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Evaluation of tropicamide-loaded tamarind seed xyloglucan nanoaggregates for ophthalmic delivery

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ABSTRACT

The present study was aimed to prepare tamarind seed nanoaggregates and its evaluation for ophthalmic delivery. The preparation of tropicamide-loaded tamarind seed xyloglucan nanoaggregates was optimized using face centred central composite experimental design, employing the concentrations of tamarind seed xyloglucan and Poloxamer-407, as independent variables. The results revealed that concentration of TSX has a significant antagonistic effect on particle size, while poloxamer displayed a significant synergistic effect on encapsulation efficiency. The optimal concentrations of TSX and poloxamer were found to be 0.45% (w/v) and 0.5% (w/v) respectively. The optimized formulation of tropicamide-loaded TSX nanoaggregates showed a significantly higher corneal permeation of tropicamide across the isolated goat cornea compared to commercial conventional aqueous formulation. The results revealed excellent mucoadhesive properties of TSX nanoaggregates. Further, the tropicamide-loaded TSX nanoaggregates formulation showed excellent ocular tolerance and biocompatibility as determined by hen's egg test chorioallantoic membrane and resazurin assay on Vero cell lines.

1. Introduction

Polysaccharides are composed of repetitive monosaccharide units linked *via* glycosidic linkages having a highest capacity for carrying biological information. Natural polysaccharides are the most abundant and cheap biomolecules in nature. Most of the polysaccharides are exogenous metabolites of bacteria out of which few are the product of total biochemical synthesis and some of them are modified by partial organic synthesis. They represent one of the most abundant industrial raw material due to their sustainability, biodegradability and biosafety. Their diverse properties and non-toxic nature make them suitable for use in pharmaceutical applications.

Xyloglucans are linear polysaccharides present widely in cell wall of higher plants. Viscosity of xyloglucan depends upon its molecular weight, the presence of glycosyl and non glycosyl subunits and their backbone (Gidley et al., 1991). Xyloglucans are present in many plants but most of the plants contain only structural xyloglucans, whereas storage xyloglucans are rarely present in plants. Storage xyloglucans differ in their molecular mass, substitution levels and distribution. Tamarind is the most common plant for storage xyloglucans (Hayashi, 1989). Tamarind seed xyloglucan (TSX) obtained from the trees of *Tamarindus indica* Linn is a non-ionic and neutral polysaccharide. TSX is a high molecular weight (720–880 kDa) galactoxyloglucan comprising of glucose:xylose:galactose:arabinose in the ratio of 4:3.4:1.5:0.3 (Freitas et al., 2005). TSX consist of β (1→4) linked glucan backbone chain which is partially substituted with α-D-xylose at O-6 position. Some of these xylose residues are substituted with β (1→2) linked galactosyl units at O-2 position. The xylose units are reported to be more hydrophobic than galactose and glucose units, as a result of the presence of these hydrophilic and hydrophobic groups the xyloglucan chain shows substantial stiffness (Buckeridge, Rocha, Reid, & Dietrich, 1992). Due to the balancing of hydrophilic and hydrophobic character xyloglucan shows solubility in water but the individual macromolecules do not hydrate fully leading to the presence of aggregated species in water. These species are present even in very dilute solutions. Thus aqueous solutions of xyloglucan show a structure-function relationship. The random coil overlap and entanglement of polymer backbone depend on the concentration of xyloglucan and its intrinsic viscosity respectively. The random-coil structure of xyloglucan is formed at higher concentrations in aqueous solutions (Morris, Cutler, Ross-Murphy, Rees, & Price, 1981).

Xyloglucan present in TSP is permitted for the use as food additive, thickening, stabilizing and gelling agent in the food industry (Glicksman, 1986). TSP has also been explored for potential commercial applications in pharmaceutical industry for controlled

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drug release (Sumathi & Alok, 2002). During earlier studies the xyloglucan nanoaggregates were loaded with anticancer drug camptothecin and were found to release the drug by first order kinetics (Tatiane, Petri, Denise, Lucyszyn, & Sierakowski, 2010).

Tropicamide is poorly water soluble antimuscarinic drug. It is commonly used for producing mydriasis with its aqueous solution (1%, w/v) during eye surgery and dilated funduscopic examination. It works by blocking the muscarinic receptors of the eye thereby controlling the pupil size and lens shape (Behar-Cohen, 2004; Blessel, Rudy, & Senkowski, 1974; Dey & Mitra, 2005; Duvvuri, Majumdar, & Mitra, 2003; Koevary, 2003). It is weakly basic drug. To increase its solubility its solutions are buffered to acidic pH (5.0). But this increases its irritation potential and lowers its bioavailability due to induced lacrimation.

In the present study formulation of tropicamide-loaded tamarind seed xyloglucan (TSX) nanoaggregates was optimized by Central Composite Design using two independent variables. The optimized batch of tropicamide-loaded xyloglucan nanoaggregates was employed for preparing ophthalmic formulation of tropicamide (1%, w/v). Tropicamide-loaded ophthalmic nanosuspension was evaluated comparatively with the commercial conventional aqueous formulation of tropicamide for *in vitro* corneal permeation. Further, the ocular tolerance and biocompatibility of nanoformulation was studied using HET-CAM study and resazurin assay, respectively. Mucoadhesive properties of the formulation were studied using mucin glycoprotein assay.

2. Experimental

2.1. Materials

Tamarind kernel powder (TKP), Poloxamer-407 and tropicamide were obtained as gift samples from Hindustan Gums and Chemicals Pvt. Ltd. (Bhiwani, India), Jubliant Phamaceuticals (Noida, India) and Optica Pharmaceuticals (Yamunanagar, India), respectively. Mucin, Schiff reagent and periodic acid were purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India). Tropicacyl® (Sunways Pvt. Ltd., Mumbai, India) was purchased from local pharmacy, Hisar. Vero cell lines were procured from National Research Centre of Equines (Hisar, India). Freshly excised goat eye was obtained from the local butcher shop (Hisar, India). Ten-day old fertilized eggs were obtained as gift samples from Indovax Pvt. Ltd. (Hisar, India). All other chemicals were of analytical grade and were used as such.

2.2. Extraction of TSX

TSX was isolated from TKP which is soluble in water as reported earlier (Rao, Ghosh, & Krishna, 1946). In brief, TKP was dissolved in cold distilled water, which was then added to 400 ml of boiling distilled water under magnetic stirring and further boiled for 20 min. The resulting suspension was kept overnight to allow settling of fibres and proteins. The above viscous solution was centrifuged at 6000 rpm (Cooling centrifuge, 4K-15, Sigma, Germany) for 20 min. The supernatant so obtained was added to twice the volume of ethanol under continuous stirring. The precipitate so obtained was lyophilized in laboratory freeze dryer (Alpha 2-4-LD Plus, Martin Christ, Germany) for 24 h at −90 °C, at 0.0010 m bar.

2.3. Optimization of formulation of tropicamide-loaded TSX nanoaggregates

A central composite design with $\alpha = 1$ was employed as per the standard protocol. The two factors, concentration of TSX and Poloxamer-407 were varied and the factor levels were suitably coded. Particle size and encapsulation efficiency were taken as response variables. Throughout the study all other processing variables were kept invariant. The present investigation involves evaluation of two factors each at three levels. In all, 13 experimental runs were carried out as shown in Table 1. The central point (0,0) was studied in pentet. All the experiments were carried out using systematic design of experiments employing Design Expert Software (Version 8.0.4, Stat-Ease Inc., Minneapolis, MN).

Table 1
Central composite design used to study effect of formulation variables on particle size (Y_1) and % encapsulation efficiency (Y_2).

S. no.	TSP (%) (X_1)	Poloxomer (%) (X_2)	Particle size (nm) (Y_1)	Encapsulation efficiency (%) (Y_2)
1	0.55	0.50	678.5	86.57
2	1.00	0.00	1260.3	36.78
3	0.55	0.50	580.2	83.23
4	1.00	1.00	745.6	88.62
5	0.10	0.50	390.8	67.89
6	0.10	1.00	445.6	64.32
7	0.55	0.50	698.7	85.49
8	0.10	0.00	399.8	27.89
9	0.55	0.00	996.7	32.61
10	0.55	1.00	675.4	76.54
11	0.55	0.50	634.5	84.41
12	0.55	0.50	608.7	86.46
13	1.00	0.50	800.8	91.62

Briefly the TSX nanoaggregates were formulated by adding tropicamide (75%, w/v of TSP) to a dispersion of TSX (0.1–1%, w/v) and Poloxamer-407 (0–1%, w/v) under magnetic stirring. Nanosuspension thus obtained was analyzed for particle size. The optimized batch of formulation was freezed at −80 °C for 4 h followed by lyophilization in laboratory model freeze dryer (Alpha 2-4 LD Plus, Martin Christ, Germany) for 24 h at −90 °C, at 0.0010 mbar using mannitol (1%, w/v) as cryoprotectant.

2.4. Characterization of tropicamide-loaded TSX nanoggregates

2.4.1. Particle size analyzer

Photon correlation spectroscopy was used for particle size determination in a suspension. Particle size distribution was also inferred. The mean particle size of optimized tropicamide-loaded TSX nanoaggregates was analyzed at 25 °C. One millilitre of the nanosuspension was scanned with 11 runs in disposable cuvette with an equilibrium time of 120 s in particle size analyzer (Zetsaizer Nano ZS90, Malvern, UK).

2.4.2. Encapsulation efficiency

The encapsulation efficiency of nanoaggregates was determined by separating the nanoparticles from aqueous medium by centrifuging the suspension at 12,000 rpm for 30 min at 4 °C (Cooling centrifuge, 4K-15, Sigma, Germany). The amount of encapsulated tropicamide in the suspension was measured by dissolving the pellet formed after centrifugation in ethanol and was sonicated (Sonoplus Bandelin) for 1 min. The solution was then analyzed for the contents of tropicamide at 257 nm by UV–vis spectrophotometer (UV 2450, Shimadzu). Encapsulation efficiency (%) was calculated by the formula given below:

$$\%\ \text{Encapsulation efficiency} = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100 \quad (1)$$

2.4.3. Morphology

Transmission electron microscopic analysis (TEM) was done using Hitachi H7500 machine. The prepared nanosuspension was dropped onto carbon coated copper grid, extra solution was removed using a blotting paper. The grid was allowed to dry for 5 min and loaded in the goniometer. The TEM micrograph was taken by applying accelerating voltage of 80 kV.

2.5. Formulation of tropicamide (1%, w/v) opthalmic nanosuspension

Required amount of the optimized lyophilized tropicamide-loaded TSX nanoaggregate powder was dispersed in Sorenson phosphate buffer (pH 7.2) and made isotonic by adding the required amounts of mannitol as tonicity modifier.

2.6. In vitro corneal permeation study

The *in vitro* corneal permeability of tropicamide-loaded TSP xyloglucan nanoaggregates was comparatively evaluated with commercial eye drop Tropicacyl® using isolated goat cornea. The study was conducted using paired corneas *i.e.* permeation of nanoformulation was studied using one cornea while contralateral cornea was used for studying permeation characteristics of marketed formulation. Whole eye ball of goat was obtained from local butcher house and corneal tissue was carefully excised along with 2–4 mm of the sclera. The excised cornea was then washed with cold normal saline to remove the proteins. Isolated cornea was mounted by sandwiching between donor and receptor compartments of the modified Franz diffusion cell in such a way that its epithelial layer faced the donor compartment. The receptor solution comprised of Ringer's bicarbonate solution (11 ml) and was maintained at 37 °C by continuous flow of water through the water basket from thermostatic water bath. The donor compartment was filled with 1 ml of the nanoformulation or marketed formulation. At the end of 2 h, 3 ml aliquot of the sample was withdrawn from the receptor compartment and the contents of tropicamide were analyzed spectrophotometrically at 257 nm. At the end of the experiment the scleral tissue was removed completely from the cornea which was then weighed and dehydrated by soaking in methanol for 24 h followed by drying in an oven at 90 °C and weighing again to calculate the % hydration (Reichl, Bednarz, & Muller-Goymann, 2004).

2.7. HET CAM study

HET CAM study is an alternative to the Draize *in vivo* rabbit eye test for the detection of ocular irritants (Luepke, 1985). The hen's egg chorioallontoic membrane bioassay was performed using 10 day fertilized eggs. Prior to use, the eggs were candled to detect the viability of the embryo. Tropicamide-loaded TSX nanoaggregates were comparatively evaluated with TSX solution and marketed formulation. Experiments were performed in triplicates using sodium chloride as a negative control and sodium hydroxide as a positive control. The CAM was treated with 500 μl of the sample and irritation levels were checked by observing for signs of irritation such as haemorrhage, lysis and coagulation at different time intervals upto 5 min. Potential irritation scores (PIS) were calculated by the formula given below:

$$\text{PIS} = \frac{(301-h)}{300} \times 5 + \frac{(301-l)}{300} \times 7 + \frac{(301-c)}{300} \times 9 \quad (2)$$

where h is the time in seconds when haemorrhage appears; l is the time in seconds when lysis appears; c is the time in seconds when coagulation appears.

2.8. Mucin glycoprotein assay

Mucoadhesiveness of optimized TSP xyloglucan nanoparticulate formulation was assessed by determining the amount of mucin adsorbed in 24 h. The optimized formulation of TSP xyloglucan was mixed with 1 mg/ml of mucin, votexed and incubated for 24 h at 37 °C. After adsorption, the solution was centrifuged at 10,000 rpm for 30 min. The supernatent so obtained was analyzed for free mucin by colorimetric method (Dhawan, Singla, & Sinha, 2004). Periodic acid/Schiff (PAS) staining was used for this assay. Periodic acid reagent was freshly prepared by diluting 10 μl of periodic acid (50%) to 7 ml by acetic acid (7%, v/v). 100 μl of the diluted periodic acid solution was incubated with 9 ml of supernatent at 37 °C for 2 h. After 2 h, 2 ml of Schiff's reagent was added and incubated for 30 min. The absorbance of the solution was measured at 560 nm. The mucin concentration was calculated using the calibration curve prepared using mucin standards by the same procedure.

2.9. Cytotoxicity screening

Tropicamide-loaded TSX nanoaggregates were comparatively screened for cytotoxicity with marketed formulation of tropicamide and TSX solution using resazurin assay. 200 μl of Vero cells with density of 1×10^6 were cultured in 96 well plate in Dulbeccos Modified Eagle Medium (DMEM) media containing 5% Foetal Bovine Serum (FBS). The experiment was performed in triplicates. Cells were allowed to incubate for proliferation at 37 °C in 5% CO_2 incubator for 24 h. The cells were further incubated for 24 h with 50 μl tropicamide-loaded TSX nanoaggregates, TSX and Tropicamide solution. After 24 h exposure of cell lines, 20 μl of resazurin (1 mg/ml) was added and the plate was incubated for 4 h in CO_2 incubator with 5% CO_2 at 37 °C for 4 h. The pink coloured resorufin produced by the reduction of resazurin by mitochondrial dehydrogenase enzyme was analyzed using ELISA plate reader at 573 nm. The % cytotoxicity was calculated by the given formula:

$$\%\ \text{Cytotoxicity} = \frac{Abs_u - Abs_t}{Abs_u} \times 100 \quad (3)$$

where Abs_u is the absorbance of cells not treated with any polymer, Abs_t is the absorbance of cells treated with tropicamide-loaded TSX nanoggregates, TSX and tropicamide solution.

3. Results and discussion

The present study was designed with the objective to optimize the concentration of TSX and poloxamer for preparing TSX nanoaggregates with minimum particle size and maximum encapsulation efficiency. TSX possesses excellent mucoadhesive properties as it has a backbone chain with branching of xylose and galactoxylose substituents which imparts it a mucin like configuration. Due to these properties it has been employed for providing sustained relief from symptoms of dry eye (Burgalassi, Panichi, Chetoni, Saettone, & Boldrini, 1999). Earlier it was reported that TSX promotes the corneal accumulation and intraocular penetration of gentamicin and ofloxacin when administered topically to healthy rabbits (Gangopadhyay, Daniell, Weih, & Taylor, 2000; Garg, Bansal, Sharma, & Vemuganti, 2001; Ghelardi et al., 2000). In addition, it has been established that TSX diminishes the effect of *in vitro* toxicity of timolol, methiolate and flouroquinolones in human conjuctival cells (Duvvuri et al., 2003). As tamarind seed xyloglucan is non-toxic in nature, increases corneal wound healing rate, increases corneal accumulation due to its mucomimetic effect and these impart a major role in ophthalmic applications. Physicochemical properties of TSX were studied earlier by Tatiane et al. who reported that TSX forms nanoaggregates with critical aggregation concentration (CAC) of 1.38 mg/ml. At a concentration 25% greater than its CAC it was loaded with a cytotoxic alkaloid which showed 42% encapsulation with sustained release of camptothecin over 24 h with first order kinetics (Tatiane et al., 2010).

The optimization of tropicamide-loaded TSX nanoaggregates were carried out using face centred central composite experimental design for estimating the extended effect of concentrations of TSP and poloxamer on two dependent variables particle size and encapsulation efficiency. The design consisting of 13 experiments

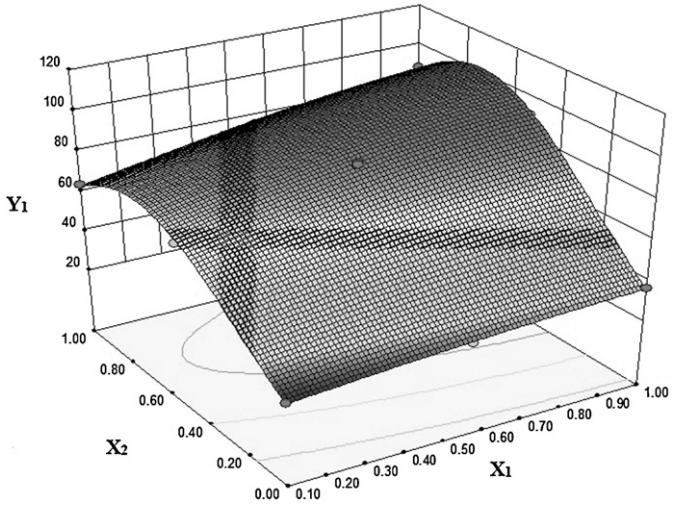

Fig. 1. Response surface plot showing combined effect of concentrations of TSX (X_1) and poloxamer (X_2) on particle size of nanoparticles.

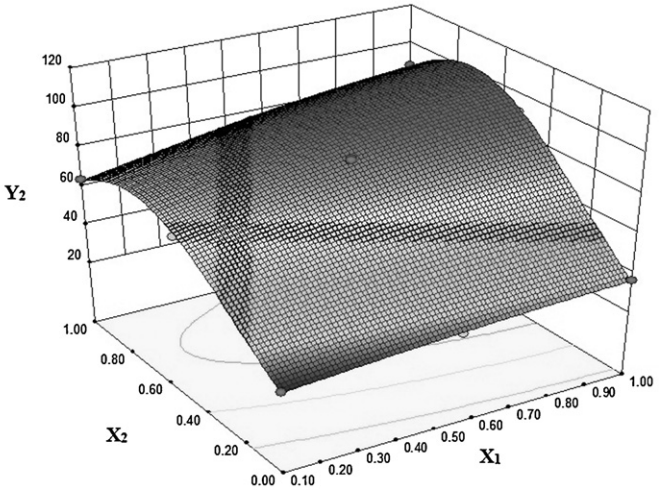

Fig. 2. Response surface plot showing combined effect of concentrations of TSX (X_1) and poloxamer (X_2) % encapsulation of drug within nanoparticles.

by two selected independent variables and corresponding data of all the experimental design is shown in Table 1.

The results of response generated by the CCD design were best fitted into the polynomial models and ANOVA test was applied to estimate the significance of the model. Both the responses particle size (Y_1) and encapsulation efficiency (Y_2) fitted best into the quadratic model with log transformation. The response of the polynomial models Y_1 and Y_2 can be represented by Eqs. (4) and (5).

$$Ln(Y_1) = 6.48 + 0.40 \times X_1 - 0.13 \times X_2 - 0.16 \times X_1 \times X_2 - 0.19 \times X_1^2 + 0.19X_2^2 \quad (4)$$

$$Ln(Y_2) = 4.44 + 0.15 \times X_1 + 0.43 \times X_2 + 0.011 \times X_1 \times X_2 - 0.048 \times X_1^2 - 0.50X_2^2 \quad (5)$$

The equations above show the coefficient of intercept, first order, second order and interaction term effects. Relative influence of each factor on the response is signified by the sign and magnitude of the main effects where negative sign and positive sign indicates the antagonistic and synergistic influences respectively.

Results of ANOVA test are represented in Table 2 which gives the details of the model summary statistics for the selected significant models. Both the responses are devoid of 'lack of fit'. It can be seen that there is a good correlation between the experimental and predicted responses as the value of 'R^2' is >0.9. Reliability of the model can be seen as the 'predicted R^2' is in a reasonable agreement with 'adjusted R^2'. Signal to noise ratio was measured by 'adequate precision'. The value of adequate precision is >4 thus indicating that model can be used to navigate the design space. Good precision and reliability of the experiment can be seen by lower values of coefficient of variation.

Fig. 1 depicts the three dimensional response surface plots constructed using the models generated by response surface methodology (RSM). It shows the combined effect of concentration of TSX xyloglucan (X_1) and poloxamer (X_2) on the response of particle size (Y_1) of nanoparticles. It can be observed at lower levels of poloxamer, increasing the concentration of TSX increases the particle size of TSX nanoaggregates. However, on increasing the concentration of poloxamer only a slight increase in particle size of nanoaggregates is seen. The increase in size of TSX with increase in concentration of TSX can be attributed to overlapping and entanglement of the xyloglucan coils but poloxamer, a non-ionic surfactant is used as a stabilizer in nanoformulation which prevents the entanglement and overlapping of these coils thereby inhibiting its aggregation (Khounvilay & Siltikijyothin, 2012). Thus, as a result on increasing the concentration of poloxamer, TSX concentration dependent increase in particle size is diminished.

Fig. 2 displays the response surface plot showing the combined effect of concentration of TSP xyloglucan (X_1) and poloxamer (X_2) on the % encapsulation efficiency (Y_2) of tropicamide in the nanoparticles. The 3D plot shows curvelinear relationship between the concentration of poloxamer and encapsulation efficiency. Further, it can be observed that the effect of poloxamer concentration on encapsulation efficiency is greater than the effect of TSX concentrations. Increase in the concentration of poloxamer increases the encapsulation efficiency which can be explained by the increase in stabilizing effect of poloxamer. Moreover, with increase in concentration of TSX there is slight increase in encapsulation efficiency which can be attributed to the greater entrapment of drug in the polymeric matrix.

A new formulation with desired responses was developed by the numerical optimization technique using desirability approach. The optimization was done with constraints for minimizing the particle size (Y_1) and maximizing the % encapsulation efficiency (Y_2). This tool provided us different sets of optimal solutions. Different experimental runs were carried out and their responses were recorded. The optimal calculated parameters were concentration of TSX 0.45%, w/v and concentration of poloxamer 0.5%, w/v. The optimized batch of tropicamide-loaded TSX nanoaggregates was found to have particle size (Y_1) of 639.8 (predicted 640.95) and % encapsulation efficiency (Y_2) of 96.57% (predicted 94.601%). The reliability of developed models is indicated by its lower % error (0.108% for particle size and 0.667% for encapsulation efficiency).

Morphology of the optimized batch of tropicamide-loaded TSX nanoaggregates was viewed under transmission electron microscopy as shown in Fig. 3. Since the prepared tropicamide-loaded TSX nanoaggregates are nanometric (10–20 nm) size and are observed to have a spherical or ovoid shape having no sharp edges thus they are not expected to cause any irritation in '*cul-de-sac*'. The particle size obtained from dynamic light scattering (DLS) was quite larger in comparison to TEM due to the fact that DLS measurements integrates the ionic environment surrounding the particle size whereas TEM analysis if focused on the particle itself (Gèze, Putaux, Choisnard, Jéhan, & Wouessidjewe, 2004).

Recently, TSP has been shown to be mucoadhesive by both *in vitro* and *in vivo* studies at the ocular surface of rabbits. It was also used for stabilizing the tear film and prolonging the retention

Table 2
Statistical summary of the quadratic response surface model.

Response factor	Model								Lack-of-fit	
	F value	Prob. > *F*	R^2	Adjus. R^2	Pred. R^2	Adeq. prec.	C.V	Std. dev.	*F* value	Prob. > *F*
Y_1	37.97	<0.0001	0.9644	0.9390	0.8122	22.780	1.28	0.083	1.41	0.3633
Y_2	793.84	<0.0001	0.9982	0.9970	0.9908	77.688	0.55	0.023	3.14	0.1489

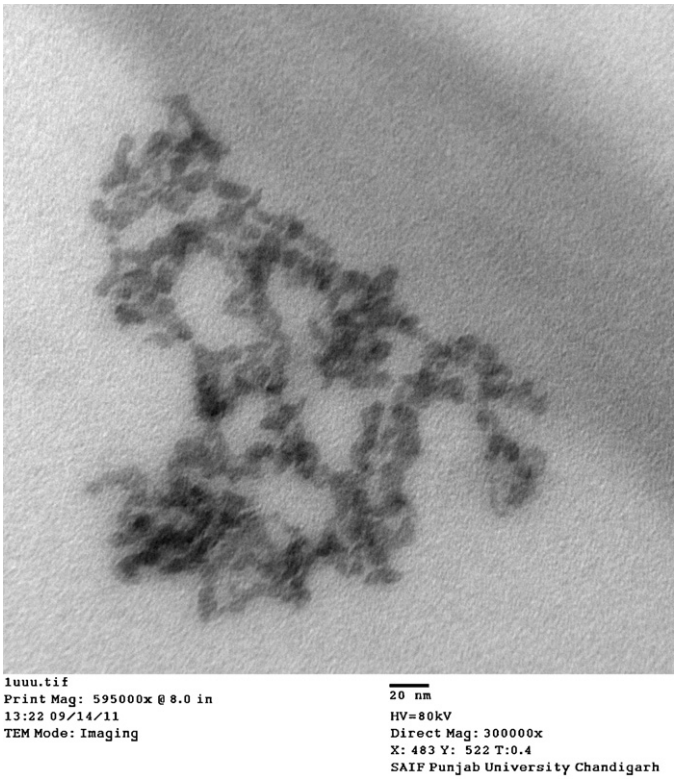

Fig. 3. TEM micrograph of tropicamide-loaded TSX nanoaggregates.

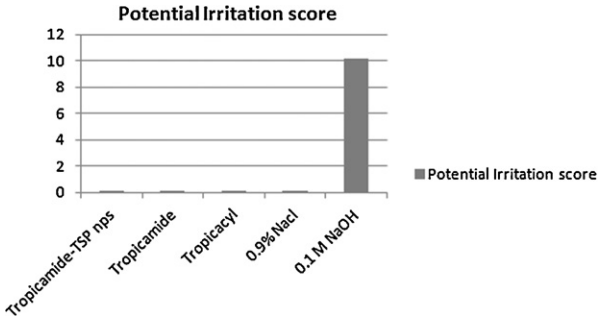

Fig. 4. Potential irritation score of various formulations tested by HET-CAM study.

of ophthalmic drugs of ketotifen fumarate and diclofenac sodium in precorneal area (Di Colo, Zambito, Zaino, & Sanso).

Table 3 shows the corneal permeation study conducted using nanoformulation and commercial formulation. To determine the ocular delivery potential of tropicamide-loaded TSX nanoaggregates for prediction of ocular drug absorption, the corneal permeation characteristics of nanoformulation was compared with that of marketed eye drop 'Tropicacyl®' using the *in vitro* goat cornea as a model. The corneal permeation study was conducted using paired corneas in order to minimize the biological variation. The results of the present study reveal that significantly higher amount of tropicamide permeated from nanosuspension formulation in comparison to marketed formulation. Earlier it was reported that drug-loaded nanoformulations provide higher corneal permeation due to endocytic uptake (Calvo, Vila-Jato, & Alanson, 1996; Gupta, Madan, Majumdar, & Maitra, 2000). The higher corneal permeation of tropicamide may be attributed to the endocytic uptake of nanoaggregates. In addition, the commercial formulation, which was used for comparative evaluation contained chlorbutol as a preservative. Chlorbutol is reported to enhance the corneal permeability. However, no preservative was added in the nanoformulation, even then it provided greater % permeation of tropicamide as compared to commercial formulation. Further, it is expected that tropicamide-loaded TSX nanoaggregates will provide higher corneal permeation in *in vivo* models, as nanoparticles would be lodged in the '*cul-de sac*' region of the eye, thereby providing sustained release of tropicamide over a prolonged period of time.

The corneal hydration levels are indicative of corneal integrity and normal corneal hydration levels are reported to be 75–80% (Maurice & Riley, 1970). The results of corneal hydration in the present study were found to be within limits thereby indicating that integrity of corneal epithelium and endothelium is not affected.

To evaluate the mucoadhesive potential of tropicamide-loaded TSX nanoparticles a colorimetric assay was performed. Because of the strong interaction between mucin and TSX, mucin is adsorbed to the surface of TSX nanoparticles. Before and after adsorption of mucin on TSP nanoparticles, the amount of mucin adsorbed was determined. The results reveal the 87.35% of the mucin was adsorbed on the surface of TSX nanoparticles. This shows excellent mucoadhesive properties of TSX nanoparticles.

Earlier reports suggest that there is a good correlation between HET-CAM study and *in vivo* Draize eye tests. HET-CAM study depends on individual interpretations of tissue reactions. Fig. 4 shows potential irritation scores. No irritation effects were observed in positive control (0.9% NaCl) and coagulation in negative control (0.1 M NaOH). The optimized formulation of tropicamide-loaded TSX nanoaggregates and Tropicacyl® were found to be non-irritant till 5 min of the study. Thus, nanoparticulate formulation shows excellent ocular tolerability.

Resazurin has been applied to nanotoxicological studies for measuring cellular redox potential. It has been said that Alamar Blue/resazurin is reduced in response to chemical reduction of the medium resulting from cell growth (Fields & Lancaster, 1993) or by mitochondrial enzymes (De Fries & Mistuhashi, 1995). Resazurin is

Table 3
Comparitive results on tropicamide-loaded TSX nanoaggregates permeation across excised goat cornea and % hydration.

Sample	% corneal permeation (mean ± S.D.) *n* = 3	% hydration (mean ± S.D.)
Tropicamide-loaded TSX nanoaggregates	0.661 ± 0.1482	78.25 ± 1.021
Tropicacyl	0.573 ± 0.1351	77.86 ± 0.8563

% Corneal permeation: *p* value = 0.0148 with significant model; % Hydration: *p* value = 0.7508 with non significant model.

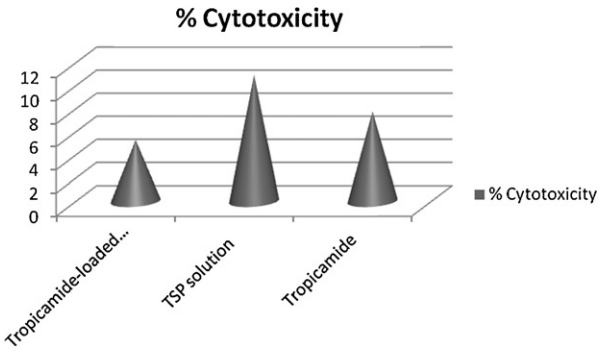

Fig. 5. Relative cytotoxic effects of tropicamide, TSX and tropicamide-loaded TSX nanoaggregates on Vero cell lines.

a cell viability indicator which uses natural reducing power of live cells for converting blue colour resazurin to fluorescent pink colour resorufin. Resazurin is non toxic compound which has the property to permeate through cells and is virtually non fluorescent. Fig. 5 compares the relative cytotoxic effects of tropicamide, TSX and tropicamide-loaded TSX nanoaggregates. Tropicamide-loaded TSX nanoaggregates showed highest % viability with 94.72% followed by tropicamide solution with 92.26% viability and least viability was observed in TSX with 89.09%.

4. Conclusion

Tropicamide-loaded TSX nanoaggregates were prepared and optimized by three-level, two factor CCD experimental design. The results attribute significant synergistic effect on two dependent variables particle size and encapsulation efficiency. Numerical optimization was used to develop an optimized formulation. The optimized formulation of tropicamide-loaded TSX nanoaggregates was prepared using 0.45% concentration of TSX and 0.55% concentration of poloxamer. The optimized nanoformulation was ovoid in shape causing no irritation in the eye. Moreover, tropicamide-loaded TSX nanoaggregates were found to have significantly higher corneal permeation across excised goat cornea in comparison to commercial formulation. The nanoaggregates were found to be less toxic and non irritant with greater mucoadhesive potential. However, *in vivo* studies are required further to validate the potential of tropicamide-loaded TSX nanoaggregates.

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