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FORMULATION AND EVALUATION OF 5 FLUOROURACIL NANOPARTICLES FOR THE TREATMENT OF COLORECTAL CANCER

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ABSTRACT

The main objective of the study is to formulate 5 Fluorouracil loaded sustained release nanoparticles with the size of around 250 nm and to increase the encapsulation efficiency of the drug. The nanoparticles were prepared by simple ionic gelation method using various concentrations of chitosan and TPP. The prepared nanoparticles were evaluated for particle size, shape, charge, encapsulation efficiency, *in vitro* drug release and *in vitro* cytotoxicity. The optimized 5FU loaded nanoparticle showed size of 232±4 nm with PDI of 0.30 ±0.07, Zeta potential of +5 ±1mv, encapsulation efficiency of 69.2% and the drug release is 97.4% at 24 hrs. These results demonstrate that the possibility of delivering 5 Fluorouracil to colorectum with enhanced encapsulation efficiency.

KEYWORDS

Chitosan, 5-Fluorouracil, Nanoparticles, Cytotoxicity and Colorectal cancer.

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INTRODUCTON

Targeting of drugs specifically to colon is advantageous for the treatment of diseases associated with the colon such as Amoebiasis, Crohn's diseases, Ulcerative colitis and colorectal cancer. A drug delivery system is most often associated with particulate carriers such as emulsion, liposomes and nanoparticles which are designed to localize drugs at the target site. The efficacy of present cancer chemotherapy is mainly limited by the toxicity associated with the anticancer drugs to normal tissues. This limitations result from the fact that anticancer drugs presently used in chemotherapy lack efficient selectivity towards tumor cells.

This necessitates the development of a novel nanoparticle delivery system to overcome these current obstacles in convention drug therapy. Nanoparticles due to their small size and target specific localization property offer numerous advantages compared to conventional dosage forms which includes reduced dose improved efficiency, reduced toxicity, patient compliance and convenience.

5-Fluorouracil a cell-cycle-phase-specific anti-neoplastic agent, is indicated in colon, rectal, breast, ovarian, cervical, gastric, oesophageal, bladder, liver, and pancreatic cancer. Fluorouracil exerts its cytotoxic activity by acting as an anti-metabolite, competing for the enzyme that is important in the synthesis of thymidine, an essential substrate for DNA synthesis[1].

Chitosan is a natural hydrophilic polysaccharide copolymer of glucosamine and N-acetyl glycosamine. It is considered as a safe excipient due to its biocompatibility, biodegradability and lack of toxicity, moreover it is cationic in nature and posses mucoadhesive property it will enhance the cellular uptake by ionic interaction[2,3].

The present study was aimed at the formulation and characterization of 5 Fluorouracil loaded chitosan nanoparticles additionally the nanoparticles have been evaluated for cytotoxicity in $Caco_2$ cell lines, to overcome the above said obstacles for better therapy of colorectal cancer.

MATERIALS AND METHOD

5-Fluorouracil was a gift sample from Sun Pharmaceuticals, Pune, India. Chitosan was purchased from sigma Aldrich USA, Glacial acetic acid was obtained from Fischer scientific, Dialysis membrane with molecular weight cut off 12000-14000 Daltons was purchased from HIMEDIA laboratories, Mumbai.

Preparation of 5fu loaded chitosan nanoaprticles[4-8]

5FU loaded chitosan nanoparticles were prepared using ionic gelation method, determinate weight of chitosan were dissolved in glacial acetic acid 1% [v/v], 5mg of 5 Fluorouracil was added to the above solution and under constant magnetic stirring followed by addition of aqueous TPP solution in a drop wise manner, then the solution was kept on constant magnetic stirring for 30 mins and sonicator [vibrasonics]. The nanoparticle suspension was centrifuged at 13,000 rpm and 4^0C for 30 minutes using Eppendr of Ultracentrifuge to remove excessive amounts of TPP and unencapsulated 5 Fluorouracil. The pellets were dispersed in deionised water. Finally, nanoparticles were lyophilized for 24 hrs using freeze dryer [lyodel] for storage in powdered form.

Physicochemical characterization of nanoparticles

Particle size and Zeta potential using photon correlation spectroscopy[9]

The average hydrodynamic diameter and polydispersity index (PDI) of the formulated nanoparticles were determined by dynamic light scattering (DLS) analysis using Zetasizer Nano ZS90 (Malvern Instruments limited, UK) 1ml of sample of nanoparticles dispersion was placed in disposable cuvettes for particle size measurements. Each experiment was conducted in triplicate. The electrophoretic mobility (zeta potential) measurements were made using the Malvern Zetasizer (Nano ZS90, Malvern Instruments) at 25ºC. Samples were diluted with double distilled water.

Transmission electron microscopy (HRTEM)

The surface morphology of the prepared NPs was determined for by using transmission electron microscopy (HRTEM). A drop of Nanosuspension was placed on a carbon film coated copper grid for TEM. Studies were performed at 80 kv using JOEL JEM 2100.The copper grip was fixed in to sample holder and placed in a vacuum chamber of the transmission electron microscope and observed under low vacuum and TEM images were recorded.

Atomic Force Microscopy (AFM)

Formulation and characterization of ant colorectal cancer drug loaded chitosan nanoparticles. The surface properties of drug loaded nanoparticles were visualized by an atomic force microscope (Nova NTEGRA prima, Russia) under normal atmospheric conditions. Explorer atomic force microscope was in tapping mode, using high-resonant-frequency (F0 = 4-150 kHz) pyramidal cantilevers with silicon probes having force constants of 0.35-6.06 N/m. Scan speeds were set at 2 Hz. The samples were diluted 10 times with distilled water and then dropped onto glass slides, followed by vacuum drying during 24 hours at 25ºC.

Height measurements were obtained using AFM image analysis software (Multimode) Scanning probe microscope (NTMDT, NTEGRA prima, Russia)

Encapsulation efficiency

Nanoparticles were separated from aqueous phase by ultracentrifugation (Eppendr of) at 13000 rpm and 4°C for 45 minutes. The supernatants were collected and evaluated for 5 Fluorouracil residues by UV. The encapsulation efficiency (EE) was determined indirectly by measurement of the amount of free 5 Fluorouracil in the supernatant after ultracentrifugation and was calculated according to the following equation:

$$EE = \frac{\text{Amount of total drug - Amount of free drug in supernant X 100}}{\text{Amount of total drug}}$$

***In vitro* release**[10]

A modified dialysis method was used to evaluate the *in vitro* release of 5 Fluorouracil-loaded chitosan NPs. Two millilitres of nanoparticles suspension (corresponding to 2 mg of 5 Fluorouracil) was placed in a dialysis bag (cellophane membrane, molecular weight cut off 10,000–12,000, Hi-Media, India) which was tied and placed into 20 ml of phosphate buffer (0.1 M, pH 7.4) maintained at 37ºC with continuous magnetic stirring. At selected time intervals, aliquots were withdrawn from the release medium and replaced with the same amount of phosphate buffer. The sample was assayed spectrophotometrically for 5 Fluorouracil at 266 nm.

***In vitro* cytotoxicity of nanoparticles**[11]

$CaCo_2$ cells were obtained from National Center for Cell Science (NCCS) Pune. 5000 $CaCo_2$ cells were seeded per plate in a 96 well TC grade plate. The cells were incubated for 24 hr at 37ºC, 5% CO_2. The culture Medium used is DMEM+ 5% FCS. The medium was removed next day and 100 µl of medium were added at the required concentrations in triplicates. The cells were incubated with pure 5 Fluorouracil drug solution, 5 Fluorouracil loaded chitosan nanoparticles and 5 Fluorouracil loaded chitosan nanoparticles conjugated with hyaluronic acid at the concentration of 10, 50 and 100 µg/ml and incubated for 24 hrs, 5 µl of MTT solution was added and incubated for 5 hrs at 37ºC. At the end of incubation period the dye was removed and 100 µl of DMSO was added. Optical density was measured in an ELISA plate reader at 540 nm Percentage toxicity was measured against control.

RESULTS AND DISCUSSION

In the present study we developed a nanoparticulate system which is composed of hydrophilic polymer chitosan possessing the following advantages like obtaining NP by mild agitations absence of organic solvents and high temperature and obtaining NP with positive charge which could enhance the cellular uptake chitosan produces low to high positive charge which could enhance the cellular uptake and has mucoadhesive property.

Conditions for formation 5 fluorouracil loaded chitosan nanoparticles

Chitosan NPs were prepared by simple scale up ionotropic gelation method similar to the method developed. Chitosan is a cationic polyelectrolyte the nanoparticles were formed by inducing the gelation by controlling its interaction with polyanion TPP which leads to reduce the aqueous solubility of CS this system based on inter and intramolecular linkages created between TPP and positive charge of charged amino groups of CS which are responsible for the successful formation of the nanoparticles. The CS/TPP ratio is rate limiting step and controls the size and size distribution of nanoparticles.

In order to obtain nanoparticles under 250 nm we studied the effect of the CS/TPP ratio on the formation of nanoparticles. The maximum concentration of CS and TPP used was up to 6 mg/ml. The particle size, PDI, drug encapsulation and zeta potential were analyzed and the results are presented in Table No.1.

Our results indicated that particle size depend on both CS and TPP concentration that the specific concentration of CS/TPP can only form the nanoparticles with smaller size.

Effect of chitosan concentration

The role of chitosan concentration (0.2, 0.4 and 0.6%) on formation of nanoparticles and its influence on particle size was evaluated. When the amount of TPP was kept constant as 0.2% and an increase in CS concentration from 0.2% to 0.6% showed a decrease in the particle size with favourable PDI value. When the amount of chitosan exceeded 0.6% of CS a highly

opalescent suspension is formed and it also leads to aggregation. Recent studies reported that when the concentration of CS is low (0.6%) it forms a low viscosity gelation medium resulting in a decrease in liquid phase dispersion, thus promoting formation of smaller particles.

Effect of tpp concentration

The role of TPP (0.2, 0.4 and 0.6%) concentration on particle size formation was studied. The increase in TPP concentration showed an increase in particle size. The TPP concentration with 0.2 and 0.6 chitosan forms particle 250 nm at the same time TPP concentration at 0.4 and 0.6% with 0.4 and 0.6% of CS concentration it showed a huge increase in particle size results in micro particles. When TPP concentration above 0.4% it results in highly opalescent suspension on storage it starts settling of particles.

Effect of sonication on particle size

The sonication time in the formation of CS-NP played a crucial role in the formation of smaller size nanoparticles. The smallest nanoparticles (232± 4 nm) were obtained with the sonication time of two minutes. While employing ultra-sonication formation of acoustic cavitations is the main cause for decreasing particle size. Acoustic cavitations by creating a large shear force on the chitosan molecules breaks the particles in to smaller ones. The increase in the sonication time from 30, 60 and 120 seconds showed the decreased particle size presented in (Figure No.1). The sonication time beyond two minutes showed no further decrease in particle size.

PARTICLE SIZE AND ZETA POTENTIAL

The nine formulations were prepared with various concentrations of chitosan and TPP. The particle size distribution of prepared CS nanoparticles was ranged from 232 ± 4 to 2857±6 nm. With increasing the concentration of CS we observed decrease in particle size and increase in zeta value. At 0.2% concentration of TPP the cross linking with chitosan is high (0.6%) this result in more compact particle structure and the neutralization degree of charged amino acid is improved leading the good net charge of the particles. Due to the compact structure and net charge the particles prepared at this concentration have a smaller size.

The zeta potential of the prepared CS nanoparticles was ranged from +3 to +6 mV. When increase in the concentration of CS the zeta value increases due to the higher degree of protonation of amino group in the CS molecule with the strong positive charge which leads to the higher zeta potential.

The optimum concentration of CS/TPP was identified as 0.6% of CS with 0.2% TPP (F3) with size of (232± 4) nm and the zeta potential showed in (Figure No.2 and 3) 5 Fluorouracil loaded CS-NP (F3) was 5 ± 1 mV which indicates the good colloidal stability of the prepared CS NP. The TEM images of the prepared 5 Fluorouracil loaded CS-NP (F3) indicate that nanoparticles were roughly spherical in shape with size of 200 nm shown in (Figure No.4). Further the morphology of the nanoparticles was also analysed using AFM and the 3D image in (Figure No.5) indicates that the particles are in sub spherical shape dense nano particles.

The encapsulation efficiency of 5 Fluorouracil loaded CS-NP were ranged from 55.7 to 69.2%. The increase in chitosan concentration from 0.2 to 0.6% increases in encapsulation was observed at constant TPP concentration of 0.2%. Out of these formulations F3 was selected as the best formulation based on particle size, zeta potential and encapsulation efficiency. The optimized formulation was selected for further studies.

***In Vitro* Release Study**

The cumulative percentage release of optimized 5 Fluorouracil loaded CS-NP (F3) was studied in phosphate buffer pH 7.4 and showed in Table No.2 and (Figure No.6). The percentage release was found to be 98.2 at 24 hrs. The release profile of 5 Fluorouracil loaded CS–NP exhibits a initial release burst release of 25% in one hour followed by the sustained release of 98% at 24 hrs. The observed burst effect was due to the dissociation of drug molecules that were loosely bound to the surface of the chitosan nanoparticles. The second part of the release was slow and sustained release of encapsulated 5 Fluorouracil at an approximately constant rate from the nanoparticles.

***In Vitro* Cytotoxicity Study**

Cytotoxicity of unloaded and 5 Fluorouracil loaded chitosan nanoparticles was evaluated by MTT assay on $CaCo_2$ cell lines, it is used extensively to screen novel compounds for cytotoxicity properties. The results of cytotoxicity were presented in (Figure No.7) There is no significant difference in cytotoxicity between pure drug 5 FU and 5FU nanoparticles at the concentration of 10 and 1* i.e. P less than 0.05 exits between pure drug 5FU and 5 FU Nanoparticles conjugated with hyaluronic acid at the concentration of 10 μg/ml, the value of P is less than 0.001 i.e. 3* exits between 5 FU drug and 5 FU nanoparticle formulation and the value of P IS less than 0.001 i.e. 3* exists between 5FU drug and 5 FU nanoparticle hyaluronic acid at the concentration of 50 μg/ml.

There is no significant difference in cytotoxicity between pure drug 5 FU AND 5FU nanoparticles at the concentration of 100 μg/ml and 3* i.e. P value less than 0.001 exits between pure drug 5 FU and 5FU nanoparticles conjugated with hyaluronic acid at the concentration of 100 μg/ml.

The data suggested that the cytotoxicity of 5 Fluorouracil loaded chitosan nanoparticles conjugated with hyaluronic acid was better than 5 Fluorouracil loaded chitosan nanoparticles and the cytotoxicity of 5 Fluorouracil loaded chitosan nanoparticles was better than the pure 5 Fluorouracil drug solution at 10, 50 and 100 μg/ml concentration. This indicates the safety of the 5 Fluorouracil loaded chitosan nanoparticles conjugated with hyaluronic acid for further use in *in vivo*.

Table No.1: Optimization of Nanoparticles of (CS-NP) on the basis of CS/ TPP ratio

S.No	Formulation code	CS %	TPP (%)	SIZE(nm)	PDI	Zeta Potential (mV)	EE (%)	Physical appearance And opacity
1	F1	0.2	0.2	494±4	0.49±0.03	+3 ±3	65.3	Opalescent suspension
2	F2	0.4	0.2	387±6	0.35±0.05	+4 ±2	67.1	Opalescent suspension
3	F3	0.6	0.2	232±4	0.30±0.07	+5 ±1	69.2	Opalescent suspension
4	F4	0.2	0.4	1274±3	0.38±0.08	+2 ±2	64.3	Highly Opalescent suspension
5	F5	0.4	0.4	1597±2	0.47±0.07	+4 ±7	62.1	Highly Opalescent suspension
6	F6	0.6	0.4	1782±3	0.58±0.05	+6± 2	60.4	Highly Opalescent suspension
7	F7	0.2	0.6	1941±2	0.64±0.03	+3 ±4	57.8	Highly Opalescent suspension
8	F8	0.4	0.6	2674±4	0.71±0.08	+4±3	56.9	Highly Opalescent suspension
9	F9	0.6	0.6	2857±6	0.84±0.04	+6±5	55.7	Highly Opalescent suspension

Table No.2: Cumulative % Drug Release of 5fu Loaded Nanoparticles

S.No	Time (hr)	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	0	0	0	0	0	0	0	0	0	0
2	1	18.4	28.4	24.7	22.5	29.3	27.6	23.8	27.8	21.4
3	2	27.8	34.2	34.8	31.7	37.4	35.9	30.6	37.2	32.5
4	4	34.7	48.3	39.8	39.6	48.2	43.7	38.2	43.4	39.7
5	6	47.9	58.5	43.2	44.2	53.9	55.2	43.5	49.3	43.6
6	8	56.2	62.7	51.9	52.5	62.3	62.9	50.7	55.2	59.2
7	10	68.3	68.8	63.1	61.7	68.4	68.5	58.3	61.1	64.4
8	12	74.1	73.4	72.8	69.1	74.4	72.8	69.7	74.2	71.8
9	16	79.3	78	77.2	74.7	79.3	78.4	78.6	79.7	77.2
10	20	84.2	85.9	83.7	84.8	83.4	84.1	83.2	84.3	83.4
11	24	88.4	89.2	98.2	87.2	86.5	90.5	86.4	89.1	90.1

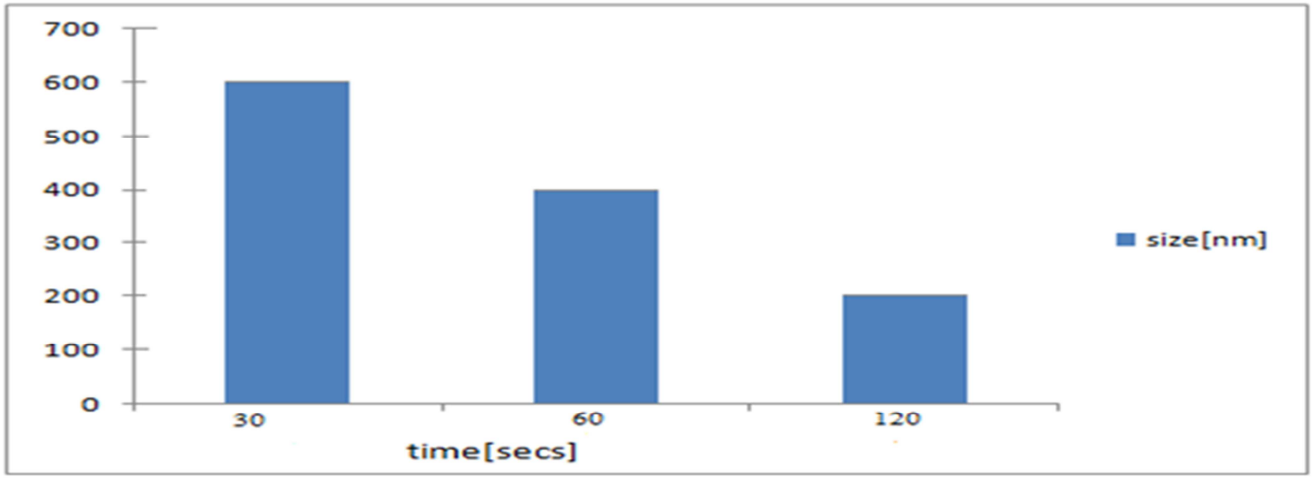

Figure No.1: Effect of sonication time on particle size

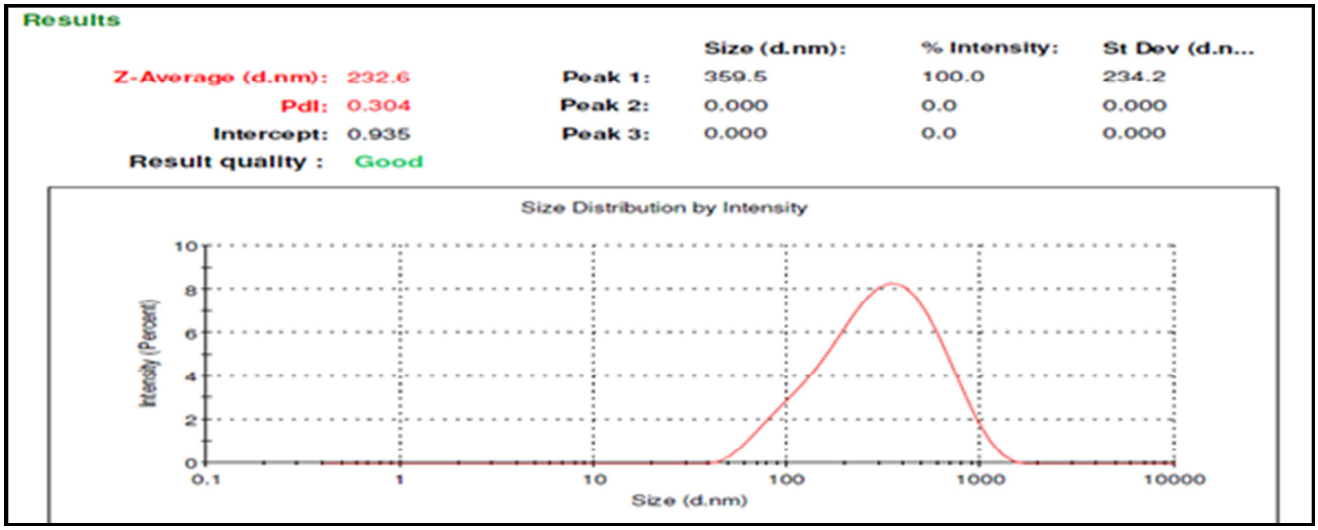

Figure No.2: Particle size of F3

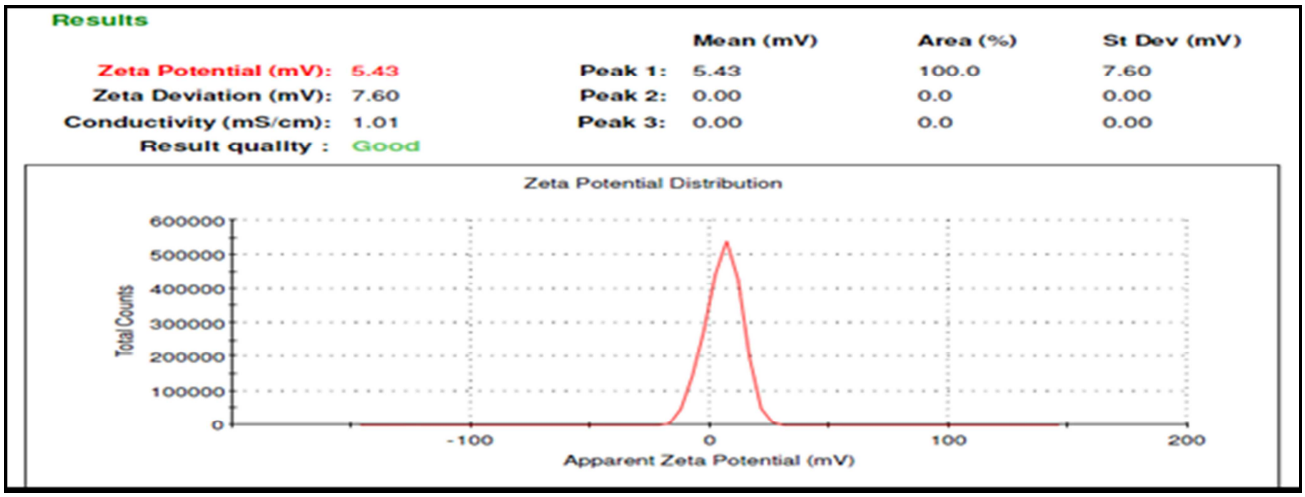

Figure No.3: Zeta potential of F3

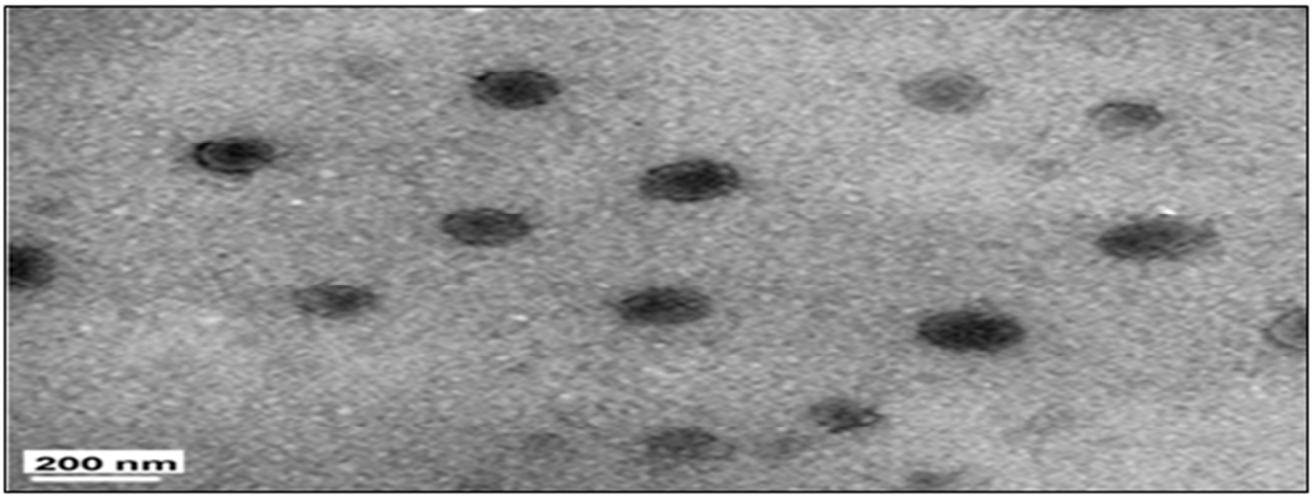

Figure No.4: TEM images of 5FU

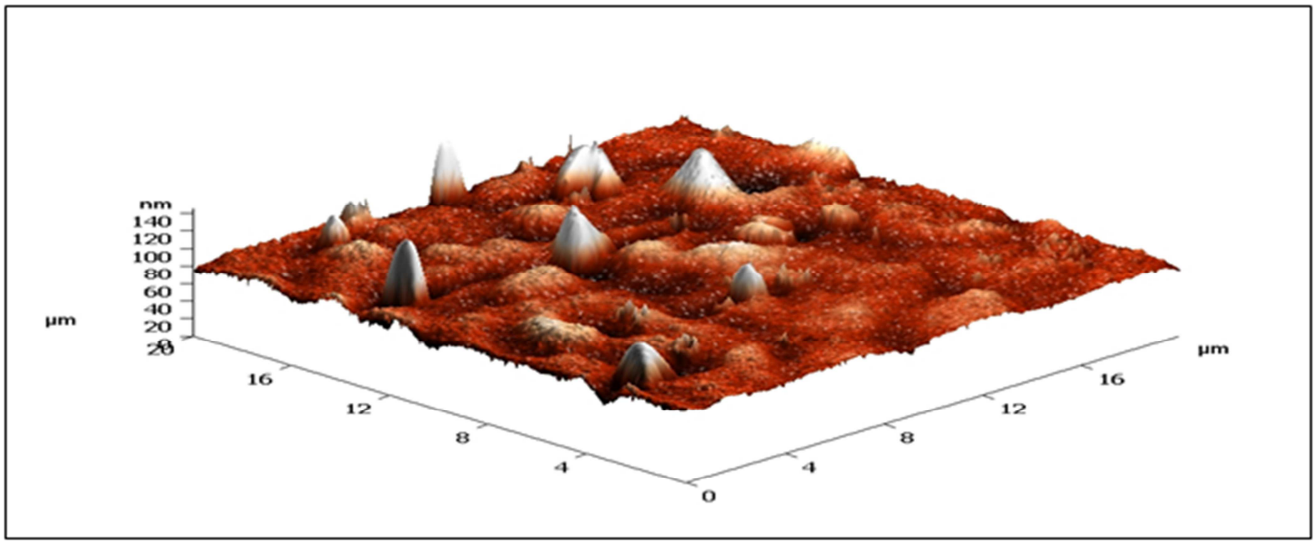

Figure No.5: 3D AFM image of 5FU

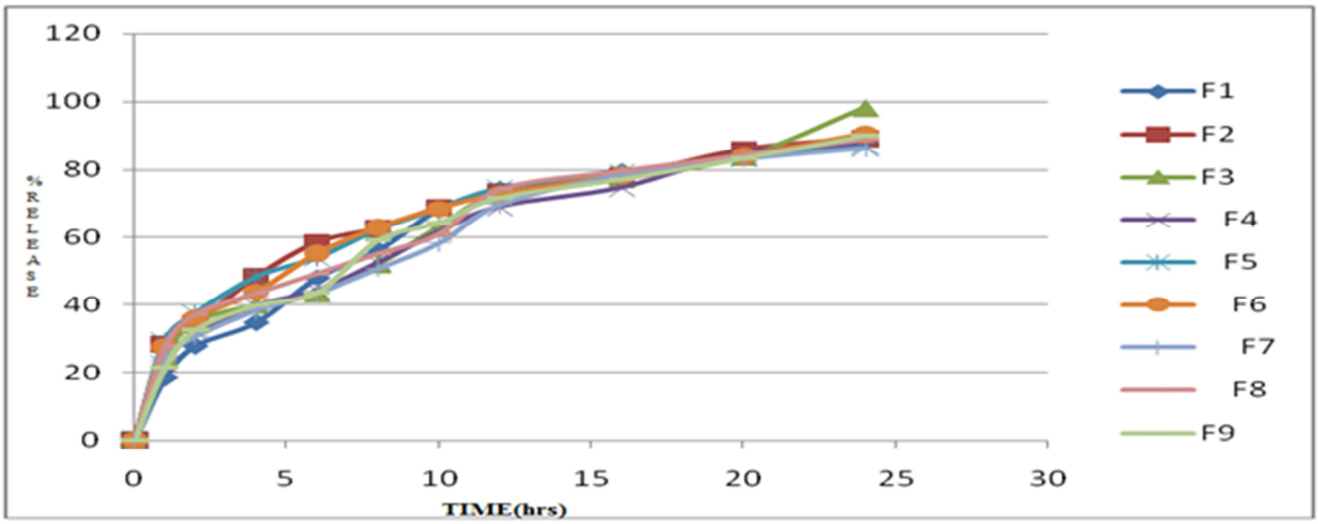

Figure No.6: Cumulative % release graph

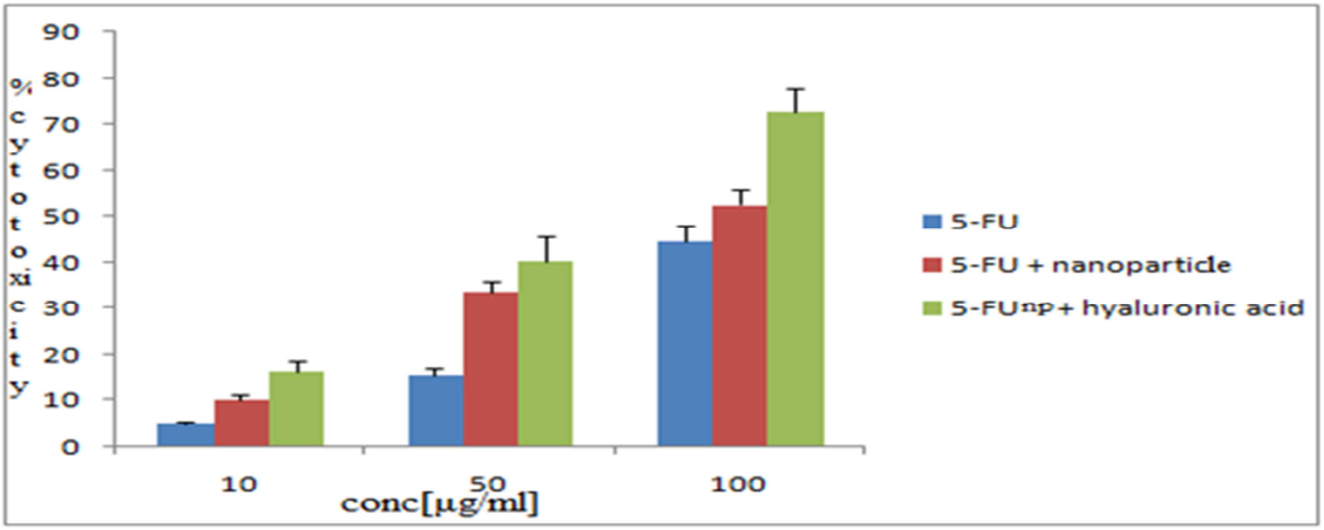

Figure No.7: *In Vitro* Cyto Toxicity Study

CONCLUSION

This study demonstrates the ionic gelation method can be used to load hydrophilic drugs and produce the size of less than 200 nm. The concentration of CS, TPP and sonication time strongly effect the particle size formation of the CS-NP. The CS-NP composed of 0.6% CS and 0.2% TPP was selected as the optimized formulation which produced smaller particle with better encapsulation. *In vitro* cytotoxicity study suggested the safety of the prepared 5 Fluorouracil loaded chitosan nanoparticles conjugated with hyaluronic acid which can be potential carrier to deliver hydrophilic drugs to target colorectum. Further *In vivo* will confirm the targeting efficiency of Fluorouracil loaded chitosan nanoparticles conjugated with hyaluronic acid to treat colorectal cancer.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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