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FORMULATION AND EVALUATION OF PH TRIGGERED *IN-SITU* OPHTHALMIC GEL CONTAINING NATAMYCIN CYCLODEXTRIN INCLUSION COMPLEX

Akshata. I. Shettar, U. B. Bolmal, A. P. Gadad

Department Of Pharmaceutics, KLE's College Of Pharmacy, Belagavi- Karnataka, India.

ABSTRACT

The objective of the present work was to formulate pH triggered *In-situ* gel of Natamycincyclodextrin inclusion complex using carbopol 940, HPMC K4M and HPMC E50LV. Natamycin possesses activity against a variety of yeast and filamentous fungi, including Candida, Aspergillus, Cephalosporium, Fusarium and Penicillium..pH triggered sol gels are shear thinning systems which show pH dependent gelation. Once instilled into the cul de sac of eye they rapidly get converted to gel increasing the precorneal contact time of drug with the corneal membrane. The prepared formulations were evaluated for various parameters such as clarity, pH, drug content, gelation capacity, gelling strength, viscosity, *In-vitro* release studies, pharmacokinetics studies, antimicrobial activity animal studies and short term stability studies. The FTIR results revealed the compatibility between the drug and polymers. Drug content was in the range 97.06% to 99.60 %. The pH was in range of 7-7.5. The viscosity of the formulations at $25^0 \pm 2^0$C and $37^0 \pm 2^0$C was in the range of 28-270cps and 49-306cps respectively. The optimised formulation NF8 was selected on the basis evaluation parameters. The *In-vitro* drug release revealed a sustained profile over a period of 6 hours and optimized formulation NF8 showing 38.39 % of release. The *In-vitro* antimicrobial study showed promising activity of NF8 against clinical isolates of *candida albicans*organisms .Short term stability study indicated that $4^0 \pm 1^0$C was appropriate storage condition for the formulations.

Keywords: Natamycin, Carbopol 940, HPMC K4M, HPMC E50LV, pH triggered in-situ ophthalmic gel.

INTRODUCTION

Eye is one of the most challenging organs due to its drug disposition characteristics. Generally, topical application of drugs is the method of choice under most Circumstances because of its convenience and safety for ophthalmic chemotherapy [1]. A significant challenge to the formulator is to circumvent (bypass) the protective barriers of the eye without causing permanent tissue damage. Conventional ophthalmic formulations like solution, suspension, and ointment have many disadvantages which result into poor bioavailability of drug in the ocular cavity. The specific aim of designing a therapeutic system is to achieve an optimal concentration of a drug at the active site for appropriate duration [2].

Natamycin is a tetraenepolyene antibiotic derived from Streptomyces natalensis. It possesses *In-vitro* activity against a variety of yeast and filamentous fungi, including Candida, Aspergillus, Cephalosporium, Fusarium and Penicillium. The mechanism of action appears to be through binding of the molecule to the sterol moiety of the fungal cell membrane. The polyenesterol complex alters the permeability of the membrane to produce depletion of essential cellular constituents. Although the activity against fungi is dose-related, natamycin is predominantly fungicidal. Natamycin is not effective *In-vitro* against gram-positive or gram-negative bacteria. Topical administration appears to produce effective concentrations of natamycin within the corneal stroma but not in intraocular fluid. Systemic absorption should not be expected following topical administration of Natamycin 5%. As with other polyene antibiotics, absorption from the

Corresponding Author:-**U. B. Bolmal** Email: udaybolmal@yahoo.co.in

gastrointestinal tract is very poor. Studies in rabbits receiving topical natamycin revealed no measurable compound in the aqueous humor or sera, but the sensitivity of the measurement was no greater than 2 mg/mL [3-5].

MATERIALS AND METHODS

Natamycin was a gift sample from Chihong Biotechnology, China. HPMC E50LV, HPMC K4M were gift samples from Colorcon Pvt. Ltd., Verna Goa. Carbopol 940 was obtained from Hi media Laboratories Pvt. Ltd, Mumbai. All chemicals and reagents were of analytical and pharmacopieal grade.

FTIR spectroscopy

TheFTIR spectrum of pure drug Natamycin , and a physical mixture of Natamycin and HP-βcyclodextrin with HPMC E50 LV, HPMC K4M and Carbopol 940 were recorded by using FTIR (IRAffinity-1, Shimadzu, Kyoto, Japan) [6].

Standard calibration curve

The standard calibration of Natamycin was carried out in STF and absorbance of aliquot solutions was measured at 303 nm by UV –Visible spectrophotometer [7].

Preparation and characterization of inclusion complex of Natamycin and 2 Hydroxy Propyl β Cyclodextrin (2HPβCD)

The complex of drug and cyclodextrin was prepared by equimolar ratio of 1:1 by using 2-hydroxy propyl β- cyclodextrin as carriers and using two methods i.e. solvent evaporation and kneading method. Molecular weight of drug: 665.73, molecular weight of HP-β-CD: 1134.98.

Solvent evaporation method

This method involves dissolving of the drug and CDs separately in to two mutually miscible solvents, mixing of both solutions to get molecular dispersion of drug and complexing agents and finally evaporating the solvent under vacuum to obtain solid powdered inclusion compound. Generally, the aqueous solution of CDs is simply added to the alcoholic solution of drugs. The resulting mixture is stirred for 24 hours and evaporated under vaccum at 45 ^{0}C. The dried mass was pulverized and passed through a 60-mess sieve.

Kneading Method

This method is based on impregnating the CDs with little amount of water or hydroalcoholic solutions to converted into a paste. Weighed amount of Natamycin is then added to the above paste and kneaded for a specified time. The kneaded mixture is then dried under vacuum at 45^{0}C for 24 hrs. The dry mass was pulverized and sieved through sieve no 100 [8, 9].

Evaluation of Drug-Cyclodextrin complex

Drug content

Prepared complex was weighed equivalent to 10mg of pure drug and diluted to 100ml with freshly prepared STF with continuous stirring for 4hrs. From this 1ml was withdrawn and diluted to 10ml using STF. The absorbance was measured at 303nm against STF as blank by using UV- spectrophotometer.

In-vitro dissolution studies

Dissolution studies were carried out in STF. Samples of 1 ml were withdrawn at various time intervals and analyzed spectrophotometrically at 303nm using UV-visible spectrophotometer, the samples withdrawn were replaced by fresh STF solutions and all the inclusion complex where compared with pure drug.

Procedure for the preparation of pH sensitive Insitu gel

1. Phosphate buffer pH 6.8 was prepared and all the three polymers i.e. Carbopol 940, HPMC K4M & HPMC E50LV were added in appropriate quantity of phosphate buffer pH 6.8 separately and allowed to hydrate overnight.
2. After 24 hours carbopol 940, HPMC K4M, & HPMC E50LV polymer solutions were mixed together according to formulation table 5 with continuous stirring for 1 hour.
3. Drug complex was dispersed in phosphate buffer pH 6.8 and added to above (step2) mixture and mixed with continuous stirring for 1 hour.
4. Tween 80 and Benzalkonium chloride were added with constant stirring until a uniform solution was obtained.
5. pH was adjusted with 0.1M NaOH.
6. Finally Phosphate buffer pH 6.8 was then added to make up the volume to 100ml.
7. The developed formulations were filled in amber glass bottles and were subjected to terminal sterilization by autoclaving at 121^{0}C for 20 minutes [10]. The composition of each formulation is shown in Table no1.

Evaluation for pH sensitive in-situ gel

Clarity

All prepared formulations were evaluated for clarity by visual observation against black and white background [11].

pH

The formulated gels were checked for pH using digital pH meter. pH meter was calibrated using standard buffer tablets

Drug content

Drug content was found out in STF. The absorbance was measured at 303 nm against STF as blank by using UV-Visual spectrophotometer.

Gel strength

It is measured by method reported by Choi [12].

Gelling capacity

The gelling capacity of the *In-situ* gel formulations was determined by placing a drop of formulation in a test tube containing 2ml of STF freshly prepared and equilibrated at 37 ^{o} C ± 2^{o}C and visually assessing the formation of gel, noting the time for gelation and the time taken for the formed gel to dissolve.

Rheological studies.

Viscosity of the formulation was determined before and after gelation by using Brookfield's digital rheometer (cap 2000+) using spindle 1.

***In-vitro* drug release of Insitu gel system**

In-vitro release studies of the formulations were carried out by using Franz diffusion cell consisting of 1ml of formulation placed in donor compartment and freshly prepared STF in receptor compartment.

Kinetics of drug release

The mechanism for the release and release rate kinetics of the dosage form was analysed, the data obtained was fitted in to Zero order, First order, Higuchi matrix and Korsemeyer-Peppas model. By comparing the R^2 values obtained, the best fit model was selected [13].

Comparison of release profile with marketed product

The *In-vitro* release study was carried out with the marketed product in order to compare its release profile with the prepared Insitu gelling system of Natamycin.

Antimicrobial studies

The microbiological studies were carried out to ascertain the biological activity of best sol-to-gel system. This was determined by using Gradient Diffusion Method (Dig well technique) [14].

Animal studies

Ocular irritancy test

Ocular irritation studies were performed on 3 female albino rabbits weighing 1-2 kg. The modified Draize technique was designed for the ocular irritation potential of the ophthalmic product. Approval of the Institutional Animal Ethic Committee was taken prior to the commencement of the study [15].

Efficacy against fungal keratitis.

Fungal keratitis was induced in rabbit eye by instilling fungal strains of *candida albicans*culture. These were done by placing 2 drops of culture in cul de sac of rabbit eye. Treatment was initiated 48 hours later. Dose of 2 drops were instilled in the cul de sac of rabbit eye once a day and animals were observed for redness, mucoidal discharge and swelling of eyelids [16].

Short term stability studies

Optimized formulation was subjected to stability studies at 4 ^{0}C and at room temperature that is 25 ^{0}C for a period of 45 days. The samples were withdrawn after 30 and 45 days and evaluated for following parameters. pH, Drug content, Gelling capacity, *In-vitro* drug release [17].

Results

IR Spectroscopy

The functional group frequencies of Natamycin were in the reported range which indicates that the obtained sample Natamycin was pure. The IR spectra of drug-polymer physical mixture indicated no interaction between Natamycin and polymers when compared with infrared spectrum of pure drug. The individual IR spectra of the pure drug and combination spectra of the pure drug and polymer are shown in the Fig: 1-3.

Standard calibration curve of natamycin

The curve was found to be linear in the Beer's range between 0-10µg/ml at 303nm. The regression coefficient (R^2) obtained was 0.998 and equation was y = 0.010x + 0.002. The standard plot of Natamycin is shown in Fig: 4.

Preparation of Complex:

Drug-Cyclodextrin Complex was successfully prepared by using two methods solvent evaporation and kneading method. The molar ratio of Drug-Cyclodextrin was taken as 1:1. Prepared complex were yellowish white in color.

Evaluation of Drug-Cyclodextrin complex

Drug Content

The drug content of the prepared Solid dispersions was found to be 73.1% for kneading method and 89.3% for solvent evaporation method which indicated that the application of the present methods for the preparation of Solid dispersions had moderate content uniformity. The maximum % drug content was found to be 89.3% for solvent evaporation method.

***In-vitro* Dissolution study**

Drug release from solid dispersions was faster than pure drug, Cumulative percent drug released after 60 minutes were 91.31% for solvent evaporation method and 86.18% for kneading method, while it was 18.36% in 60 minutes for pure drug Natamycin.

From the in-vitro dissolution profile, it can be seen that solvent evaporation method containing Drug-HP-β-CD (1:1) show higher dissolution rate compared with other method. All the results of *In-vitro* dissolution studies are shown in Fig: 5.

Preparation of sol gel formulation

The sol gels were successfully prepared by using drug-cyclodextrin complex.

Evaluation of formulated sol gel

Clarity, pH and visual appearance

All the prepared formulations were free flowing liquid at room temperature, clear, and free of any particulate matter. The pH was within the acceptable range and hence would not cause any irritation upon administration. The results are tabulated in Table No 2.

Determination of drug content

Drug content was determined by UV spectrophotometer at 303nm using STF as the diffusion medium. Drug content of formulations NF1-NF9 were in the range of 97.06% to 99.60 % indicating uniform distribution of drug. All the results are shown in Table No 2.

Gel strength

It was seen that with increase in carbopol 940 there was increase in the gel strength. This may be due to reversible ionization at pH 7.4, physiological condition, to form a stiff gel network, which swells and forms large aqueous pores. Gel strength observed for the *In-situ* gel formulations is as shown in Table No 3.

Gelling Capacity

All the formulations showed instantaneous gelation when contacted with simulated tear fluid (STF). Formulation NF3, NF4, NF8 and NF9 showed immediate gelation as compared to others and remained for extended period of time. This may be due to increase in concentration of HPMC K4M and HPMC E50LV. The gelling capacity of all pH sensitive formulations NF1 to NF9 is shown in Table No 3.

Rheological studies

It was observed that with increase in pH there was drastic increase in viscosity. All the formulation exhibited Pseudo plastic behavior i.e. with increase in shear rate they showed decrease in viscosity. Further it was seen that with increase in the concentration of HPMC there was increase in the viscosity of formulation. Also it was found that there was no significant effect of tear fluid on the viscosity of gel confirming efficacy of gel to remain stable in cul de sac of eye. Viscosity of all formulation is depicted in Fig: 6 and 7.

***In-vitro* diffusion studies**

The *In-vitro* drug release studies were carried out for all the formulations using STF as the dissolution medium. It was found that the drug release for all the formulations were in the range of 29.33% -51.67%. It was seen that with increase in concentration of HPMC there was a sustained drug release from the formulation.

Above results indicated that the structure of gel functioned as a barrier to drug release. The *In-vitro* release profiles of formulations are shown in Fig: 8.

Kinetics of drug release

The diffusion exponent (n) values for formulations NF4, NF5 and NF6 were less than 0.5 and showed fickian mechanism and of drug release and NF1, NF2, NF3, NF7, NF8 and NF9 values were more than 0.5 and showed non-fickian mechanism of drug release.

From the above results it was seen that Formulation NF8 showed optimum gelling capacity and highest drug content. Viscosity of the formulation was around 295.90cp which is not less neither too dense. Formulation showed release rate of 38.39 % showing sustained drug release for the period of 6 Hours and formulation remain stable in gel form at physiological temperature of eye. It also followed non-fickian mechanism for the drug release. And hence formulation NF8 was choosen as optimised formulation.

The optimized formulation was used for the further evaluation parameters such as antimicrobial activity, comparison study, animal studies and stability studies.

Comparison between release profiles of optimized formulation with marketed formulation.

The *In-vitro* release profile of NF8 was compared with marketed formulation. The cumulative percentage drug release after 60 min was found to be 38.39% for the optimized NF8 formulation and 91.50% for Marketed formulation. Results indicated that, the drug release was significantly prolonged by using an *In-situ* gelling system. The *In-vitro* diffusion release comparative plot was depicted in Fig: 9.

Antimicrobial activity

The drug was active against the selected *candida albicans*organism as indicated by zone of inhibition. The zone of inhibition was 24mm for optimized formulation and 22mm for marketed formulation. The results of the antimicrobial efficacy test are shown in Fig: 10.

Animal studies

Ocular irritation studies

The formulation NF8 was non irritating with no ocular damage. There were no signs of unwanted reactions like redness, mucous formation or inflammation. The results are shown in Fig: 11

Efficacy against induced fungal keratitis

Symptoms associated with keratitis were reduced faster with sol-gel formulation as compared to marketed formulation. Sol gel system took 5 days to cure the infection while marketed formulation took 8 days. The results are shown in Fig: 12.

Short term stability studies

The physical appearance of the formulation NF8 stored at room temperature changed slightly. The viscosity was

found to increase when stored at room temperature but not in significant amount. The drug content of the formulation stored at 4 ^{0}C showed slight variations but of that stored at room temperature varied significantly. The pH of formulations did not vary much over the period of 45 days at either temperature conditions. From the above results it can be interpreted that the prepared sol gel NF8 can be best stored at 4 ºC ± 1ºC. The results of the stability studies are as shown in Table 4.

Table 1. Composition of *Insitu* Gel Formulation

Formulation ingredient	Formulation code								
	NF1	NF2	NF3	NF4	NF5	NF6	NF7	NF8	NF9
Natamycin complex equivalent to (% w/v)	5	5	5	5	5	5	5	5	5
Carbopol 940 (% w/v)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
HPMC K4M (% w/v)	0.2	0.4	0.6	0.8	-	-	-	-	-
HPMC E50LV(% w/v)	-	-	-	-	0.4	0.5	0.6	0.7	0.8
Tween 80(% w/v)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Benzalkonium Chloride (% w/v)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Sodium Hydroxide 0.1M	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Phosphate buffer 6.8 pH (qs to ml)	100	100	100	100	100	100	100	100	100

Table 2. Appearance, Clarity, pH & drug content of NF1-NF9 Formulation

Formulation code	Appearance	Clarity	pH (mean± SD)	Drug content % (mean± SD)
NF1	Translucent free flowing liquid	Clear	7.4 ± 0.1	98.20 ± 0.198
NF2	Translucent free flowing liquid	Clear	7.2 ±0.2	97.06 ±0.524
NF3	Translucent free flowing liquid	Clear	7.0 ±0.251	97.40 ± 0.343
NF4	Translucent free flowing liquid	Clear	7.1 ± 0.057	98.16 ± 0.396
NF5	Translucent free flowing liquid	Clear	7.4 ±0.1	97.24 ± 0.686
NF6	Translucent free flowing liquid	Clear	7.4 ±0.251	99.44 ± 0.524
NF7	Translucent free flowing liquid	Clear	7.3 ±0.208	98.89 ± 0.198
NF8	Translucent free flowing liquid	Clear	7.5 ±0.305	99.60 ± 0.687
NF9	Translucent free flowing liquid	Clear	7.5 ±0.057	99.00 ± 0.396

Data are expressed as mean. (n=3) ±SD

Table 3. Gelling strength & gelling capacity of Nf1-Nf9 formulations

Formulation code	Gelling strength (Seconds)(mean ± SD)	Gelling capacity
NF1	61 ± 1.527	+
NF2	68 ±1.154	++
NF3	76 ±2.081	+++
NF4	62 ± 0.577	+++
NF5	86 ±1.732	+
NF6	95 ±2.645	+
NF7	84 ±1.527	++
NF8	97 ±0.577	+++
NF9	108 ±1.154	+++

Data are expressed as mean. (n=3)±SD

+ gel forms after few minute, disperses rapidly; ++ Immediate gelation, remains for few hours;

+++ Immediate gelation, remains for extended period of time.

Table 4. Short Term Stability Studies For Optimized Formulation Nf8

Parameter	Initial value	30 days		45 days	
		4^0C	25^0C	4^0C	25^0C
pH	7.5	7.4	7.4	7.2	7.2

Drug content	99.60%	99.59%	99.40%	99.55%	99.39%
Drug release	38.39%	38.26%	37.96%	38.10%	37.95%
Viscosity(25◦C ± 1◦C)(5 rpm)	140	139.80	137.20	139	136.98
Viscosity (37 ◦C ± 1◦C)(5 rpm)	295.90	295	295.10	295.52	295.85

Fig 1. FTIR spectra of NATAMYCIN pure drug

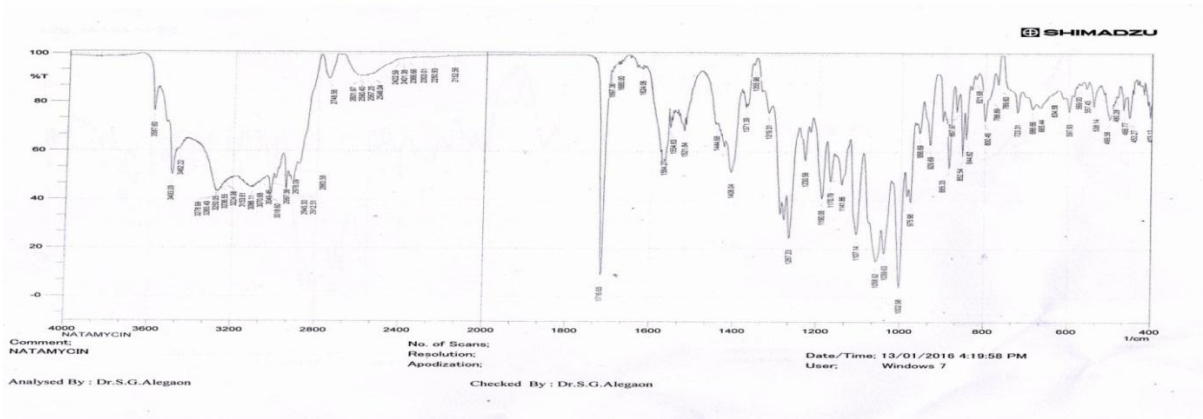


Fig 2. FTIR spectra of physical mixture 1

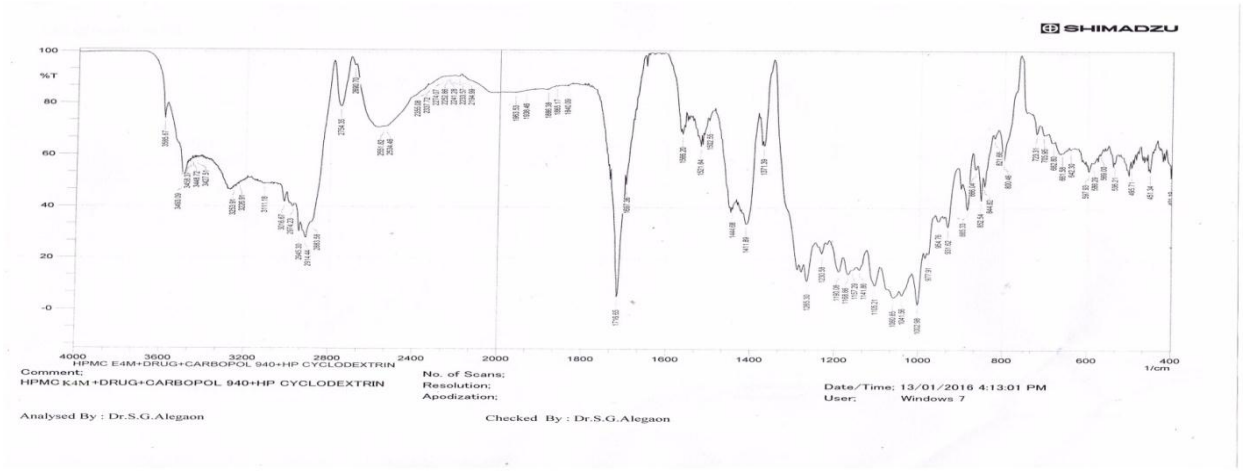


Fig 3. FTIR spectra of physical mixture 2

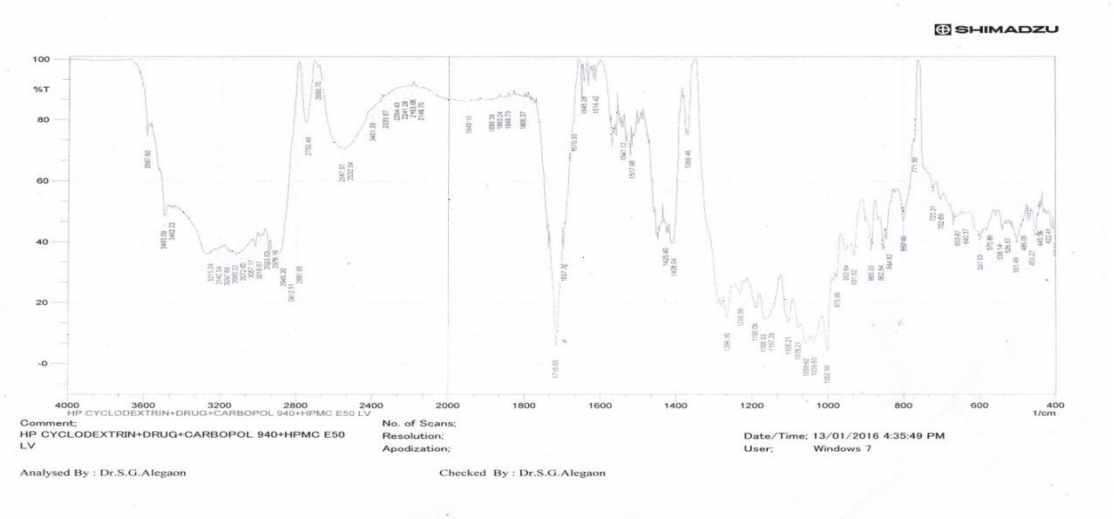


Fig 4. Standard calibration curve of Natamycin at 303nm

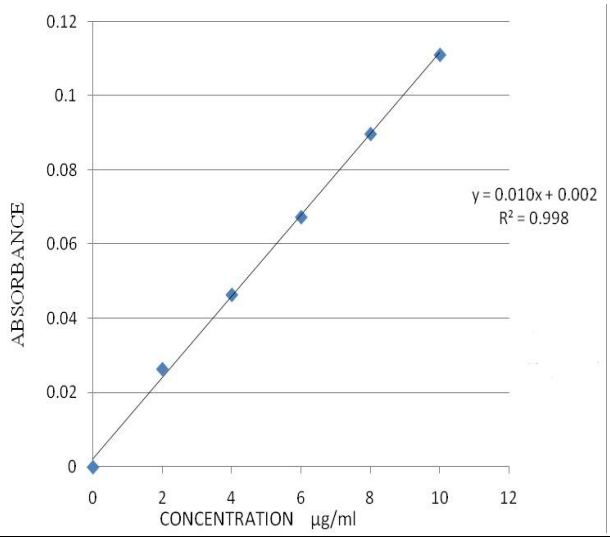


Fig 5. Comparison graph of in-vitro dissolution of solid dispersion product and pure drug

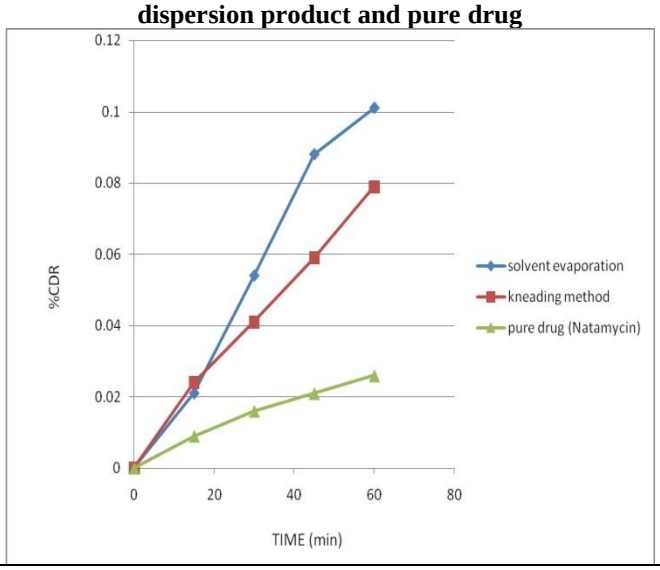
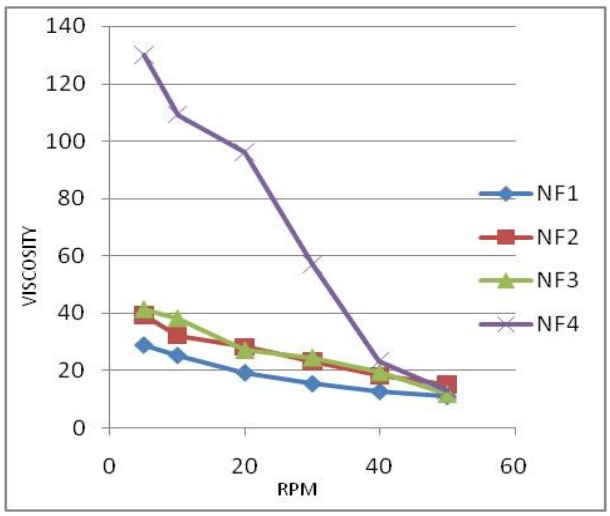


Fig 6. Viscosity of formulation NF1-NF9 at 25°C Viscosity at 25 °C ±1 °C (After dilution with STF)



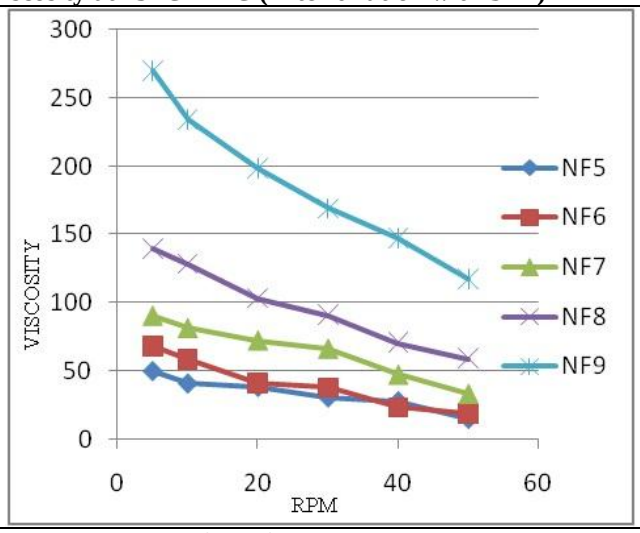
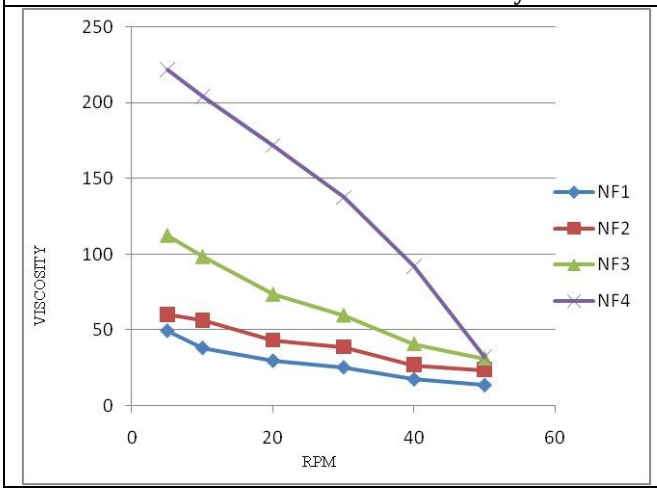


Fig 7. Viscosity of formulation NF1-NF9 at 37°C ±1°C Viscosity at 37°C ±1 °C (after dilution with STF



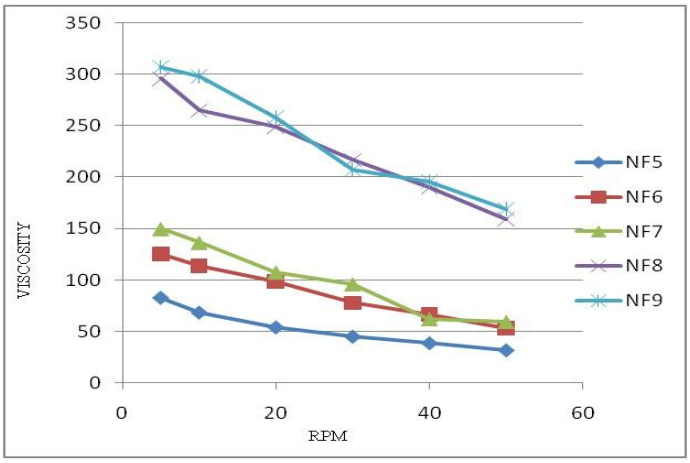


Fig 8. Comparative diffusion profile of formulations F1-F9

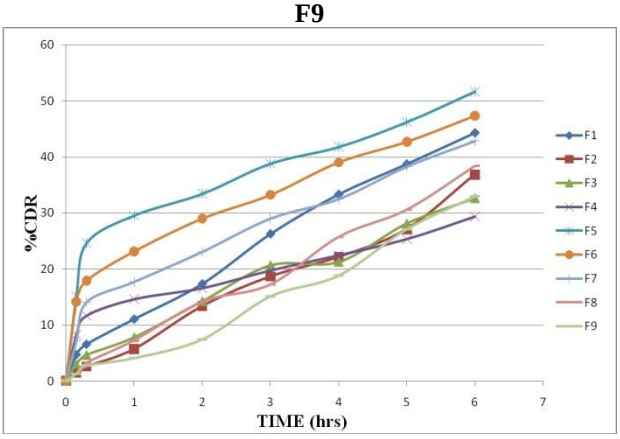


Fig 9. Comparative diffusion profile of optimized formulation NF8 and marketed formulation

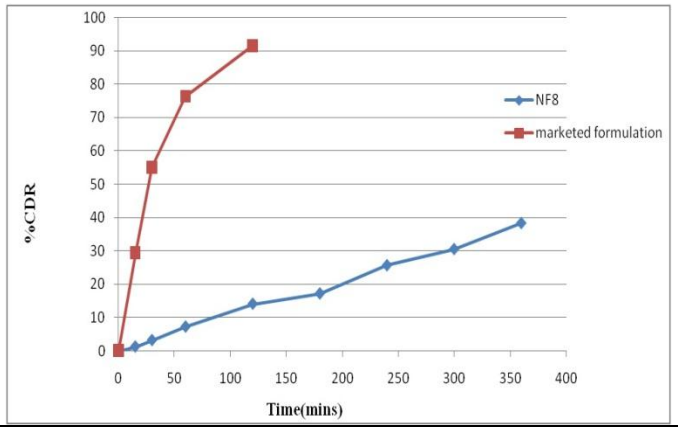
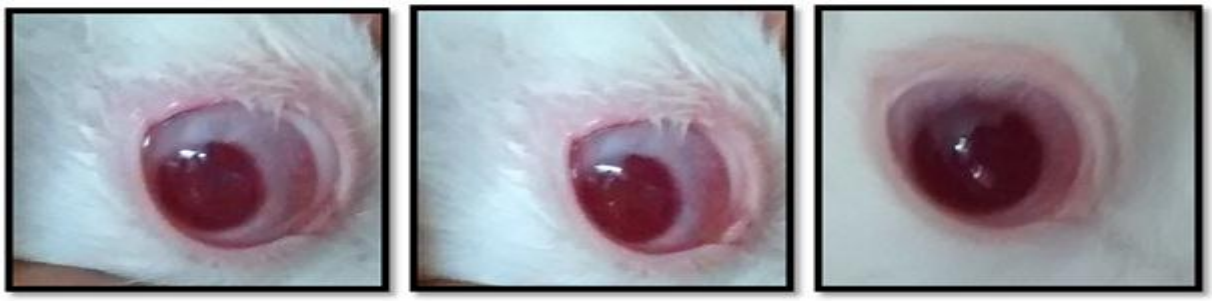


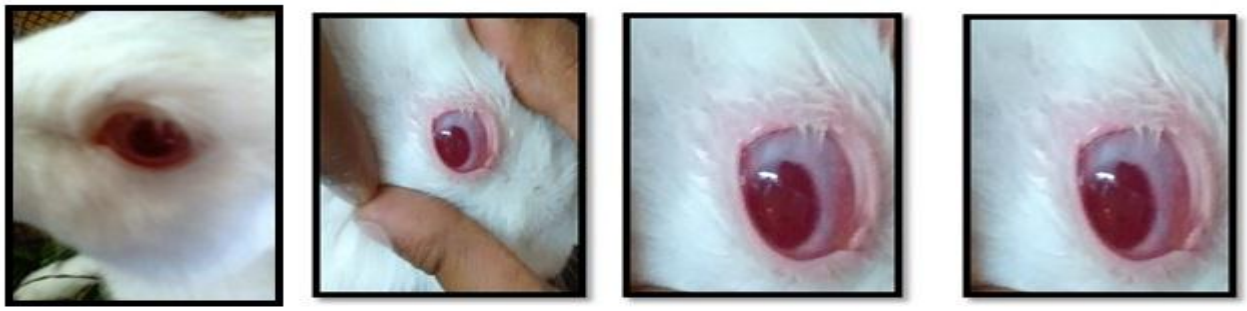
Fig 10. Antimicrobial activity against *Candida Albicans* for optimized NF8 and marketed formulation

Fig 11. Ocular Irritancy test for optimized formulation NF8



After 1hour | After 24hour | After 7 days

Fig 12. Efficacy against induced fungal keratitis



Infection induced eye | control eye | After 5 days treatment with NF8 | After 8 days treatment with marketed formulation

CONCLUSION

From the experimental results it can be concluded that: -

- IR spectroscopy studies of Natamycin and physical mixture of polymers revealed that, Natamycin is compatible with all the polymers used.
- Ophthalmic *In-situ* gelling system of Natamycin was successfully formulated using Carbopol 940 pH-sensitive polymer as gelling agents, along with HPMC K4M and HPMC E50LV as viscosity enhancing agent.
- The clarity of the prepared formulations was found satisfactory.
- The pH of all formulations was found to be satisfactory in the range of 7 - 7.5.
- The drug content of the prepared formulation was within the acceptable range, and ensures dose uniformity. The formulation NF8 showed maximum drug content.
- Formulation NF3, NF4, NF8 and NF9 showed immediate gelation and remained for extended period of time when contacted with simulated tear fluid (STF).
- It was observed that with increase in pH there was drastic increase in viscosity. All the formulation exhibited Pseudo plastic behavior i.e. with increase in shear rate they showed decrease in viscosity.
- Formulation NF4, NF8 and NF9 showed sustained drug release for a period of 6 hour. Formulation NF8 showed most sustained drug release.
- The results of *In-vivo* release studies revealed that, all the hydrogel formulations showed better sustained drug release when compared with conventional eye drops.
- The results of the ocular irritation studies indicate that the optimised formulations NF8 was non-irritant and excellent ocular tolerance was noticed.
- The short term stability studies indicated decrease in the drug content on storage at room temperature. Thus it was concluded that $4^0 \pm 1^0$C is appropriate storage condition for the formulations.
- Present work was a satisfactory preliminary study in developing *In-situ* gelling system of Natamycin. Further detailed investigations needed towards the optimization of concentration of gelling and viscofying agent to formulate the *in-situ* gelling system for ophthalmic delivery. The *in-vitro-in-vivo* correlation need to be established to guarantee the bioavailability of prepared formulations.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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