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Original Article

CAMPTOTHECIN LOADED POLY (METHACYCLIC ACID-CO-METHYL-METHYACRYLATE) NANOPARTICLES: FABRICATION, CHARACTERIZATION AND CYTOTOXICITY STUDIES

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ABSTRACT

Objectives: The present investigation was aimed to fabricate Camptothecin loaded Poly (Methacyclic acid-co-methyl-methyacrylate) nanoparticles to characterize the prepared nanoparticles and to evaluate the cytotoxicity of prepared Camptothecin.

Methods: Camptothecin loaded polymeric nanoparticles were prepared by nanoprecipitation method by sonication approach. The prepared nanoparticles were evaluated for particle size, particle size uniformity, surface area, zeta potential, surface morphology, encapsulation efficacy, drug loading and *in-vitro* drug release. To evaluate the potential anticancer efficacy of nanoparticulate system, *in-vitro* cytotoxicity studies on human colon cancer cell line (HT-29) were carried out using MTT assay.

Results: Camptothecin loaded polymeric nanoparticles were successfully prepared by nano precipitation method using sonication approach. Nanoparticles prepared using sonication method were with an average particle size 100 to 200 nm, particle size uniformity found to be<0.3 and zeta potential>20mV. Prepared Camptothecin loaded polymeric nanoparticles were spherical in shape and showed excellent encapsulation efficiency and drug loading. The release profile was found to be pH dependent. It was observed that polymer coated Camptothecin nanoparticles gave no release in simulated gastric fluid, negligible release in simulated intestinal fluid and maximum release in colonic environment. The drug release followed Hixson Crowell cube root law model. Prepared Camptothecin loaded polymeric nano formulations displayed enhanced cytotoxicity against HT-29 cells in comparison with pure Camptothecin.

Conclusion: In summary, Camptothecin loaded Poly (Methacyclic acid-co-methyl-methyacrylate) nanoparticles may be considered as an attractive and promising formulation. Thus, the results indicate the potential for *in-vivo* studies for the developed pH sensitive nanoparticles of Camptothecin to establish their clinical application.

Keywords: Camptothecin, Cytotoxicity, Drug release, MTT assay, Nanoparticles, Particle size.

INTRODUCTION

Colon cancer is a multifunctional disease related with high morbidity and mortality in western countries. The death rate of peoples with colon cancer around the world is about 715000 every year and is rising steadily over time [1, 2]. Most chemotherapeutic approaches available now-a-days are intended to treat the cancer are non-specific and are associated with various toxicities to healthy cells in addition to tumor cells [3]. The concept of colon targeting of active drug delivery is attractive because after the conventional oral administration the drugs are aimed at increasing its aqueous solubility, reduction in the stomach and intestine degradation and to achieve site specific delivery [4-6]. Colon targeted drug delivery would ensure directs treatment at the disease site, lowering the dose and reducing the systemic side effects [4, 7].

Camptothecin (CPT) naturally occurring quinolone alkaloids shows a significant anticancer activity with a broad spectrum of human malignancies. Camptothecin is an inhibitor of the DNA-replicating enzyme topoisomerase I which is believed to act by stabilizing a topoisomerase I-induced single strand break in the phospho diester backbone of DNA, thereby preventing relegation. Despite of its promising activity, the clinical applications are hampered by its poor water solubility, low stability in physiological medium, severe systemic toxicity and low antineoplastic activity [8]. Accordingly, a novel drug delivery system is imperative to overcome the internal defects. In recent years, a nano structured materials such as nano particles have been considered as potential carriers for hydrophobic drug delivery that may resolve the aforementioned problems [9, 10].

Now-a-days, significant research has been done using nanoparticles as an oral drug delivery vehicle. Polymeric nanoparticles are actively investigated as drug carriers to reduce drug toxicity and degradation, so as to deliver therapeutic agents to several sites of action, to promote stable, selective and specific targeted therapy [11, 12].

However, recent development in the field has rekindled interest in targeting approach [13]. Colon targeted delivery system of Camptothecin includes coating with pH dependent polymer, formulation of the timed release system, use of the pro drug, exploitation of carriers that degrade specifically by colonic bacteria and bioadhesive system [4]. In the present investigation, an attempt has been made to formulate Camptothecin loaded nanoparticles using a pH dependent polymer to deliver Camptothecin to the colon.

Poly (Methacyclic acid-co-methyl-methyacrylate), a pH dependent enteric polymer which does not dissolve in the stomach and intestine pH, but dissolves in colonic pH due to ionization of its carboxyl functional group was used to selectively deliver the drug to colon [14]. Therefore the polymer protects drug from degradation in the stomach and intestine and the dosage form increase the solubility of the drug, sustain the drug release and protects the drug from degradation from the colonic environment [1, 4].

The present investigation was aimed to prepare Camptothecin loaded nanoparticles and characterization of the prepared nanoparticles such as particle size, particle size uniformity, surface area, zeta potential, surface morphology, encapsulation efficiency, drug loading and *in-vitro* drug release behavior. In addition, cell culture studies were conducted in order to evaluate the cytotoxicity of prepared Camptothecin on HT-29 cell line.

MATERIALS AND METHODS

Materials

Camptothecin was commercially purchased from S. M Herbals, India. β-cyclodextrin and poloxamer (Grade 188) were procured from Sigma Aldrich, India. Poly (methacylic acid-co-methyl methyacrylate) was obtained from Evonik Industries, India. Dimethyl sulphoxide and propanol were obtained from E-Merck specialities Pvt. Ltd., Mumbai, India. 3-(4,5–dimethyl thiazol–2–yl)–

5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. All other chemicals and reagents used were of analytical grade and used without further purification.

Methods

Fabrication of polymeric nanoparticles

Drug loaded polymeric nanoparticles were prepared by a novel nanoprecipitation method. However, this method is reported to yield nanoparticles with broad distribution [15]. Hence, few modifications to the conventional nanoparticles method were implemented to yield narrow particle size distribution [16]. Poly (methacylic acid-co-methyl methyacrylate) based nanoparticles drug delivery system was developed using the nanoprecipitation method by sonication method (table 1).

Briefly, about 100 mg of Poly (methacylic acid-co-methyl methyacrylate) polymer with 10 mg of Camptothecin were dissolved in 10 ml of dimethyl sulphoxide, which was transferred at once into 25 ml of distilled water containing 62.5 mg of β-cyclodextrin and 125 mg of poloxamer 188 under the sanction (Ultrasonic cleaner, Lark). Drug loaded nano particles were formed spontaneously but the sonication process was continued for 50 min [17]. The formulation was stored at refrigerated condition until further use.

Table 1: Fabrication of drug loaded polymeric nanoparticles using sonication approach

Trials	A (mg)	B (mg)	C (mg)	D (mg)	E (ml)	F (ml)	G (mins)
1	10	100	62.5	125	10	25	50

A: Concentration of Drug; B: Concentration of Polymer 100; C: Concentration of β-Cyclodextrin; D: Concentration of Poloxamer 188; E: Volume of Organic Phase; F: Volume of Aqueous Phase; G: Sonication Duration.

Characterization of prepared polymeric nanoparticles

Particle size, particle size uniformity, surface area and zeta potential

The average particle size, particle size uniformity and surface area of prepared Poly (methacylic acid-co-methyl methyacrylate) nanoparticles were measured based on laser light scattering principle using master sizer (Malvern Instrument, UK). Briefly, prepared Camptothecin loaded polymeric nano particle formulations was added drop wise into the water maintained in the sample dispersion unit of particle size analyzer, where the nanoparticles scattered using simple shaft pump a stirrer and recirculated continuously around the measurement zone of particle size analysis. The experiments were performed in triplicate [18].

Zeta potential of prepared polymeric nanoparticles was measured using the Zetasizer (ZEN3600, Malvern). Aggregation of nanoparticles in formulation reduces the physical stability of nanosuspension and leads to decreased oral bioavailability. The charge on the nanoparticles play a significant role in aggregation. Higher number of either positive or negative charge repels each other which in turn prevents aggregation. Zeta potential around±30 mV was considered acceptable [19]. Briefly, about 1 ml of polymeric nanoparticles was diluted approximately using ultra-pure water (Milli Q Academic Milli Pore). Diluted samples were loaded separately in a disposable zeta cell and measured for zeta potential.

Particle surface morphology analysis

The morphological examination of the prepared polymeric nanoparticles was performed by Field Emission Scanning Electron Microscopy (FESEM) and Transmission Electron Microscopy (TEM). FESEM (Hitachi SU 660) was used to analyze the morphology by an accelerating voltage of 15 kV. The spot size in FESEM is smaller than conventional SEM and it can produce a very high resolution image. The TEM is also used to analyze the shape of the prepared nanoparticles. Briefly, the prepared Camptothecin loaded polymeric nanoparticles were dropped onto Formvar-coated copper grids and air dried. The samples were then negatively stained with 1% uranyl acetate for 10 min and air dried again. The samples were then imaged using transmission electron microscope (Hitachi H7500, India) at 20 000 magnifications [18].

Encapsulation efficiency and drug loading estimation

Camptothecin loaded polymeric nano formulations were centrifuged using a cooling centrifuge (C-24, Remi) for 45 min at 19000 rpm at-20 °C and supernatant was separated. To 1 ml of supernatant, an equal volume of methanol was added[20]and sonicated (Ultrasonic cleaner, Lark) for 5 min followed by filtration through 0.45 µm membrane. Samples were analyzed using the developed HPLC methods as mentioned below. Estimated amount of free drugs was expressed as W_{free}. The experiments were performed in triplicate.

For the analysis, Shimadzu HPLC system was used with the best chromatographic conditions equipped with C18 column (ODS 250 mm X 4.6 mm with 5 micron pore size, Phenomenax) using a mobile phase combination of 0.5% W/V of ammonium acetate aqueous solution and acetonitrile (85:15, v/v) in an isocratic mode elution with a flow rate of 1 mlmin-1 at the column oven temperature of 35 °C and the samples were analyzed by PDA detector at a wavelength of 368 nm [22-24].

Encapsulation efficiency (EE) and drug loading (DL) were estimated as follows

$$EE\ (\%) = \frac{[\text{Drug Content } (W_{total})] - [\text{Drug in the supernatant } (W_{free})]}{[\text{Drug Content } (W_{total})]} \times 100$$

$$DL\ (\%) = \frac{[\text{Drug Content } (W_{total})] - [\text{Drug in the supernatant } (W_{free})]}{[\text{Weight of the polymer used in the formulation } (W_{polymer})]} \times 100$$

***In-vitro* drug release study**

The release characteristics of Camptothecin from the prepared nanoparticle formulation were investigated using USP dissolution apparatus 2 (Electrolab, Mumbai, India) maintained at 37° C±0.5° C with a rotating speed of 100 rpm. To achieve simulated gastrointestinal transit condition the release profile of nano formulation was studied with the dissolution medium of changing pH at various time intervals. Initially, the dissolution medium was maintained at pH 1.2 with 350 ml of 0.1N HCl for 0-2 h. At the end of the second hour, the pH of the dissolution medium was raised to 4.5 by the addition of 250 ml of solution composed of 3.75 g of KH_2PO_4 and 1.2 g of NaOH and the total volume of dissolution medium was 600 ml. At the end of the fourth hour, pH of the medium was raised to 7.4 by addition of 300 ml of phosphate buffer concentrate (2.18 g of KH_2PO_4 and 1.46 g of NaOH in distilled water) [25, 26]. At predetermined time intervals, 5 ml of sample was withdrawn and replaced with fresh dissolution media. The collected samples were filtered through 0.45 µm membrane filter (Millipore). After appropriate dilution, the concentration of drug in the sample was analyzed using HPLC.

The data obtained from in-vitro release studies were kinetically analyzed to find out the mechanism of drug release rate kinetics of the dosage form. The obtained data were fitted with the zero order, First order, Higuchi, Hixson-Crowell erosion equation, Korsmeyer-Peppas equation.

***In-vitro* cytotoxicity studies**

Cell line and culture medium

Prepared polymeric nano formulation was evaluated for cytotoxicity using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

(MTT) assay on HT-29 (Human colon carcinoma) [27]. HT-29 (Human colon carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 g/ml) and amphotericin B (5 g/ml) in a humidified atmosphere of 5% CO_2 at 37° C until confluent. The cells were dissociated with a TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

MTT assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 105 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10 000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, wash the monolayer once with medium and 100 µl of different test concentrations of test drugs, subsequent concentrations of DMSO (1000, 500, 250, 125 and 62.5%) maintained as control was added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 d in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h intervals. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm [28].

RESULTS AND DISCUSSION

Colon cancer is being one of the most leading cancers and treating colon cancer can be most efficacious by the direct delivery of the drug to the affected area. This selective delivery also may reduce the dose of the therapeutic agent to be delivered [7]. Targeting to the colon relies on protecting the moiety to be targeted from being released in the stomach, at the same time exploring the unique features of the colon for maximizing the drug release [1].

In the present investigation, the pH-dependent approach was exploited for the specific delivery of Camptothecin to the colon. As mentioned earlier, the selective dissolution of Poly (Methacyclic acid-co-methyl-methyacrylate) at the colonic pH by the ionization of its carboxylic functional groups was exploited to achieve the desired action.

Fabrication of polymeric nanoparticles

Camptothecin loaded Poly (Methacyclic acid-co-methyl-methyacrylate) nanoparticles were prepared based on nanoprecipitation principle under the influence of sonication. Organic phase contains Camptothecinand Poly (Methacyclic acid-co-methyl-methyacrylate) in water miscible organic solventdimethyl sulphoxide. Aqueous phase contains poloxamer 188 as a surfactant and β-cyclodextrin as a stabilizer. Addition of organic phase in to aqueous phase under the influence of sonication results in rapid miscibility of dimethyl sulphoxide with water, which increases the polarity of the dimethyl sulphoxide and decreases the solubility of Camptothecin leading to initiation of crystal nucleation. Concurrently, sonication process produce bubbles, whose size is near the resonant size for the applied frequency and begins to oscillation on linearly and finally collapse resulting in production of extremely high temperature, high pressure and shockwave, which inhibits the crystal growth of Camptothecin. However, developed Camptothecin nanocrystals form complex with β-cyclodextrin, which increases the stability and solubility of Camptothecin in the aqueous phase. Negatively charged particles repel each other and develop an electrostatic force, which maintains the nanoparticles in Brownian motion and overcomes the Vander Waals force of attraction and gravitational force resulting in the prevention of nanoparticle aggregation and sedimentation.

Particle size, particle size uniformity surface area and zeta potential

After fabrication, prepared Camptothecin loaded polymeric nanoparticles were stored at room temperature for one month to identify any aggregation and post-formulation degradation. Prepared Camptothecin loaded polymeric nanoparticles were characterized for particle size, particle size uniformity, surface area and zeta potential as per the procedure mentioned above. The results were summarized in table 2 and characterization spectrum was displayed in fig.1 and fig.2. In sonication method, prepared polymeric nanoparticles have shown an average particle size of 150 nm with particle size uniformity of 0.210, surface area of 51.7 m^2g^{-1} and zeta potential of-21.8 mV.

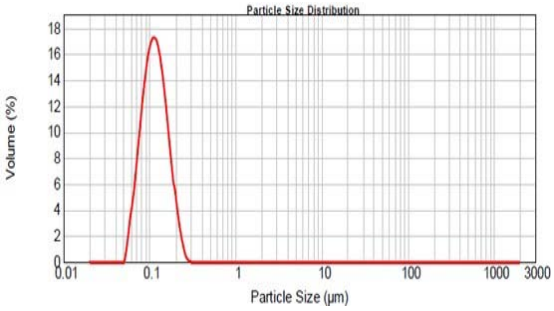


Fig. 1: Particle size spectrum of Camptothecin loaded polymeric nanoparticles prepared using sonication approach

Table 2: Characterization of Camptothecin loaded polymeric nanoparticles

Trial	Average particle size (nm)	Particle size uniformity	Surface area (m^2g^{-1}±SD)	Zeta potential (mV)
1	150±0.19	0.210±0.01	51.7±0.42	-21.8±2.50

(n=3)

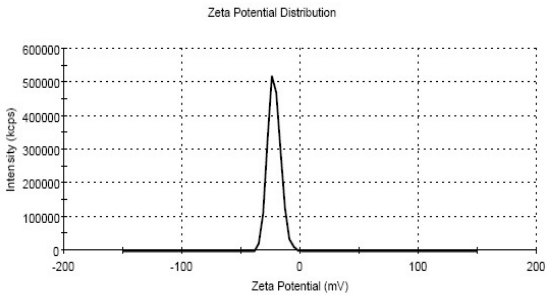


Fig. 2: Zeta potential spectrum of Camptothecin loaded polymeric nanoparticles prepared using sonication approach

Particle surface morphology analysis

Particle surface morphology decides the basic function of particles, degradation, release of drug from polymer matrix, transport of particles in the body, internalization of the drug. Prepared Camptothecin loaded polymeric nanoparticles were imaged using field emission scanning electron microscopy (fig. 3) and transmission electron microscope (fig. 4).

Prepared Camptothecin loaded polymeric nanoparticles were spherical in shape. Hence, Camptothecin encapsulated in the polymer matrix will also be in spherical shape and expected to enhance the basic function, release of Camptothecin from the polymer matrix, transport of Camptothecin in the body and internalization of Camptothecin by many folds than the free Camptothecin.

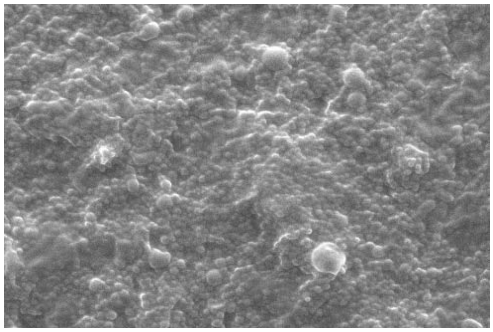


Fig. 3: Particle surface morphology of Camptothecin loaded polymeric nanoparticles prepared using FESEM

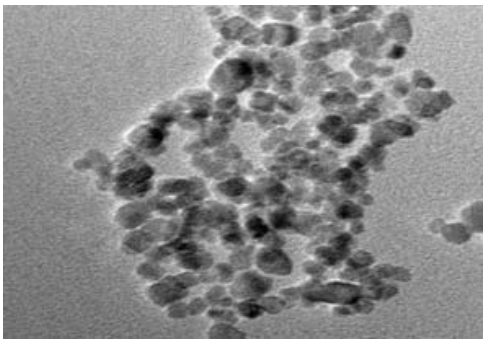


Fig. 4: Particle surface morphology of Camptothecin loaded polymeric nanoparticles prepared using TEM

Encapsulation efficiency and drug loading estimation

The amount of Camptothecin encapsulated in polymeric nanoparticles determines the effectiveness of prepared nanoformulations. Hence, encapsulation efficiency and drug loading estimation were performed. The results were summarized in table 3. In sonication method, encapsulation efficiency and drug loading were found to be 92.85% and 9.12%. Sonication approach displayed excellent encapsulation efficiency and drug loading only an insignificant amount of Camptothecin were seen as a free drug. Hence, prepared Camptothecin loaded polymeric nanoparticles is expected to display superior pharmacological activities.

Table 3: Encapsulation efficiency and drug loading of the prepared Camptothecin loaded polymeric nanoparticles

Trial	Encapsulation efficiency (%)	Drug loading (%)
1	92.85±0.40	9.12±0.17

(n=3)

***In-vitro* drug release**

In-vitro drug release from the nanoparticles was assessed in simulated gastrointestinal conditions. The pH condition used was pH 1.2 for a period of 2 h (stomach), pH 4.5 for 2 h (duodenum) followed by pH 7.4 (distal ileum and colon) for the remaining period of the study using a USP dissolution test apparatus (Apparatus type 2) [29]. The cumulative percent drug release curve shows (fig. 5) that the drug release was less than 10% up to 4 h and the drug release increased when the pH of the medium was adjusted to 7.0.

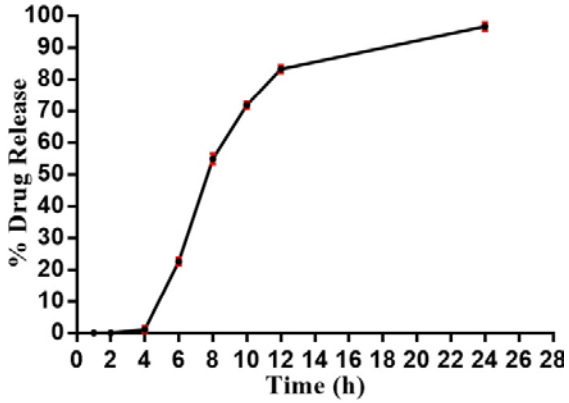


Fig. 5: *In-vitro* release profile of Camptothecin loaded polymeric nanoparticles(n=3)

Poly (Methacyclic acid-co-methyl-methyacrylate) is an anionic polymer; the ratio of free carbonyl groups to the ester groups is approximately 1:2. It exhibits a dissolution threshold pH slightly above 7.2 [30]. Due to the pH sensitive property, it was selected to avoid the rapid dissolution of Camptothecin during the initial transit of the nanoparticles through gastric cavity and the upper small intestine. It was observed that polymer coated Camptothecin nanoparticles gave no release in the simulated gastric fluid, negligible release in the simulated intestinal fluid and maximum release in the colonic environment.

Kinetic analysis of release data

The results of *in-vitro* release profile obtained from the formulations were plotted to know the mechanism of drug release. The data were treated according to zero order release, first order release, Higuchi model, Korsemeyer-Peppas model and Hixson Crowell cube root law. The release rate kinetics data of the formulation is shown in table4. It is concluded that the formulation gave a good fit to the Hixson Crowell cube root law. The diffusion exponent (n) value was greater than 0.89, this result indicated that the release of drug from the polymer matrix formulations was found to be super case-II transport, i.e., drug release by both diffusion and relaxation of polymer chain.

Table 4: Determination coefficients (r2) and release exponent (n) of kinetic data analysis of Camptothecin release from polymeric nanoparticles

Trial	Zero order	First Order	Higuchi model	Korsmeyer-Peppas model		Hixson-Crowell cube root law r^2
	r^2	r^2	r^2	r^2	n	
1	0.7244	0.7198	0.629	0.515	1.4019	0.9317

***In-vitro* cytotoxicity studies**

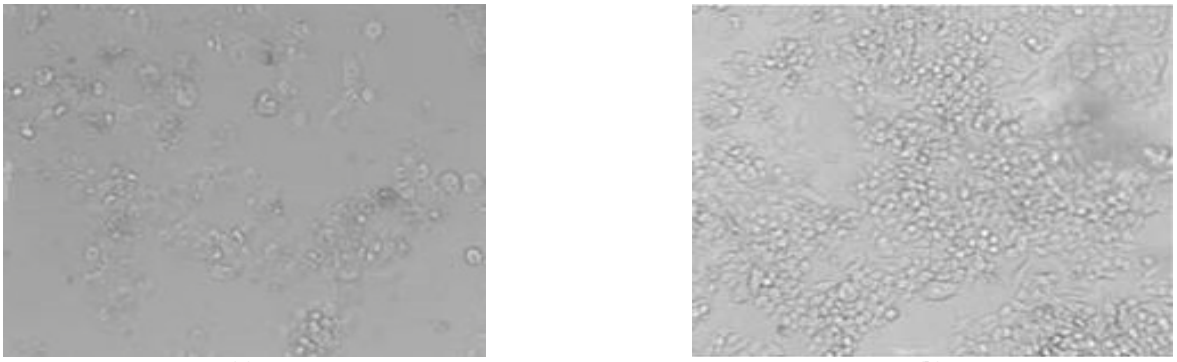
Prepared Camptothecin loaded polymeric nano formulations were studied for its *in-vitro* anticancer efficacy against human colon cancer HT-29 cell lines, and the results were summarized in table 5 and fig. 6.

Pure Camptothecin displayed very poor cytotoxicity on HT-29 cell at 1000 µg/ml(CTC_{50}:>1000 µg/ml). Camptothecin loaded polymeric nano formulation displayed good cytotoxicity on HT-29 cells at 1000 µg/ml (CTC_{50}: 155.00 µg/ml). However, prepared Camptothecin loaded polymeric nano formulations displayed enhanced cytotoxicity against HT-29 cells in comparison with pure Camptothecin.

Table 5: *In-vitro* cytotoxicity of prepared Camptothecin loaded polymeric Nanoparticles on HT-29 cells

Sample	Test concentration (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
Pure camptothecin	1000	20.18±0.24	>1000
	500	16.21±0.29	
	250	14.12±0.25	
	125	12.24±0.19	
	62.5	9.57±0.15	
Camptothecin loaded polymeric nanoformulation	1000	96.98±0.2	155.00±8.7
	500	61.20±1.0	
	250	54.06±2.4	
	125	48.73±0.6	
	62.5	41.68±2.8	

(n=3)



(a) **(b)**

Fig. 6: *In-vitro* cytotoxicity of pure camptothecin (a) and camptothecin loaded polymeric nanoparticles (b) on HT-29 cells

CONCLUSION

Poly (Methacyclic acid-co-methyl-methyacrylate) nanoparticles was prepared using the nano precipitation method by sonication approach. Prepared plain and Camptothecin loaded polymeric nanoparticles were characterized for particle size, particle size uniformity, surface area and zeta potential. Nanoparticles prepared using sonication method were with an average particle size<100 nm, uniformity<0.3 and zeta potential>20mV. Prepared Camptothecin loaded polymeric nanoparticles were spherical in shape. Hence, Camptothecin encapsulated in the polymer matrix will also be in spherical shape and expected to enhance the basic function, release of Camptothecin from the polymer matrix, transport of Camptothecin in the body and internalization of Camptothecin by many folds than the free Camptothecin. Sonication approach displayed excellent encapsulation efficiency and drug loading and only an insignificant amount of Camptothecin were seen as a free drug. Hence, prepared Camptothecin loaded polymeric nanoparticles is expected to display superior pharmacological activities. It was observed that Poly (Methacyclic acid-co-methyl-methyacrylate) coated Camptothecin nanoparticles gave no release in the simulated gastric fluid, negligible release in the simulated intestinal fluid and maximum release in the colonic environment. It is concluded that the formulation gave a good fit to the Hixson Crowell cube root law. Prepared Camptothecin loaded polymeric nanoparticles were studied for its *in-vitro* anti-cancer efficacy against human colon cancer cells using MTT assay. Prepared Camptothecin loaded polymeric nano formulations displayed enhanced cytotoxicity against HT-29 cells in comparison with pure Camptothecin. Thus, the results indicate the potential for *in-vivo* studies of the developed pH sensitive nanoparticles of Camptothecin to establish their clinical application.

CONFLICT OF INTERESTS

Declared None

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