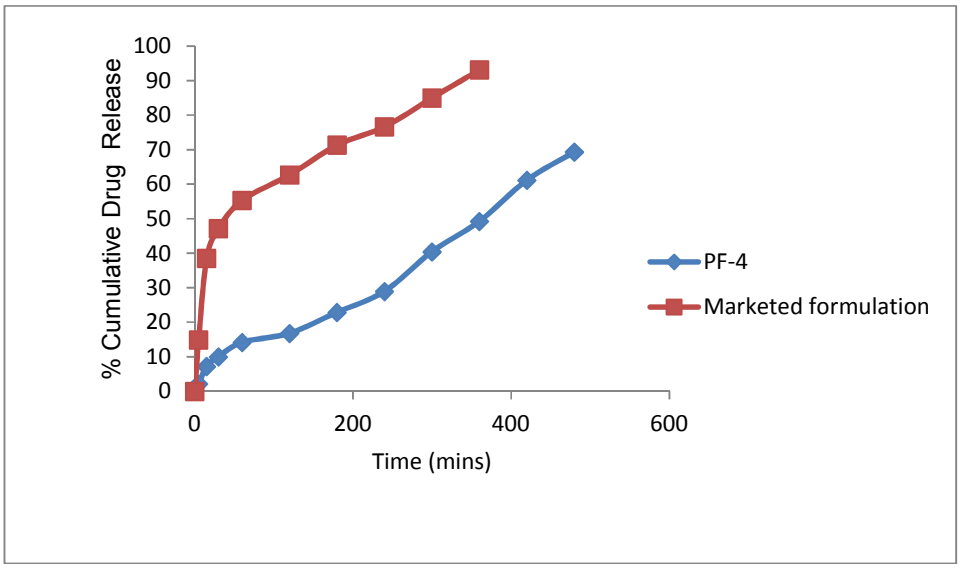

Fig. 14. *Ex vivo* release profile of optimized formulation (F4) and marketed formulation

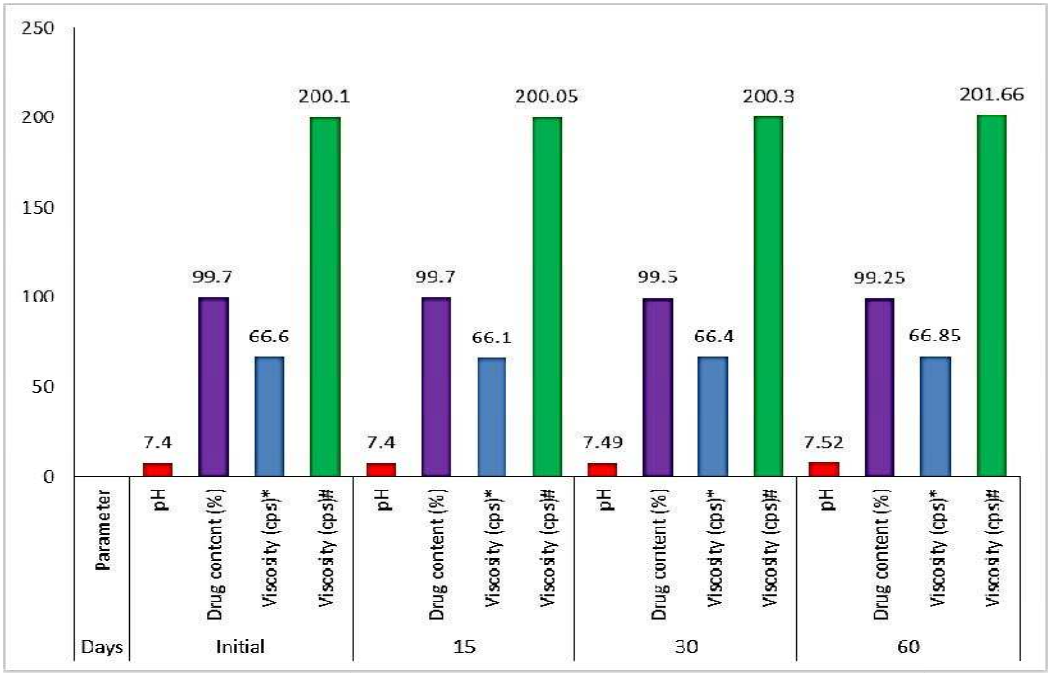

Fig. 15. Comparison of data from stability studies for optimized formulation (F4) at 4□C/100% RH

** Viscosity at 25±1□C and 5 rpm, # Viscosity at 37±1□C and 5 rpm*

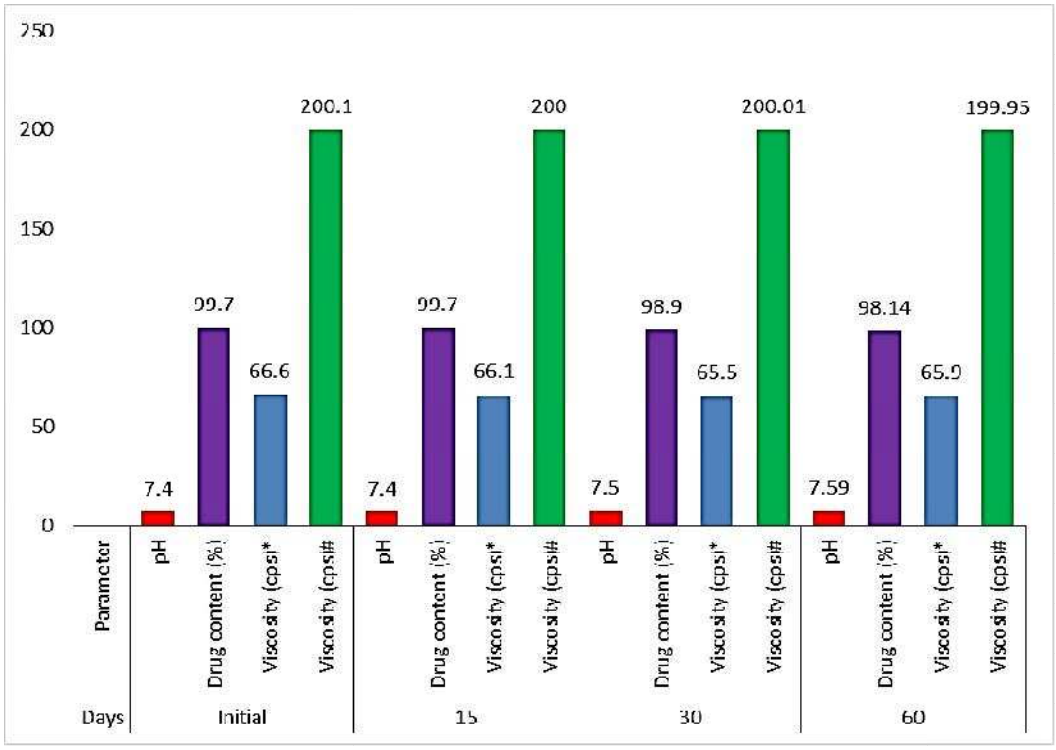

Fig. 16. Comparison of data from stability studies for optimized formulation (F4) at 25□C/60% RH

** Viscosity at 25±1□C and 5 rpm, # Viscosity at 37±1□C and 5 rpm*

4. CONCLUSION

It can be concluded that the natamycin nanocrystal loaded *in situ* gel was successfully formulated. The prepared formulation was characterized for various parameters. The Natamycin nanocrystal loaded *in situ* gel formulations was prepared using various polymers such as Carbopol 940 and HPMC, which release the drug in a sustained manner and maintain prolonged therapeutic effect. The results reveal that our formulation can be a promising alternative to the conventional eye drops of natamycin in the treatment of FK. Moreover, method of preparation is simple, cost effective and could be easily scaled up for commercial manufacture.

The effectiveness of the optimized formulation investigated needs to be further confirmed in animal studies or extended to human studies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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