Carboxymethyl Modification of Tukhm-Malanga Polyuronide and its Evaluation for Nanoparticulate Delivery of Ofloxacin

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ABSTRACT

In this study, polyuronide was isolated from Lallementia royleana seeds, and its carboxymethyl modification was carried out by Williamsons' synthesis. The degree of carboxymethylation substitution was found to be 1.65. The outcomes of the characterization studies performed on carboxymethylated derivative pointed to the increased flow properties and elasticity, decreased swelling property, viscosity and anionic nature. The nanoparticulate suspension of modified polyuronide was prepared by its interaction with chitosan using ofloxacin as a model drug. The concentrations of modified polyuronide and chitosan were found to affect the particle size. The polyelectrolyte nanosuspension formulated using carboxymethylated polyuronide (0.02%,w/v) and chitosan (0.01%,w/v) showed particle size, polydispersity index, zeta potential and entrapment efficiency of 402.3 nm, 0.315, 22.3 mV and 96.50%, respectively was selected for in vitro release study. The selected batch of nanosuspension provided a sustained release of 87.10% of ofloxacin over a time period of 12 h following first-order kinetics with mechanism of release being diffusion through the matrix.

Keywords: *Lallementia royleana*, Carboxymehtylation, Polyuornide, Nanoparticles, Ofloxacin.

INTRODUCTION

Tukhm-malanga polyuronide (TP) is a high molecular weight polyuronide native to Asia (including Afghanistan, Iran and India) and in some regions of Europe [1]**.** Tukhm-malanga seeds which are obtained from an important medicinal herb *Lallemantia royleana* (Family: *Lamiaceae*) [2] are a good source of fiber and flavouring agent and can be taken as food additive because of swelling properties [1]. The seeds are black in color. It is also known as Balangu [3]. Tukhm-malanga is widely distributed in Asia, Africa, central and southern America [4] and is included in beverages and ice deserts [2]. Tukhm-malanga seeds in contact with water swells rapidly and shows a thick layer of polyuronide i.e., TP on their surface [4]. TP is an anionic polyuronide with glucomannan and possesses high viscosity and pseudoplastic behavior [2]. TP has already been evaluated as edible film forming agent [5], suspending agent [6], superdisintegrating agent [7], stabilizer [8], thickening agent [9, 10], dermal penetration enhancer and sustained drug release agent [3]. In food industry TP, can be used as stabilizer, fat replacer and crystal growth controller.

Tukhm-malanga seeds possess carbohydrates (45.25%), crude fiber (1.29%), ash (3.63%) oil (18.27%) and protein (25.60%) [1]. The carbohydrate present mainly are arabinose, galactose and rhamnose with little amount of xylose and glucose, mannose, fructose, glucuronic acid, galacturonic acid [1,10,11]. *Lallemantia royleana* seeds possess certain medicinal properties and are used for the relieving joint inflammation, rheumatism, joint pain, abscesses inflammations, osteoarthritis, Fever, common cold and as expectorant [12]. TP has various advantages over other polyuronides i.e., low cost, biocompatibility, biodegradability and hydrophilic nature. In current study, carboxymethylation of TP has been carried out by Williamson's synthesis and the degree of substitution was determined by acid wash method. The characterization of carboxymethylated Tukhm-malanga CTP was done by Fourier transform infrared spectroscopy (FT-IR), thermogravimetric analysis (TGA), X-ray diffraction and scanning electron microscopy (SEM) studies. Rheometer was used to study the rheology of CTP. The polyuronide was then investigated for its pharmaceutical usage by preparing nanoparticulate delivery system using ofloxacin sodium as a model drug. The CTP nanoparticles loaded with Ofloxacin were characterized by FT-IR and SEM and evaluated for particle size, yield %, entrapment efficiency and *in vitro* release behavior.

MATERIALS AND METHODS

Materials

Tukhm-malanga seeds were purchased from local market of Hisar, Haryana (India), and were authenticated as seeds of *Lallemantia royleana* Benth. by raw material, herbarium and museum division of CSIR-National Institute of Science Communication and Information Resources, (New Delhi, India) vide NISCAIR/RHMD/Consult/2017/3132-81-2, dated 26.12.2017. Ofloxacin was obtained as a gift sample from Ranbaxy Research Laboratories (Gurgaon, India). Acetone, calcium chloride, chitosan, glacial acetic acid, magnesium chloride and methanol were procured from Sisco Research Laboratory, (Mumbai, India). Monochloroacetic acid and dialysis membrane were purchased from Hi-Media Laboratories Pvt. Ltd., (Mumbai, India). All other chemicals used were of reagent grade.

Isolation of polyuronide

The polyuronide was extracted as per the procedure reported earlier with slight modifications [1]. Briefly, 10 g of seeds were soaked in 1L of purified water (pH-8.0) at temperature of $80 \pm 2°C$, under mechanical stirring for 2 h. The dispersion was filtered

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using cotton cloth, and the filtrate was treated with acetone to precipitate the polyuronide. The polyuronide so obtained was dried at 60°C for 24 h and stored in desiccator.

Carboxymethyl modification of Tukhm-malanga Polyuronide (CTP)

Carboxymethyl functionalization of TP was carried out as reported earlier [13-15]. Briefly, 5 g of TP was dispersed in 100 mL of ice cold sodium hydroxide (45% w/w) under continuous stirring for 60 min, followed by addition of 100 mL of monochloroacetic acid (75% w/v). The temperature of the reaction mixture was maintained at 70ºC for 2 h. The reaction mixture was then cooled and poured into 1 L of methanol. The precipitate so obtained was filtered and treated with glacial acetic acid till the washings were neutral. The product was washed with 1×300 mL portions of methanol (80% v/v), filtered and dried in oven at 45ºC.

Characterization of TP and CTP

Physical characterization

The characterization of TP and its carboxymethyl derivative for swelling index, bulk density, tapped density, Hausner's ratio and Carr's index was carried out as per standard protocol [13, 16, 17]. Further compression behaviour of TP and CTP was determined. Briefly, the powder samples of TP or CTP (500 mg) with magnesium stearate (1%, w/w) were taken polybags and blended for 5 min (A and B), and 30 min (C). The powder blends were then compressed into tablets by applying a pressure of 75 kg/cm^2 in IR hydraulic press (PCI analytics, Mumbai, India) for 2 s (A and C) and 30 s (B). The tablets thus prepared were stored in desiccators for overnight. After 24 h, the crushing strength of tablets was determined using digital tablet hardness tester (VTHT 500, VinSyst Digital Tester, Mumbai, India).

Degree of substitution

The classical acid was method was employed to determine the degree of carboxymethyl substitution [18]. An accurately weighed 10 g of CTP was dispersed in 100 mL of hydrochloric acid reagent with the aid of stirring for 3-4 h, followed by filtration and washing the residue with methanol (70% v/v), till the washings were neutral. The residue so obtained was dried to constant weight in an oven at 80ºC.

An accurately weighed 1 g of the dried CTP was dispersed in 20 mL of methanol (70%, v/v) in a flask and stirred for 30 min. To this an aliquot of 50 mL of distilled water and 30 mL of sodium hydroxide (0.5 N) was added and stirred for 3 h to dissolve the sample completely. The excess of sodium hydroxide present in the reaction mixture was back titrated with hydrochloric acid (0.5N), determining the end point of titration potentiometrically. The degree of carboxymethyl substitution (DS) in CTP was calculated using the following equation.

$$DS = \frac{0.162A}{1 - 0.058A} \quad (1)$$

Where, A is the milli-equivalents of sodium hydroxide required per gram of the sample.

FTIR spectroscopy

The powder samples of TP or CTP were blended with KBr and analysed using diffuse reflectance spectroscopy accessory (DRS 8000) in FT-IR spectrophotometer (Shimadzu, IR Affinity-1S, Japan) in range of (4000 cm^{-1} to 400 cm^{-1}).

Thermal analysis

The thermogravimetric analysis of the samples of TP and CTP was done using (EXSTAR TG/DTA 6300, Hitachi High Technologies Corporation, Japan) with air flow of 200 mL min^{-1}.

X-ray diffraction

The X-ray diffraction patterns of TP and CTP were studied by scanning powder samples in X-ray diffractometer (Bruker D8-Advance, Germany) from 0-100º diffraction angle (2θ) under the following measurement conditions: source, nickel filtered Cu-Kα radiation; voltage 40 kV; current 30mA; scan speed 0.018 min^{-1}; division slit 0.15º; receiving slit 0.15 mm.

Morphology

The morphological features of TP and CTP were studied employing SEM (Carl Zeiss Ultra Plus, UK). The samples were coated with gold and then placed in sample holder. The photomicrographs were taken at an accelerating voltage of 15 kV at different magnifications.

Viscosity

The rheological behaviour of aqueous dispersions of TP and CTP (5% w/v) was investigated using rheometer (Modular Compact Rheometer, Anton Paar, Austria) employing measuring cell P-PTD 200 with fixture of PP50, keeping the gap of 1 mm between plates at different shear rates from 10-100 sec^{-1}.

Evaluation of CTP for nanoparticulate drug delivery

Preparation of ofloxacin-loaded CTP nanoparticles

An aqueous solution of CTP (0.02-0.08%, w/v) containing ofloxacin (0.01%, w/v) was prepared in HPLC grade water with aid of sonication. This ofloxacin containing CTP solution was added to chitosan solution (0.01-0.03%, w/v), drop wise using # 16G needle under bath sonication. The dispersion so obtained was further probe sonicated (Q55, Q Sonica, USA) at amplitude of 40% for 1 min.

Characterization of ofloxacin-loaded CTP nanoparticles

Particle size and zeta potential determination

The average particle size, particle size distribution and zeta potential of ofloxacin-loaded chitosan-CTP polyelectrolyte nanoparticles were measured using dynamic light scattering technique employing Zetasizer (Nano ZS90, Malvern Instruments Ltd, UK). The measurements of average particle size and zeta potential were done in disposable sizing cuvette and disposable folded capillary cell, respectively. The measurements were done in triplicate at temperature of 25ºC keeping equilibration time of 120 s in automated mode.

Entrapment efficiency

The amount of ofloxacin entrapped in chitosan-CTP polyelectrolyte nanoparticles was determined by measuring the amount of free ofloxacin present in clear supernatant, obtained by centrifuging the nanoparticulate suspension at 15000 rpm for 35 min in cooling centrifuge (C-24, Remi Instruments, Mumbai, India). The contents of free ofloxacin in supernatant were determined spectrophotometrically by measuring the absorbance at λ_{max} of 293 nm. The entrapment efficiency (EE) was calculated as follows:

$$EE(\%) = \frac{OFLOX_T - OFLOX_F}{OFLOX_T} X100 \quad (2)$$

Where $OFLOX_T$ is the total amount of ofloxacin present in the formulation whereas $OFLOX_F$ is the unentrapped ofloxacin present in the supernatant.

***In vitro* release study**

In vitro release behavior of ofloxacin from the selected batch of ofloxacin-loaded CTP-chitosan polyelectrolyte nanoparticles was comparatively evaluated with conventional ofloxacin solution of equivalent concentration by dialysis sac method [15]. The release rate studies were conducted in USP type II dissolution apparatus (TDL-08L, Electrolab, Mumbai, India). The ofloxacin-loaded nanoparticulate formulation or conventional ofloxacin solution (3 mL) were taken in dialysis sacs (cut off 10kDa)**.** The sacs were then immersed with the help of sinkers in 90 mL of phosphate buffer (pH 7.4) as release media maintained at 37 ± 0.5ºC and rotated at 25 rpm. The contents of ofloxacin released at various time intervals were determined spectrophotometrically by measuring the absorbance of the withdrawn samples at 293 nm.

RESULTS AND DISCUSSION

Tukhm-malanga polyuronide was isolated by precipitating the aqueous dispersion of swollen seeds using acetone. Polyuronide was further functionalized by carboxymethylation to modify its physicochemical properties. Carboxymethylation of polyuronide provided a modified polyuronide (CTP) with a yield of 57%. The degree of carboxymethyl substitution of CTP, as measured by acid wash method was found to be 1.65 carboxymethyl groups/g of polymer.

The results of physical characterization studies (Table 1) indicate that carboxymethylation of TP diminishes its swelling and imparts good flow characteristics. The results of compressibility behaviour study revealed that the tablets of TP blends prepared

during compression characteristic study had crushing strength of 38.4 N, 36 N and 6.2 N for A, B and C blends, respectively. On the other hand the tablets of CTP blends were found to be laminated after 24 h of storage indicating that CTP is highly elastic in nature.

Table 1: Physical-characteristics of tukhm-malanga polyuronide and its modified derivative.

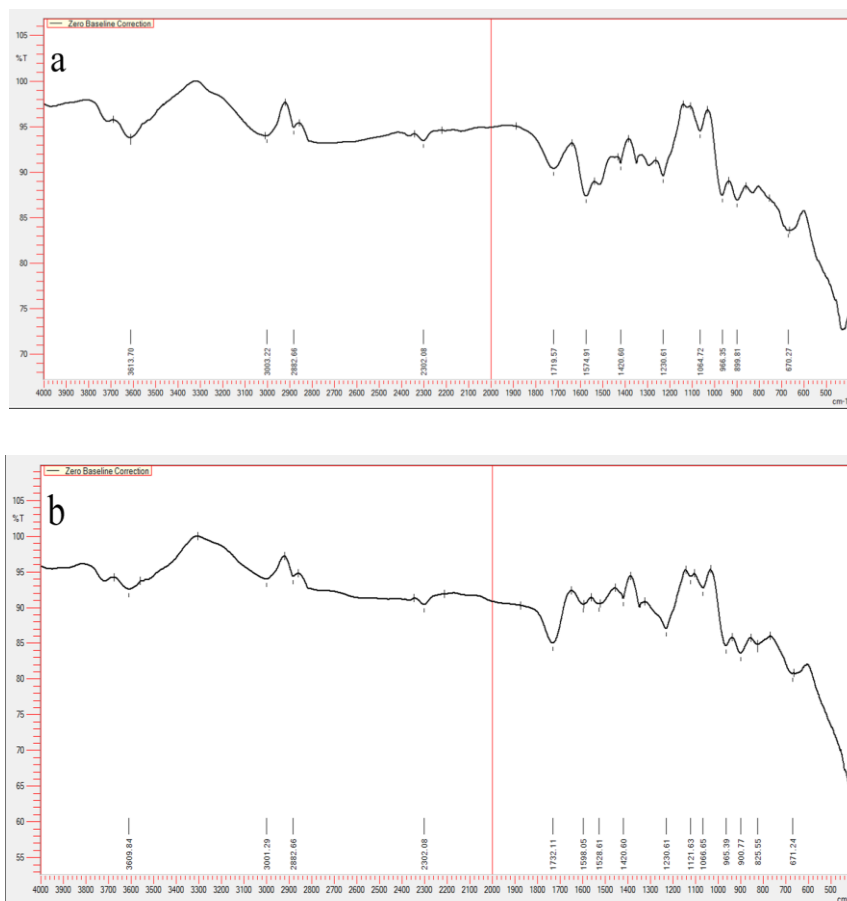| Physical Property	TP	CTP
Swelling Index	10.2	3.66
Bulk Density (g/mL)	0.519	0.768
Tapped Density (g/mL)	0.618	0.865
Hausner's ratio	1.19	1.12
Compressibility Index (%)	16.01	11.21
Angle of Repose (º)	18.6	4.97

Figure 1: a) FT-IR spectrum of TP b) FT-IR spectrum of CTP.

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Figure 1 shows the FTIR spectrum of TP and CTP. A peak at 3613.70 cm^{-1} in the spectrum of TP can be attributed to O-H stretch of alcohol. The peaks obtained at 3003.22 and 2882.66 cm^{-1} can be the result of C-H stretch of alkanes while the peak at 1719.57 cm^{-1} may be due to C=O stretch of carbonyl group. The peaks at 1574.91, 1420.60 and 1230.61 cm^{-1} can be ascribed to asymmetric stretching of carboxylate ion, C-H bending of alkanes and –CH stretch deformation. The stretching of C-O of primary alcohol gives peak at 1064.72 cm^{-1} and bending of C=C results a peak at 966.35 cm^{-1}. Polymer backbone bending is represented by peaks at 899.81 cm^{-1} and 670.27 cm^{-1}. Peak in the spectrum of CTP (Figure 1b) at 3609.84 cm^{-1} may be due to stretching of hydroxyl groups (O-H). The peaks at 3001.29 and 2882.66 cm^{-1} may be due to C-H stretching. The peak at 1732.11 cm^{-1} can be attributed to carboxymethyl group. Asymmetric stretching of carboxylate ion gives peaks at 1598.05 and 1528.61 cm^{-1}. The peaks at 1420.60 cm^{-1}, 1230.61 cm^{-1} and 1066.65 cm^{-1} are due to the bending of –CH group, C-H stretch deformation and C-O stretching of primary alcohol. The peaks at 965.39 and 900.77 cm^{-1} represents C=C bending. Other peaks at 825.55 and 671.24 cm^{-1} may be due to polymer backbone bending.
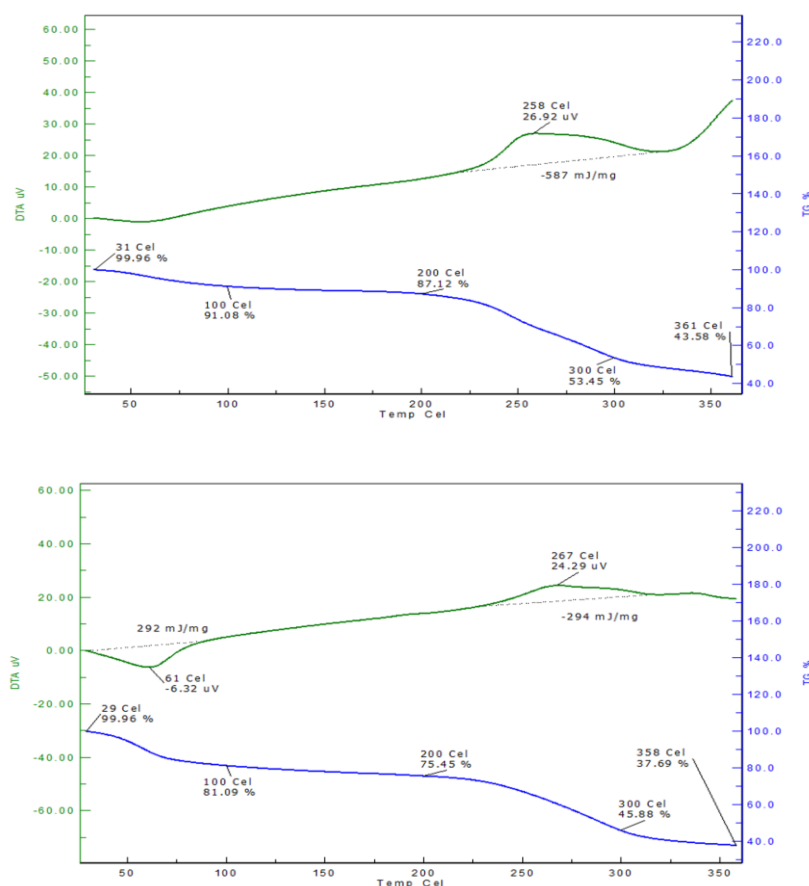

Figure 2: TGA and DTA graph of a) TP and b) CTP.

Figure 2 presents the thermal curves of TP and CTP. The TGA curve of TP shows three stages of degradation. In first stage as the temperature is increased from 30°C to 100°C, there occurs a weight loss of 9% which can be attributed to the loss of physically bound and structural water. On further increasing the temperature to 200°C, there occurs degradation with weight loss of 4%

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leads to a mass loss of 9% w/w approximately. As the temperature was raised to 200°C, further loss of 4%w/w mass occurred. The third stage of degradation which begins around 225°C is marked with significant loss of mass which may be attributed to polymer chain degradation and rupture of C-C and C-O bonds of saccharide ring.

Thermal degradation curve of CTP also exhibits three stages, with first stage due to loss of bound and structural water represented by 19% loss up to 100°C, followed by second stage characterized with weight loss of 6% up to 200°C. Similar to TP, the third degradation stage of CTP begins around 225°C with much greater weight loss due to polymeric chain degradation. Further at the end of the thermal degradation study at 360°C, 43% and 32% of residual mass of TP and CTP respectively is left. The results thus reveal that TP is thermally more stable than CTP.

Figure 3 displays the X-ray diffraction curve of TP and CTP. The diffraction pattern of TP and CTP are of typical amorphous substances. It can be observed that the diffraction curve of TP shows a small peak at 22.24° (2θ), while the CTP shows a diffraction pattern having a characteristic peak at 22.13° (2θ). However, the peak of CTP is of slightly higher intensity than that of TP indicating increase in degree of crystallinity of TP on carboxymethylation.
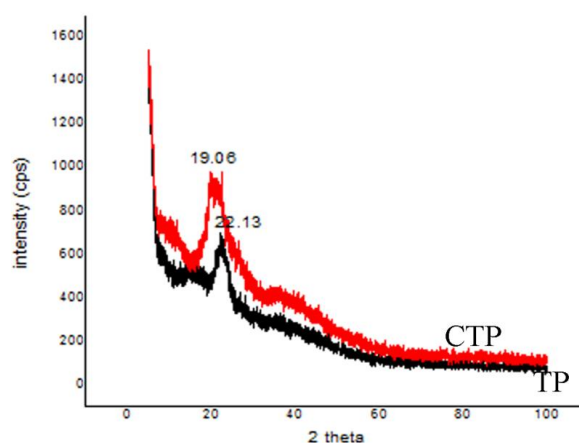

Figure 3: XRD diffraction curve of TP and CTP.

Figure 4 exhibits the scanning electron photomicrographs of TP and CTP. The micrographs of TP (4a and 4b) reveal that the TP particles are polyhedral in shape with slightly rough surface. On other side the CTP particles are polyhedral and porous with presence of needle shaped crystalline structures on its surface.
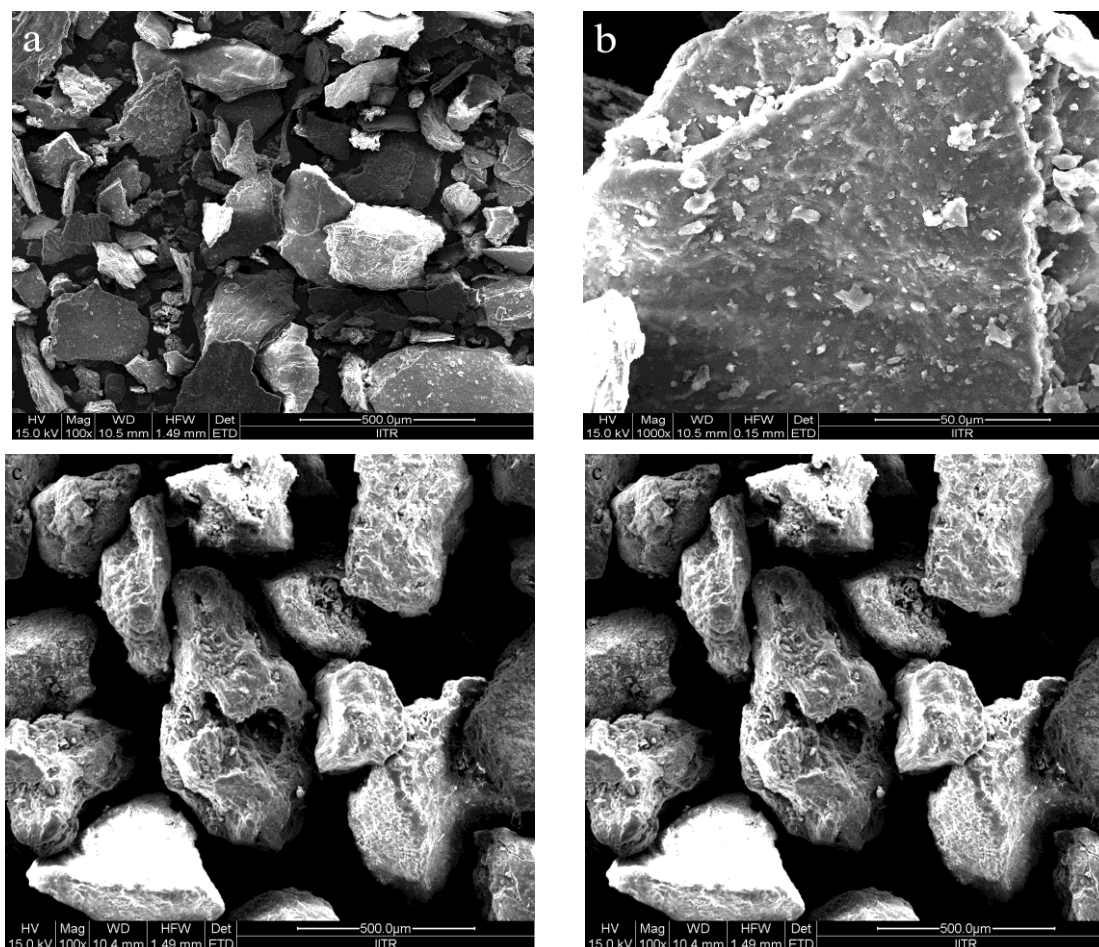
Figure 4: a) Shape of TP b) Surface of TP c) Shape of CTP d) Surface of CTP

Figure 5 depicts the effect of shear rates on viscosity of TP and CTP. It can be observed that there is significant fall in the viscosity of TP on carboxymethylation, which may be the result of columbic repulsion between anionic polyuronide backbones chains. Further, moderate to negligible shear thinning is observed for CTP, indicating the Newtonian flow characteristics of CTP.

Carboxymethylation of polysaccharides is reported to impart anionic characteristics on the macromolecule. This modification paves the way for the interaction of carboxymethylated derivative with cationic polyelectrolytes. The preliminary interaction studies of aqueous dispersions of CTP (0.5% w/v) with aqueous solutions (0.05% w/v) of magnesium chloride, calcium chloride and chitosan (0.5% w/v in 2% v/v acetic acid) revealed the interaction of CTP with these cations to form ionically gelled polyelectrolyte particles. However, the interaction between CTP and chitosan was more intense, which provided the particles with smaller size and narrow particle size distribution. Thus, the ionic interaction between CTP and chitosan was further explored for preparing polyelectrolyte nanoparticulate formulation using ofloxacin as a model drug.
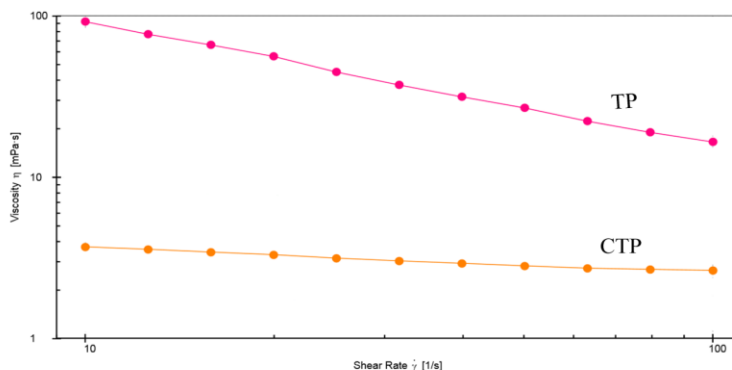
Figure 5: The effect of shear rates on viscosity of TP and CTP.

Table 2 summarizes the result of particle size analysis, zeta potential and entrapment efficiency of various batches of ofloxacin-loaded CTP nanoparticulate suspension prepared by varying the concentrations of CTP and chitosan. The results reveal that as the concentration of CTM is decreased from 0.08 to 0.02% the particle size decreases, while increasing the concentration of chitosan at constant CTM concentration was found to decrease the particle size. Further it can be observed that the nanosuspensions having smaller particle size have also narrow size distribution as indicated by their polydispersity index. The size of polyelectrolyte nanoparticles depend upon a number of factors like the concentration and charge on polyelectrolytes, pH of the medium, order of mixing etc [19] Moreover, the zeta potential of nanosuspension increases with the increase in concentration of chitosan, a cationic polymer. However, no significant effect of varying the concentration of CTM or chitosan was observed on entrapment efficiency of ofloxacin in the nanoparticles. The batch of nanosuspension prepared with 0.02% (w/v) CTP and 0.01% (w/v) chitosan with minimum particle size and optimum zeta potential range was selected for conducting *in vitro* release study.

Table 2: Particle size, polydispersity index and zeta potential of various batches of nanoparticles.

Concentration (%,w/v)		Average Particle size (d. nm)*	Polydispersity Index (PdI)*	Zeta Potential (mV)*	Entrapment Efficiency %
CTM	Chitosan				
0.08	0.01	1975 ± 216.5	0.602 ± 0.130	9.69 ± 3.09	97.94
0.08	0.02	1239 ± 133.7	0.863 ± 0.025	14.9 ± 0.896	97.87
0.08	0.03	522.2 ± 10.02	0.251 ± 0.043	28.8 ± 2.21	97.16
0.05	0.01	1730 ± 492.5	0.846 ± 0.121	9.67 ± 1.07	98.69
0.05	0.02	492.4 ± 8.04	0.253 ± 0.008	26.4 ± 1.70	98.06
0.05	0.03	422.0 ± 5.006	0.279 ± 0.016	29.7 ± 2.17	97.61
0.02	0.01	402.3 ± 3.81	0.315 ± 0.037	22.3 ± 1.79	96.50
0.02	0.02	440.5 ± 64.96	0.476 ± 0.190	32.1 ± 4.96	97.46
0.02	0.03	441.2 ± 22.84	0.361 ± 0.030	37.2 ± 7.64	95.93
Note: *Values are mean ± SD (n=3)					

In vitro **release**

The release study of ofloxacin from CTP nanoparticles was conducted in comparison to solution formulation to study the limiting effect of a dialysis membrane. Figure 6 shows the comparative release profile of ofloxacin from both the formulations. It can be observed from the plot that the nanoparticulate formulation provided a prolonged release of ofloxacin with 87.10% of the drug getting released over a period of 12 h, while the solution formulation released almost 100% of ofloxacin within 4 h.
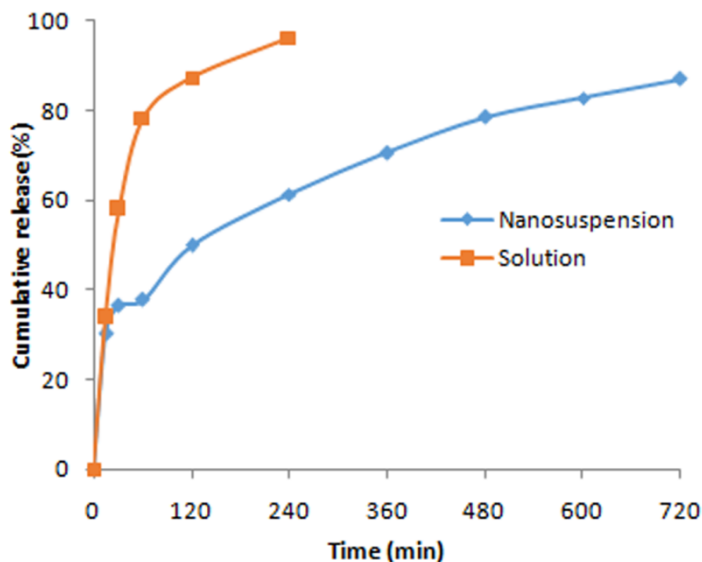

Figure 6: *In vitro* release of ofloxacin from nanoparticulate formulation.

Table 3: Release kinetics of ofloxacin from CTM-chitosan polyelectrolyte nanoformulation.

Variables	Model				N
	Zero-order	**First-order**	**Higuchi**	**Korsmeyer-Peppas**	
R^2	0.821	0.972	0.906	0.981	0.247

To estimate the kinetics and mechanism of drug release, the release data was fitted into various release kinetic equations. The release data was found to fit best into first order release kinetics. Further, the value of '*n*' (n=0.247) the release exponent of Korsemeyer-Peppas equation indicates that the primary mechanism of release of ofloxacin from CTM-chitosan polyelectrolyte nano-suspension is diffusion through the polymeric matrix (Table 3).

CONCLUSION

The polyuronide isolated from the tukhm-malanga seeds was functionalized by carboxymethylation to make it useful for drug delivery applications. The carboxymethylation of tukhm-malanga polyuronide led to decrease in its viscosity and improved its interaction with cationic polymers. The interaction between the carboxymethylated polyuronide and chitosan was utilized for

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making nanoparticulate formulation of ofloxacin. The results indicated that nanoparticulate formulation provided a sustained release of ofloxacin. However, further studies *in vivo* are needed to fully utilize it in drug delivery applications. In conclusion, carboxymethyl modification of polyuronide of tukhm-malanga improves its functionality for pharmaceutical applications.

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