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Short communication

Development and *in vitro*, *ex vivo*, *in vivo* investigation of curcumin loaded nanoparticles in management of dry eye disease

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ABSTRACT

Dry eye disease is the most common ocular complication so far. The present treatment of DED is associated with a high frequency of administration, which affects patient compliance. We have formulated the curcumin-loaded nanoparticles by using HPMC E15 and Poloxamer 407 to target the peroxisome proliferator-activated receptor-γ (PPAR- γ). Nanoparticles were optimized by using a 32-factorial design to understand the impact of concentration of polymer (Poloxamer) and high pressure homogenizer (HPH) cycles on particle size, polydispersity index (PDI), and percent entrapment efficiency (%EE). Nanoparticles of particle size 170.2 nm, 0.341 PDI, and 90% EE with desirability 1 were selected as the optimized batch. The nanoparticles were further characterized for zeta potential, XRD, TEM, etc. In vitro drug release studies showed more uniform and sustained drug release (64.23 ± 3.81% CDR at 10 h). Histological study of formulation-treated goat cornea showed the non-irritating potential of formulation (*ex vivo*). Fabricated nanoparticles showed promising results in DED while investigating on mice.

1. Introduction

The dry eye disease (DED), also popularly called the dry eye syndrome, is the most common multifactorial ocular complication associated with a lack of tear secretion and/or rapid evaporation [1–4]. As per the latest survey, globally, 300 million people experience the DED, of which females are more prone than males [5,6]. The prevalence of DED increases with age (>50). The young population has also entered the DED zone due to increased screen time and long-term usage of a few medications, including antihistamines, nasal decongestants, blood pressure medications, birth control pills, antidepressants, and contact lenses [3,7]. Current treatment of DED includes the instillation of artificial tears, which need to be administered frequently (every 30 min), or NSAIDs, cyclosporine, and corticosteroids, which lead to systemic side effects. To improve DED, it is necessary to establish some alternative treatments.

Through our former investigation, we confirmed the involvement of the PPAR-gamma receptor in the management of DED [7,8] by using pioglitazone-PLGA nanoparticle-loaded gel prepared by using Poloxamer-HPMC and Carbopol-HPMC. The goal of the current study is to create curcumin nanoparticles for the treatment of DED by utilizing HPMC and Poloxamer. Curcumin is a PPAR-gamma agonist obtained from Curcuma longa. It has proven useful in the treatment of inflammation, which is one of the major symptoms associated with the DED. Additionally, activation of PPAR-gamma receptors also leads to increased tear secretion and inhibition of NO formation, which affects the normal function of the tear gland [9]. However, poor solubility and bioavailability have rendered the involvement of curcumin in DED unnecessary. This problem can be overcome by formulating the nanoparticles. HPMC is the safest, biodegradable, and biocompatible

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Table 1
Optimization of nanoparticles with Coded levels and translation in actual units along with responses.

Run	FC	Coded levels of variables		Particle Size (nm) Y1	PDI Y2	%EE Y3
		Factor X_1 (Conc. Of Poloxamer 407 mg)	Factor X_2 (HPH Cycles)			
1	F1	60(-1)	10(-1)	194.1 ± 2	0.317 ± 0.03	85 ± 2
2	F2	60(-1)	12(-1)	163.5 ± 5	0.301 ± 0.06	85.7 ± 1
3	F3	60(-1)	14(-1)	138.01 ± 7	0.297 ± 0.08	88.5 ± 3
4	F4	80(0)	10(0)	201.6 ± 3	0.360 ± 0.05	89.01 ± 2
5	F5	80(0)	12(0)	170.2 ± 2	0.341 ± 0.06	90 ± 3
6	F6	80(0)	14(0)	148.01 ± 3	0.321 ± 0.07	90.03 ± 1
7	F7	100(+1)	10(+1)	281.8 ± 4	0.605 ± 0.08	91 ± 2
8	F8	100(+1)	12(+1)	260.01 ± 3	0.581 ± 0.03	92.30 ± 3
9	F9	100(+1)	14(+1)	247.2 ± 5	0.523 ± 0.06	95.6 ± 1

Where; −1: Low, 0: middle; +1: High.

polymer. It is inert, mucoadhesive, and possesses good encapsulation capacity along with sustained release ability. It becomes viscous when it comes into contact with water. Several researchers showed an improvement in the ocular bioavailability of drugs by using HPMC. Both HPMC and Poloxamer serve as lubricants [8].

All these properties increased our attention as we formulated the nanoparticles of curcumin using HPMC E15 and Poloxamer 407. For optimization of nanoparticle preparation, a 3^2-factorial design was used. Nanoparticles were characterized by *in vitro*, *ex vivo* and *in vivo* parameters.

2. Materials and methods

2.1. Materials

Curcumin, HPMC E15 and Poloxamer 407 were obtained from the Reve Pharma Pvt. Ltd. (India), S.D. Fine Chemicals (India) and BASF Ltd. (India) respectively.

2.2. Fabrication of nanoparticles

Curcumin loaded nanoparticles were prepared by using precipitation method. Curcumin (100 mg) was dissolved in acetone and added gradually in aqueous phase (under the probe sonicator) made up of HPMC E15 (100 mg), Poloxamer 407 and SLS (5 mg). The dispersion was then subjected to a high pressure homogenizer i.e. HPH (ShearJet HL 60, Dyhydromatic), with varying cycles and a fixed pressure of 15 kpsi. Particle size and Polydispersity Index were assessed for each batch using a Zetasizer Nano ZS 90 (Malvern Ltd., UK). Furthermore, all prepared batches were heated on a magnetic stirrer for an overnight period to allow the organic solvent to evaporate. The obtained suspension was then run through a 0.45 μm Millipore membrane filter (Bedford, Massachusetts, USA). All filtrates were cold centrifuged and supernatant was used to calculate entrapment efficiency [10,11].

2.3. Optimization and evaluation

The independent variables viz. the concentration of Poloxamer:X1 and number of HPH cycles:X2 were optimized by using 3^2 factorial design by evaluating at three levels as shown in Table 1. The particle size (Y1), PDI (Y2) and percent entrapment efficiency i.e. %EE (Y3) were selected as the responses. The average particle size and size distribution determines the stability, efficacy as well as the compatibility with ocular site. Smaller particles (below 200 nm) are more compatible with eye. PDI below 0.4 is associated with the stability. Entrapment efficiency was determined by spectrophotometrical analysis of the supernatant (obtained after the cold centrifugation at 429 nm. The %EE was calculated by using formula 1. The obtained data was analyzed statistically by using ANOVA after addition of resulting data into Design Expert software (v13). The desirability search approach was used to finalize the optimized batch. Optimized batch further was spray dried and used for further evaluation.

The optimized batch was evaluated for zeta potential (Zetasizer) which is measurer of the charge on the colloidal particle and its magnitude. The spray-dried nanoparticles were assessed for percent drug loading efficiency and product yield by using formula 2 and 3. The morphology and size of fabricated nanoparticles were further confirmed by using TEM analysis. Prepared nanoparticles were also subjected for comparative XRD study. The diffractogram of drug and nanoparticles was captured with a Brucker AXS D8 Advance at 2 θ angle ranges between 3 and 50°.

$$E.E.\ (\%) = \frac{Total\ amount\ of\ the\ drug - Amount\ of\ the\ free\ drug}{Total\ amount\ of\ the\ drug} \times 100 \quad (1)$$

$$DL\ (\%) = \frac{Total\ amount\ of\ the\ drug - Amount\ of\ the\ free\ drug}{\text{Weight of spray dried nanoparticles}} \times 100 \quad (2)$$

$$\%\ Yield = \frac{Total\ weight\ of\ spary\ dried\ nanoparticles}{Drug + polymer\ weight + dispersing\ agent} \times 100 \quad (3)$$

The *in vitro* drug release study of optimized nanoparticles was carried out for 10 h by using phosphate buffer (pH 7.4) for 10 h. Dialysis membrane was soaked in dissolution medium for 8 h and one of the open end was sealed carefully. Through the remaining open end, 1 ml formulation (2 mg/ml) was transferred. The sac was suspended the beaker containing 100 ml dissolution medium after the tight sealing of open end of sac. The whole system was positioned on magnetic agitator at a temperature of 37 ± 0.50 °C and 50 rpm. At the time interval of 1 h, 5 ml aliquot was pipetted and analyzed at 429 nm on UV spectrophotometer to calculate percent cumulative drug release. The sink condition was maintained throughout the experiment [12].

The histological investigation of goat eye cornea (*ex vivo*) was carried out to confirm ocular irritation potential as per the procedure described by Jain GK et al. (2011). Eyes of a goat that had just been sacrificed were collected from the local slaughter house and cornea was isolated. Corneas were incubated for 300 min at 37 ± 0.5 °C in colloidal dispersion, saline solution: negative control and 0.1% w/w Sodium dodecylsulfate solution: positive control. After being cleaned with saline solution, the corneas were preserved in formalin (8% w/w). Corneas were placed in melted paraffin after the dehydration in alcohol gradient. Tissue blocks were formed and cross-sections were cut. Corneal structure was observed under the motic microscope followed by staining with haematoxylin and eosin [13].

The Schirmer's test on Swiss albino mice (*in vivo*) was performed to check the efficacy of prepared formulation in DED after the approval by CPCSEA, New Delhi. The comparative study was performed by forming 4 groups as Group I: Control, Group II: untreated, Group III: Treatment (3 mg/ml) and Group IV: Standard (cyclosporine eye drop 5 mg/ml) each having n = 6. To induce DED, 0.2 mg/ml benzalkonium chloride (BAC) solution was administered topically (twice a day for 7 days) in all groups except group I. Treatment was performed by administration of drop of assigned formulation at the time interval of 3 h after confirmation of DED by Schirmer's Test. Effectiveness of formulation was checked by measurement of tear secretion by placing a strip under the lower lid of eye [8].

Table 2
Summary of results of regression analysis and ANOVA for measured responses.

Responses	Model	R^2	SS	DF	MS	F value	P value	Model Significance
Y1	Quadratic	0.9989	3364.88	2	1682.44	206.61	0.0006	Significant
Y2	Quadratic	0.9982	0.0187	2	0.0094	119.25	0.0014	Significant
Y3	Linear	0.9411	78.54	2	39.27	47.91	0.0002	Significant

Where; Y1: Particle size; Y2: PDI; Y3: %EE; SS: Sum of Square; DF: Degree of freedom; MS: mean square; P: probability.

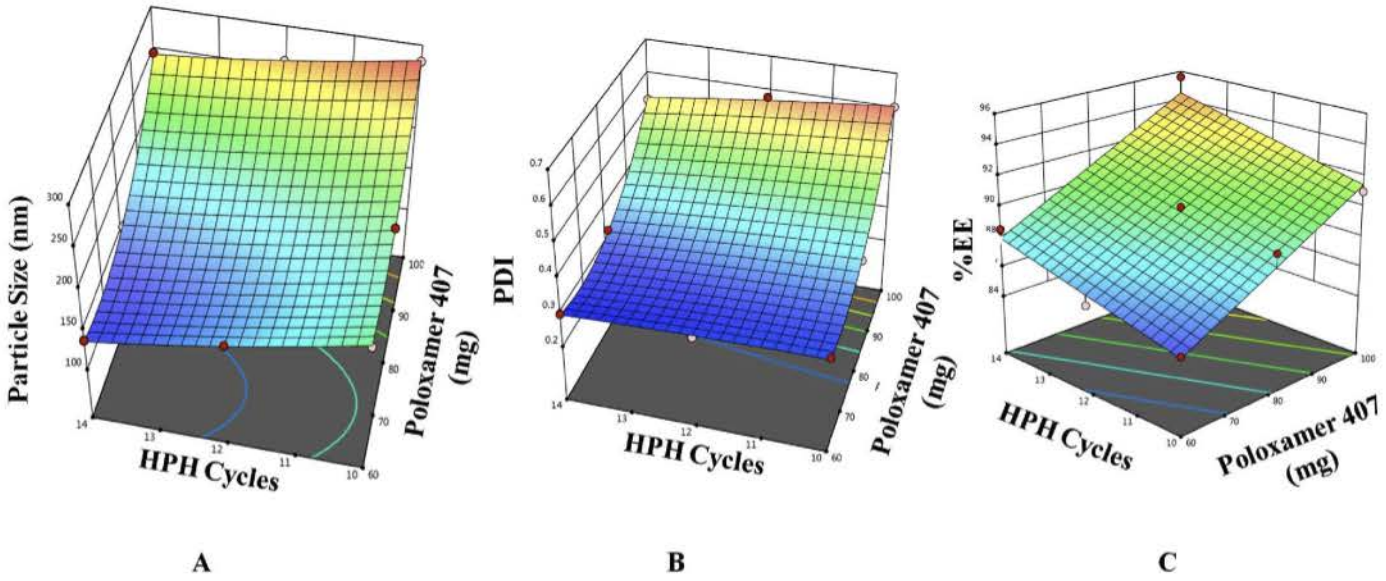

Fig. 1. Three dimensional (3 D) response surface plot for response A) Particle Size B) PDI and C) %EE.

3. Result and discussion

Curcumin loaded nanoparticles were prepared by precipitation method. A 3^2 factorial design was applied to optimized the concentration of Poloxamer 407 and HPH cycles against the Particle Size (Y1), PDI (Y2) and %EE (Y3) as the dependent variables. Responses Y1, Y2 and Y3 were found to be in the range of 148.01 ± 3 to 281.8 ± 4, 0.297 ± 0.08 to 0.605 ± 0.08 and 85.0 ± 2 to 95.6 ± 1% respectively (Table 1). The statistically analyzed data is summarized in Table 2. As the value of 'p' is below 0.05, it confirms the significance of model for selected parameters.

The software suggested following polynomial equations for the responses;

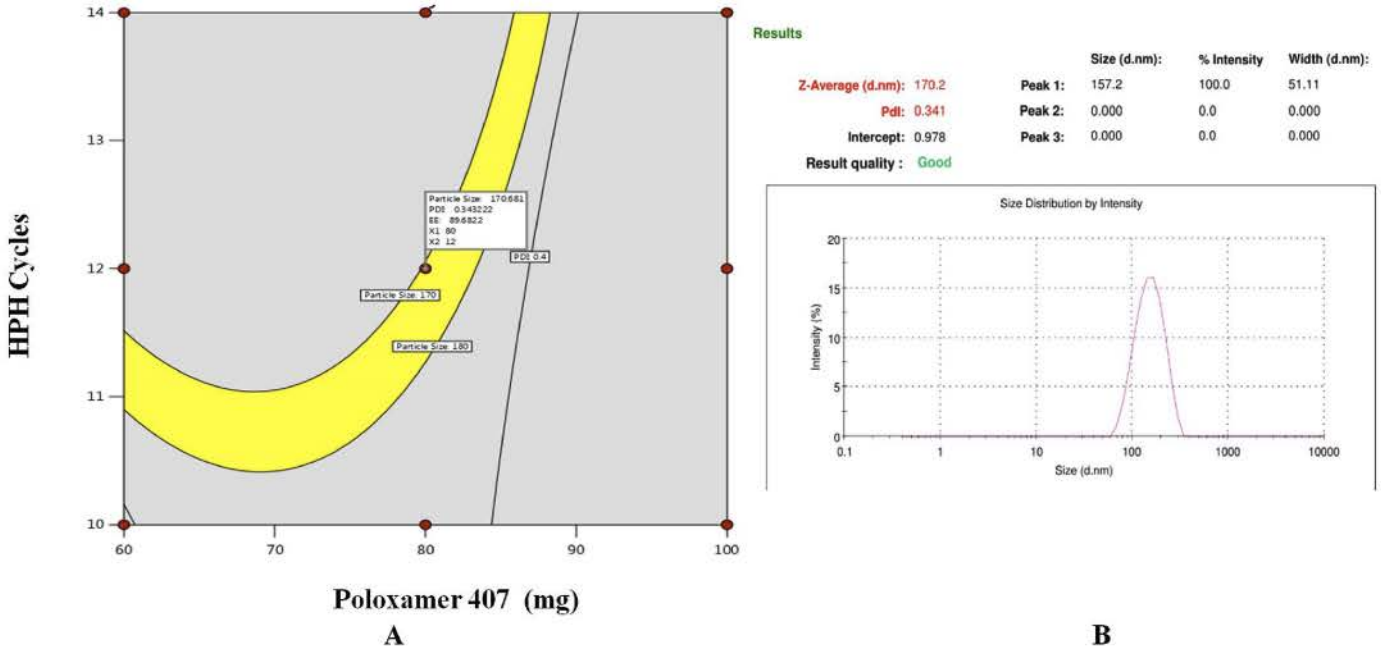

Fig. 2. A) Desirability plot B) Particle size and PDI.

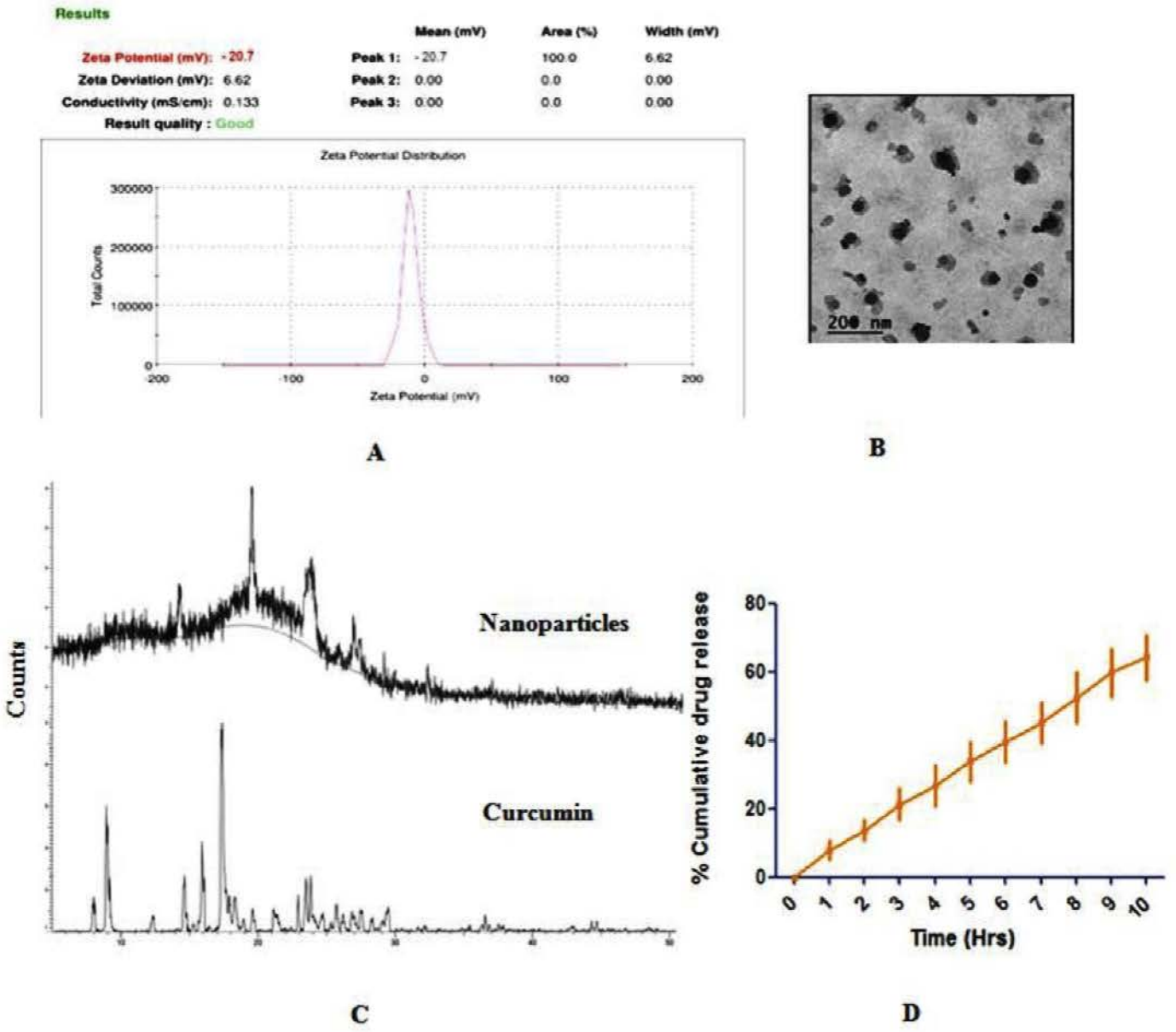

Fig. 3. A) Zeta potential, B) TEM analysis C) Comparative X-ray diffractogram, D) *In vitro* drug release study.

$$Y1: 170.68 + 48.90X1 - 24.05X2 + 5.37\ X1X2 + 40.83 \times 1^2 + 3.88 \times 2^2 \quad (1)$$

$$Y2: 0.3432 + 0.1323X1 - 0.0235X2 - 0.0155X1X2 + 0.0967 \times 1^2 - 0.0038 \times 2^2 \quad (2)$$

$$Y3: 89.68 + 3.28X1 + 1.52X2 \quad (3)$$

The Poloxamer concentration showed positive impact on the all responses (Eqs. (1)–(3)). As the concentration of Poloxamer increases the particle size as well as PDI also increases which might be because of increase in viscosity of system which reduces the impact of shearing because of HPH. With the increasing concentration of polymer, the entrapment of drug also increases as the high concentration of polymer leads to maximum encapsulation of drug. Similar results were observed by Kakad et al., 2021 [11]. On the other hand HPH cycles showed negative impact on particle size as well as PDI and positive impact on % EE (Eqs. (1)–(3)). As the cycles increases, shearing of material increases which reduces the particle size as well as PDI and at the same time increases the loading of drug in polymeric system [10,12]. The effect of independent variables on responses further can be correlated by 3D response surface plots as shown in Fig. 1. An optimized batch was selected by desirability search approach. Particle size below 200 nm, PDI near to 0.3 and good entrapment efficiency were the selection parameters. Impact on drug release was also considered at the time of selection of optimized batch. Batch F5, having 170.2 nm particle size, 0.341 PDI and 90% entrapment efficiency having desirability 1 was selected as the optimized batch (Fig. 2).

The charges on colloidal particles and its magnitude which measured in terms of zeta potential, plays critical role in the stability of the system. It should be ±20 mV and above to maintain nano particles in Brownian motion [12]. Optimized batch showed zeta potential −20.7 mV (Fig. 3-A). As particle size is below 200 nm, PDI is near to 0.3 and zeta potential is above the −20 mV, it clearly indicates the stability and suitability of fabricated nanoparticles for ocular administration. Spray dried nanoparticles showed the 8.12% drug loading efficiency, whereas some material loss was observed while calculating the product yield (71.1%) due to spray drying process.

The surface morphology and the particle size was confirmed by performing TEM analysis. The average particle size was observed as

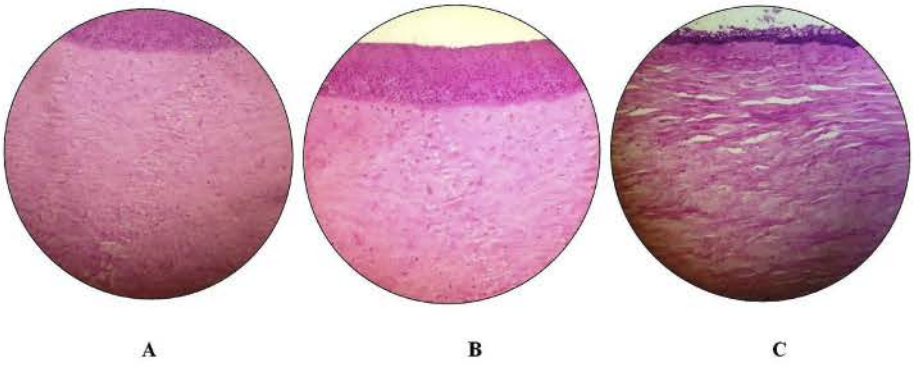

Fig. 4. Histological section of goat eye cornea A) negative control, B) formulation treated cornea, C) positive control.

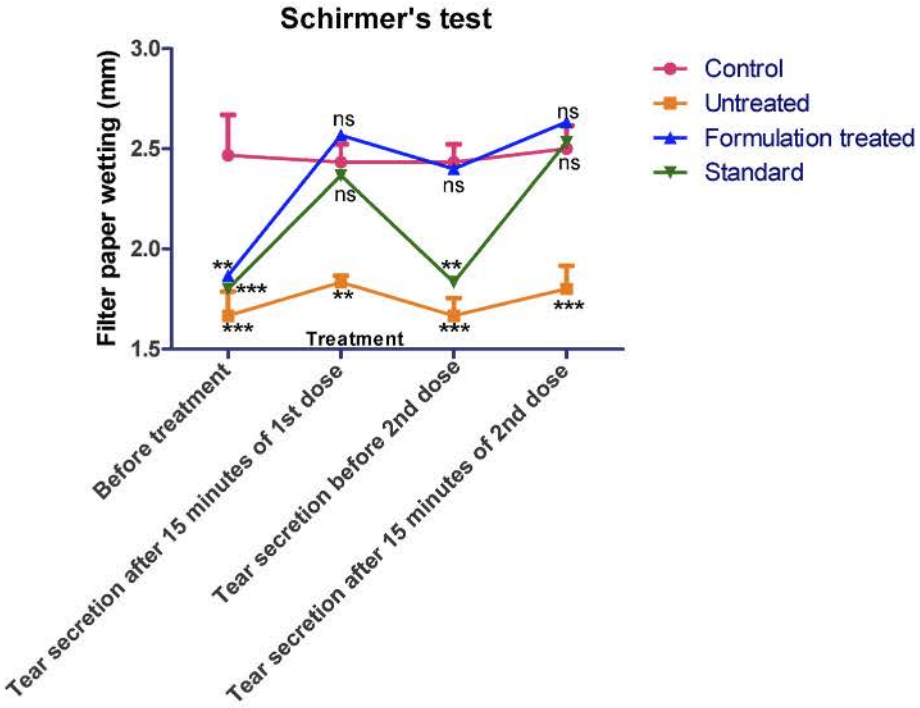

Fig. 5. Comparative effect of curcumin nanoparticles on tear secretion in dry eye disease (n = 6; Mean ± SEM)
Data expressed as mean ± sem and analyzed by Two way analysis of Variance followed by Bonferroni's post test where *** P < 0.001, **P < 0.01 and ns = non-significant.

below 200 nm with the spherical shape (Fig. 3-B). This morphology is essential to prevent scratching effect to the corneal surface. Moreover; TEM analysis confirms the encapsulation of drug in polymeric shell. Fabricated nanoparticles were further subjected to the XRD study (Fig. 3-C). The sharp peaks of drug were vanished in the diffractogram of nanoparticles which confirms the molecular dispersion of drug. Results further substantiate the finding of TEM analysis. The curcumin nanoparticles were subjected for *in vitro* drug release study for 10 h. An optimized batch showed the 64.23 ± 3.81% cumulative drug release at the end of 10 h. More uniform and sustained drug release was noted as shown in Fig. 3-D which might be due to swelling of polymers (HPMC and Poloxamer) as when it come in contact with solvent.

Prior to preclinical investigation, the ocular irritation potential was confirmed by histological investigation of goat cornea after 5 h of incubation in formulation. The formulation treated cornea was found to be intact as like saline solution. (Fig. 4 A and B). In contrast, the corneal structure of the positive control (treated with SDS) was severely damaged along with significant sign of necrosis (Fig. 4-C). Results clearly indicate the non-irritating nature of prepared formulation which made it more suitable for further *in vivo* exposure.

Table 3
Summary of results of ANOVA by comparing each group with the control group.

Treatment phase	Tear secretion (Mean ± SEM)			
	Control	Untreated	Formulation	Standard
Before treatment	2.46 ± 0.20	1.67 ± 0.12***	1.86 ± 0.09**	1.80 ± 0.11***
Tear secretion after 15 min of 1st dose	2.43 ± 0.08	1.83 ± 0.03**	2.56 ± 0.03^{ns}	2.36 ± 0.09^{ns}
Tear secretion before 2nd dose	2.43 ± 0.09	1.67 ± 0.08***	2.40 ± 0.06^{ns}	1.83 ± 0.15**
Tear secretion after 15 min of 2nd dose	2.50 ± 0.11	1.80 ± 0.11***	2.63 ± 0.08^{ns}	2.53 ± 0.07^{ns}

where *** P < 0.001, **P < 0.01 and ns = non-significant.

The Schirmer's test on BAC induced DED in mice was performed to investigate effectiveness of prepared system. Initially, DED was confirmed after 7 days of BAC treatment (Fig. 5: before treatment). After confirmation of DED, treatment was initiated. The Schirmer's test was

performed 15 min after the first dose, 3 h after the first dose and 15 min after the second dose. Results showed tear secretion of formulation treated group quiet near to the control group throughout the treatment period in comparison to the standard (Fig. 5). For all groups the two-way ANOVA followed by Bonferroni post-test study was applied in which group II, III and IV was compared with the control. The statistical result is summarized in Table 3. Formulation treated group showed no significance difference in tear secretion in comparison to the control group after the administration of dose. Even the tear secretion was maintained before the second dose. On the other hand standard formulation showed dropdown in tear secretion before the administration of second dose. Results reveal the advantage of prepared formulation over the conventional treatment.

In comparison to our previous published work (even though drug is different), the current research is associated with some key advantages as; i) use of HPMC and Poloxamer make the system economical in comparison to previous work which involved the use of PLGA polymer and dispersion of it polymeric system prepared by either Carbopol or Poloxamer with HPMC, ii) the drug release is more in comparison to nano-particles loaded gel which is because of limited barriers that the drug needs to cross prior to entry in dissolution medium, iii) better zeta potential with the nanoparticles which eliminated the need of structured vehicle, iv) comparatively results are quite promising without use of gelling system.

The current findings demonstrate the usefulness of Curcumin nanoparticles prepared by Poloxamer 407 and HPMC E15 in treatment of dry eye disease. Prepared nanoparticles showed notable improvement in tear fluid secretion. Fabricated nanoparticles showed adequate results when characterized for different parameters. Henceforth; use of curcumin nanoparticles prepared by HPMC and Poloxamer can be consider as the feasible alternative to the current treatment.

Author statement

Umesh D. Laddha: Project lead, supervision, conceptualization, methodology, writing original draft, reviewing and editing, Project lead, supervision and project administration.

Shubham S. Chikhale: Methodology, writing, reviewing and editing.

Neelam L. Dashputre: Methodology, writing, reviewing and editing.

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Kailas K. Moravkar: Methodology, writing, reviewing and editing.

Declaration of competing interest

There is no any conflict of interest.

Data availability

Data will be made available on request.

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