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RESEARCH ARTICLE

Design and characterization of Gaur gum coated chitosan nanoparticles for delivery of 5-Fluorouracil for effective treatment of colon cancer

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ABSTRACT

The present study was aimed to develop gaur gum (GG) coated chitosan nanoparticles as vector to provide successful delivery of 5-fluorouracil (5-FU). Guar gum anchored chitosan nanoparticles were prepared by cross linked with bifuctional PEG (NH2-PEG-COOH) for the treatment of colon cancer. Nanoparticle preparation was done by nanoprecipitation method to protect the drugs in gastric fluids and achieve a targeted drug delivery to colon. The characterization of GG coated chitosan nanoparticles was done by Fourier Transform Infrared Spectrophotometry (FT-IR), scanning electron microscopy (SEM), atomic force microscopy (AFM), average particle size in (Zeta seizer), Particle size analyzer. The drug polymer interactions were checked by Differential scanning calorimetry (DSC). The amount of 5-FU loading estimated by high performance liquid chromatography (HPLC) and in vitro drug release characterized. These studies revealed that controlled release behavior of Gaur gum coated chitosan NPs could be a potential alternative pharmaceutical formulation in treatment of colon cancer..

INTRODUCTION

Chitosan, a natural cationic polysaccharide, is prepared industrially by the hydrolysis of the aminoacetyl groups of chitin, a naturally available marine polymer. Chitosan is a non-toxic, biocompatible and biodegradable polymer and has attracted considerable interest in a wide range of biomedical and pharmaceutical applications including drug delivery, cosmetics, and tissue engineering. The primary hydroxyl and amine groups located on the backbone of chitosan are responsible for the reactivity of the polymer and also act as sites for chemical modification. However, chitosan has certain limitations for use in controlled drug delivery and tissue engineering. These limitations can be overcome by chemical modification. Thus, modified chitosan hydrogels have gained importance in current research on drug delivery and tissue engineering systems.Biocompatible, biodegradable hydrogels have been designed using natural polymers that are susceptible to enzymatic degradation, or using synthetic polymers that possess hydrolyzable moieties. Of these, hydrogels using the natural polymer, chitosan, have received a great deal of attention due to their well documented biocompatibility, low toxicity and degradability by human enzymes (1, 2, 3).

Cancer has several physiological obstacles (4, 5), like vascular endothelial pores, heterogeneous blood supply, heterogeneous architecture etc. For a medication to be productive, it is very essential to overcome these obstacles. Cancer corresponds to an immense biomedical challenge (6), for drug delivery. Cancer treatment is very much reliant on the method of delivery. In the past, cancer patients used numerous anticancer drugs, but these drugs were found to be less effective and had major side effects. Nanoparticles have exerted a pull on the attention of scientists due to their multifunctional behaviour. The treatment of cancer using targeted drug delivery nanoparticles is the newest achievement in the medical field. Nanomaterials are at the cutting edge of the rapidly emerging area of nanotechnology. The possibilities of nanoparticles in cancer drug delivery are infinite with innovative applications constantly being explored.

MATERIALS AND METHODS

Materials

5-FU was procured from as a generous gift samople from Neon Pharmaceutical, Andheri, Mumbai, India. Gaur gum was purchased from CDH, Delhi, India. Chitosan, Pluronic F-68 dialysis membranes, 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were bought from Himedia, Mumbai, India. Acetonitrile, and acetone were purchased from Spectrochem Mumbai, India. All other chemicals used were of analytical reagent grade and were utilized as they received.

Methods

Synthesis of Gaur gum-chitosan Copolymer

Gaur gum (20 mg) was dissolved in 10 ml of distilled water under the temperature 30°C to 120°C and maintain the pH 5.0 with the addition of formic acid and magnetically stirred (Remi, Mumbai, India) for 24 hrs and then fivefold additional 250 mg of bifunctional PEG (NH2-PEG-COOH) was added with constant stirring, followed by addition of 250 mg of EDAC. The reaction was carried out at room temperature for 12 hrs. Chitosan (50mg) was dissolved in 10 ml of distilled water and then added to the polymeric solution of gaur gum and then magnetically stirred (Remi, Mumbai, India) for 24 hrs. The acquired copolymer named GCNPs was dried under vacuum. Synthesis of GCNPs was authenticated by IR spectroscopy. IR Spectra of GCNPs were recorded with a FT-IR spectrophotometer (Cary- 630 FTIR, Agilent Technologies).

Preparation of Gaur gum-chitosan nanoparticles (GCNPs)

GCNPs (10 mg) was dissolved in 10 ml of distilled water. 5-FU (30 mg) was dissolved in 50 ml of acetone and pluronic F-68 (125 mg) was added into UA solution. GCNPs solution was added dropwise in pluronic solution with continuous stirring on magnetic stirrer for 2 hrs. Nanoparticles were developed on solvent interface. Then, the solvent was evaporated at room temperature. The resultant suspension of nanoparticles was filtered through 0.45 μm membrane filter (Millipore) and centrifuged for 40 min at 10,000 rpm (Remi, Mumbai, India). The supernatant was discarded and GCNPs NPs were lyophized and kept for further use. GCNPs was lyophized kept for further use. Moisture content of lyophilized NPs was determined by Karl-Fischer Titrator (Dolphin Instruments, Mumbai (MH) India.

Characterization of GCNPs.

Morphology

The detailed surface characteristics of the selected 5-FU loaded GCNPs formulation was observed using a scanning electron microscopy (SEM) (JELO 5400, Japan). The samples for

SEM were prepared by lightly sprinkling the NPs powder on a double adhesive tape which stuck to an aluminium stub. The stubs were then coated with gold to a thickness of about 300Å using a sputter coater. The shape and surface morphology of nanoparticle was investigated by Atomic Force Microscopy, AFM (AIST-NT Smart SPM 1000, CA). AFM of the nanoparticles was carried out at glass substrate in AC mode.

Particle size and zeta potential

A properly diluted dispersion of GCNPs was filled in the chamber of a laser diffraction particle size analyzer (DTS Ver. 4.10, Malvern Instruments, WR14 1XZ, UK) and the average particle size and PDI were determined. The zeta potentials of the GCNPs were determined by laser doppler anemometry using a Malvern Zetasizer (DTS Ver. 4.10; Malvern Instruments, WR14 1XZ, UK).

DSC analysis

DSC analysis was performed by employing DSC 60 instrument (Shimadzu, Tokyo, Japan). The samples (Gaur gum, Chitosan, 5-FU and 5-FU loaded GCNPs) were weighted into aluminum pan and closed with a pin-holed lid. Thermograms were observed under nitrogen atmosphere from ambient to 350ºC at a heating rate of 10ºC per min.

XRD analysis

XRD diffraction analysis was executed in order to exemplify the crystalline nature of polymer and drug. Powder X-ray diffraction patterns of samples (Gaur gum, Chitosan, 5-FU and 5-FU loaded GCNPs) were attained by using power X-ray diffractometer (Bruker, Munich, 123 Germany) a nickel-filtered Cu-Ka radiation (a voltage of 40 kV and a current of 20 mA). The 124 scanning rate was 2θ/min over a 2θ range of 0–40º and with an interval of 0.02º.

Entrapment efficiency

Ten milligrams of 5-FU loaded GCNPs dissolved in acetone. The mixture was initially centrifuged and then supernatant was diluted by using methanol-PBS (pH 7.4) mixture. Subsequently, the quantity of entrapped drug was analyzed with HPLC (Agilent Technologies, 1220 infinity LS, UK). The HPLC system (Agilent Technologies, 1220 infinity LS, UK) consisted of variable wavelength detector and a zorbax 5µ C18 column (250 × 4.60 mm) was utilized for the

analysis of drug. The mobile phase was water: methanol (95:5 v/v) pumped at a flow rate of 1 mL/min at 25°C. The mobile phase was out-gassed under vacuum prior to use (7).

In-vitro release study

Ten milligrams of 5-FU loaded GCNPs suspended in 2 mL of PBS (pH 7.4) and successively placed into a dialysis tube (MWCO 2000 Da) separately. The dialysis tube was placed into 50 mL of aqueous recipient medium of PBS (pH 7.4). The release media was stimulated at 100 rpm at 37 ± 2°C. A whole-media change method was used to prevent the drug saturation in the drug release study. At particular time intervals, the whole medium (50 mL) was substituted with the same volume of fresh PBS (pH 7.4) (50 mL). The samples were then analyzed by HPLC.

Hemolytic toxicity

Whole human blood was collected and stored in HiAnticlot blood collection vials as described in our previous paper (8, 9). The human blood was centrifuged then red blood cells were separated and resuspended in normal saline solution (10% hematocrit). One ml of the red blood cell suspension was individuall incubated with 5 mL of distilled water (taken as 100% hemolytic standard) and 5 mL of normal saline (taken as blank for spectrophotometric evaluation). Further, 1 mL of adequately diluted GCNPs (negative control), 5-FU loaded GCNPs and plain 5-FU were added to 5.0 mL of normal-saline and interacted with RBC suspension. The suspension was centrifuged for 10 min at 2000 rpm and the absorbance of supernatants was measured at 540 nm, which was used to evaluate the percentage hemolysis using distilled water as 100% hemolytic standard.

Cellular cytotoxicity study

Cellular cytotoxicity was analyzed by tetrazolium dye-based MTT assay b adhering to the procedure which was stated earlier (8, 10), and colon-26 (C26) cellline was maintained in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics at 37ºC in a humidified incubator containing 5% CO2. The cells were treated with drug loaded nanoparticles in various concentrations of 10, 20, 30 and 40 µg/ml for 24 hrs. The amount of 5-FU loaded GCNPs needed to prepare molar equivalents of 5-FU. Control was taken without any drug treatment. Afterward, MTT was added and plates were then incubated for another 3 hrs, the media was pipetted off and 250 IL DMSO was added. The absorbance of individual wells was noted at 570

nm via an ELISA plate reader at 25 ºC. Average values from triplicate were subtracted from average value of control and the survival fraction of cells was calculated by the formula.

Animal handling

120-200 gm albino rats of both sex which have been acclimatized in the animal facility of Guru Ramdas Khalsa Institute of Science and Technology, Pharmacy, Jabalpur were placed in standard cages where they were maintained on standard animal pellets (obtained from Nuvita Feed Ltd., Kampala). The National Institute of Health Guide for the care and use of Laboratory Animals approved by the Institutional Ethical Committee was adopted for the animal protocol in this study. Ethical Committee registration number is 147/PO/a/11/CPCSEA.

Blood level study

Blood level studies of sustained release formulations were finally taken up to determine the release and performance in vivo. The formulation were predialyzed and lyophilized. The formulation having 200µg was administered i.v. to the rats. The formulated nanoparticles were used to detect blood level of drug at various time intervals. Six animals was taken and divided into three groups. To first group 200µg drug (5-FU) solution was administered i.v.to the rats. To second group GCNPs having equivalent drug content of 200µg were dissolved in PBS (pH 7.4) administered while third group was kept as control. The concentration of drug in blood was derermined at various time intervals. The concentration of dug in blood samples were measured by UV spectrophotometer at 267nm. The blood samples was withdrawn at various time intervals and blood was clotted and washed by vortexing with normal saline and washings were centrifuged at 2000 rpm for 15 minutes. The serum was deproteinized by acetonitrile. The sample was centrifuged and supernatant were analysed for drug concentration. The concentration of dug in blood samples were measured by UV spectrophotometer at 267 nm. The standard curve of serum was plotted between absorbance and concentration (11).

Statistical analysis

Investigational results were expressed as mean ± SD (number of experiments). Statistical evaluations were obtained by t-test. $P < 0.05$ was considered to be noteworthy. All experiments were repeated three times.

RESULT AND DISCUSSION

The intention behind the present investigation was to analyze the delivery of 5-FU from GCNPs which were formulated by using Gaur gum-chitosan copolymers. These NPs had tumor specific targeting capability. In order to accomplish cost effective tumor specific targeted carrier.

FTIR and NMR spectroscopy

Synthesis of gaur gum-chitosan copolymer was validated by FTIR spectroscopy. The FTIR spectroscopic graph of gaur gum-chitosan copolymer is depicted in Figure no. 1. In FTIR spectra of gaur gum-chitosan copolymer, peaks at 1023, 1100, 1110 and 1420 cm^{-1} verified the presence of chitosan. 531, 1036, 1045, 1443, 1495, 1623 and 3350 cm-1, confirmed the presence of GG Typical peaks at 587, 935 and 2723 cm-1 illustrated presence of bifunctional PEG. Typical peak of N-H stretch of amide at 3450 cm-1 and C=O stretch at 1623 cm-1, were used to authenticate the formation of gaur gum-chitosan copolymer.

Morphology

The shape of NPs was visualized under AFM and SEM, and photomicrographs were acquired. The NPs were found to be spherical in shape and having smooth surface which were of nanometric size range (91.17-130 nm) as monitored by AFM and SEM (Figure no. 2a and 2b).

Particle size, zeta potential determination and drug entrapment efficiency

The average particle size of GCNPs were found to be 91±1.17 nm and particles size distribution of GCNPs were found 74.1% of 98.4 nm, 20.5% of 81.35 nm and 5.4% of 87.64 nm and zeta potential of GCNPs was found to be -12.4mV. The foremost challenge lies in the division between single particles and agglomerates comprising two or three single particles. The larger sizes are attributed to the particles that were counted as single particles when no difference between the single particle and agglomerate was possible from the micrograph. The effect of surfactant concentration on size distribution has exclusive importance in GCNPs. Thermodynamic effect of surfactant addition in emulsion formation has been known since the earlier times. (12).

As the conclusions, the drug entrapment efficiency and particle size of GCNPs were majorly affected by polymer concentration and concentration of surfactant. With increasing concentration of polymer (10 to 20 mg) and Pluronic F-68 concentration (1-2%), alters the NPs characteristics and is used in various concentrations and therefore some batches were prepared. The results are depicted in Table no. 1, particle size was increased from 91±1.17 to 130±1.50 nm and drug encapsulation efficiency of NPs was decreased from 93.15±0.50% to 76.21±1.19%. It may be attributed that the viscosity of dispersed phase increases, which causes weaker dispersibility of the GCNPs solution into the aqueous phase (13).

The higher concentration of surfactant decreases the size of the NPs. When the amount of Pluronic F-68 was increased it was found that the granulometric distribution became restricted. This phenomenon can be supposed from the stabilizing function of a surfactant upto a definite concentration, more increase in amount of surfactant imparts little or no effect on particle and entrapment efficiency. It was also perceived that when a small concentration of surfactant is used, it leads to aggregation of the particles and the size of particles may increase (14).

Polydispersity index of all GCNPs prepared during optimizing process variable were found 0.046±0.07. Samples with the broad size distribution have polydispersity index values > 0.7 (15).

DSC analysis

DSC thermogram of Gaur gum, Chitosan, 5-FU and 5-FU loaded GCNPs was carried out and thermograms were displayed in Figure no. 3 (a, b, c and d). In the case of 5-FU, an endothermic peak was spotted at 280ºC (Figure no. 3a). In case of Gaur gum, an endothermic peak was obtained at 110ºC (Figure no. 3b). In case of Chitosan, small endothermic peak was observed at 100ºC (Figure no. 3c). In case 5-FU loaded GCNPs. However, the endotherm of 5-FU is detected at 280ºC in the DSC thermogram of drug loaded NPs, indicating that the drug exists in crystalline form inside the nanoparticles (Figure no. 3d).

XRD analysis

The XRD of Gaur gum, Chitosan, 5-FU and 5-FU loaded GCNPs are illustrated in Figure no. 4. In the XRD of 5-FU (Figure no. 4d), intense peak were observed between 2θ values of 6, 18, 22 and 24. However 5-FU loaded GCNPs has displayed typical intense peaks between 2θ values of 6, 7, 18, 19, 22 and 24 which revealed the increase in crystallinity and 5-FU associated with the crystalline form (Figure no. 4a).

In-vitro drug release

The release of 5-FU from the gaur gum-chitosan NPs demonstrated that the NPs have sustained release property (Figure no. 5). The conclusions established the fact that there was a prominent time prolongation of 5-FU release from NPs system. GCNPs are able to release 93.61% 5-FU in 32 hrs.

Hemolytic toxicity

Hemolytic toxicity study was executed to evaluate the hemotoxic effect of the GCNPs. The plain 5-FU, 5-FU loaded GCNPs have exhibited hemolytic toxicity upto 27.16±1.19% and 3.91±1.17% individually. The 5-FU concentration for nanoparticulate formulations was determined on the basis of the drug content. Feasibly, the encapsulation of drug molecules in the NPs and consequential delayed release results in a significant reduction in the hemolytic toxicity. Moreover, 5-FU loaded GCNPs demonstrated lesser hemotoxicity in comparison with plain 5-FU. This might be because of the hydrophilic nature of gaur gum and chitosan which brings about the system more hemocompatable.

In-vitro cell line study

The growth inhibition of NPs on colon-26 (C26) cell was examined by MTT assay. The conclusions evidently indicated a dose-dependent cytotoxicity that was reduced by cellular viability upon increasing the concentration of 5-FU. The subsistence fraction of cells upon incubation of plain 5-FU and NPs formulation in different concentration is displayed in Figure no. 6. After incubation of 5-FU loaded GCNPs it exhibited the inhibitory effect on cell growth, the percentage control growth was decreased with the increasing of the concentration of 5-FU. Moreover, the cell viability gets reduced when the concentration of 5-FU either in free form or inside the NPs was enhanced. In the concentration range of 10–80µg/ml, NPs were cytotoxic to a greater extent when compared to plain 5-FU. Cytotoxic effect of 5-FU loaded GCNPs in C26 was discovered to have greater inhibitory effect. These findings

established the fact that GCNPs (cytotoxicity caused by 5-FU to the tumor cells was dose dependent and had a superior cytotoxic effect on the tumor cells as compared to plain 5-FU.

Blood level study

In blood level study in case of plain drug solution higher concentration of drug was found in blood during one hour 36.32±0.19% and then declined to 4.9±0.76% with increasing time. In case of gaur gum coated chitosan nanoparticles initial drug concentration in blood was 4.73±1.19% and then increased to 30.32±0.47% with time and then stabilize for some time after then decline slowly upto 26.29±0.5% in 24 hrs shown in Figure no. 7. By the coated gaur gum-chitosan NPs, % 5-FU level was stabilized to significant amount of requirement which slowly localized the coated system and sustained the release of drug.

CONCLUSION

The GCNPs loaded with 5-FU were acquired lucratively by nanoprecipitation technique with high 5-FU entrapment efficacy and low particle size. GCNPs have sustained the release of 5-FU for period of upto 32 hrs and also discovered to be more hemocompatable. The conclusions of cytotoxicity analysis revealed that the cytotoxicity of 5-FU loaded GCNPs seems to be less cytotoxic to C26. On the basis of these findings, the preparations formulated in this work could be favorable for in vivo 5-FU drug delivery systems.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Table 1: Ingredients and concentration using in the formulation of GCNPs.

S. No.	Internal phase		External phase			% Entrapment efficiency	Particle size (nm)
	Gaur gum	Chitosan	Drug (5-FU)	Pluronic F68	Acetone		
1	10	50	30	250	25	89.53±0.5	94.58±1.50
2	20	50	30	500	25	93.15±0.50	91±1.17
3	10	50	30	250	25	76.21±1.19	130±1.50
4	20	50	30	500	25	79.16±1.17	119.68±0.7

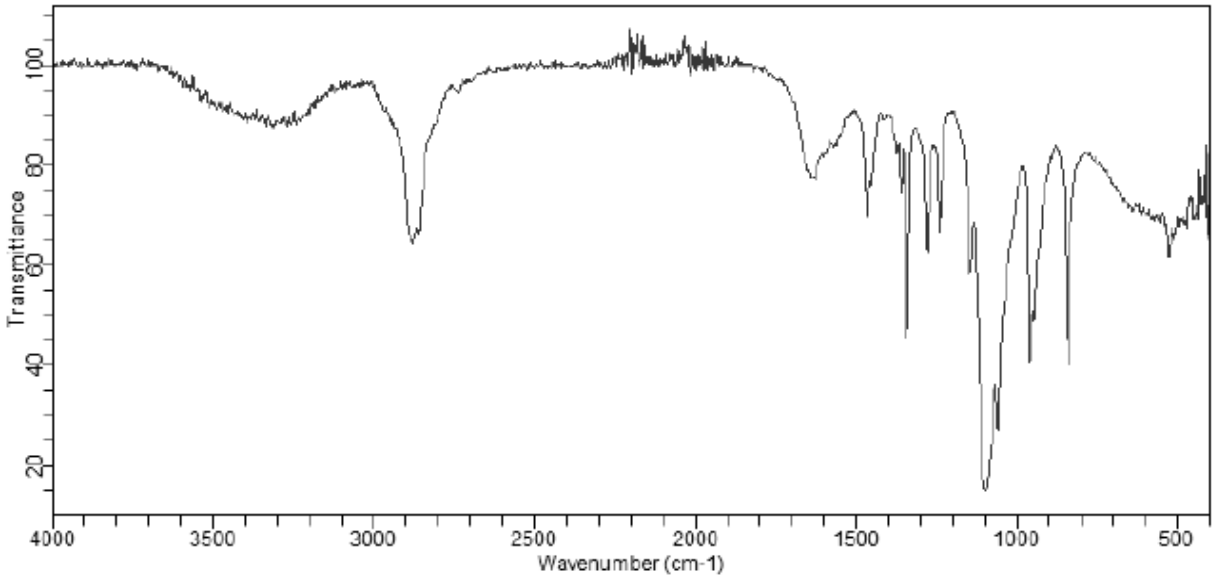

Fig.1: FTIR of GCNPs.

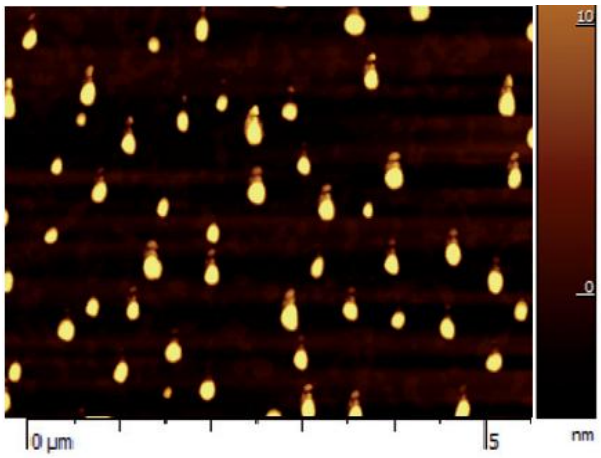

Fig.2a: AFM image of GCNPs.

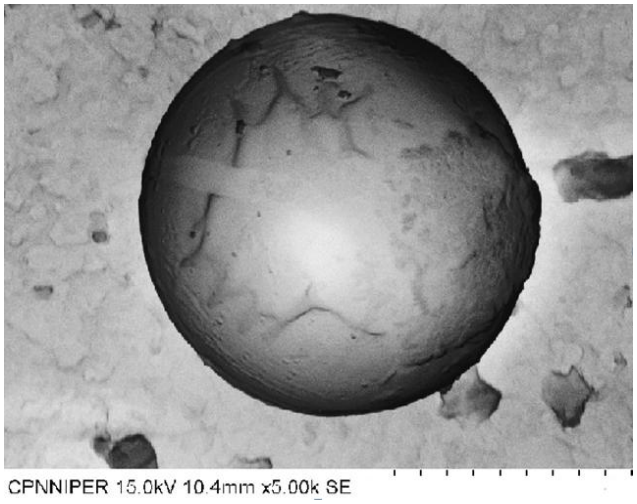

Fig.2b: SEM image of GCNPs

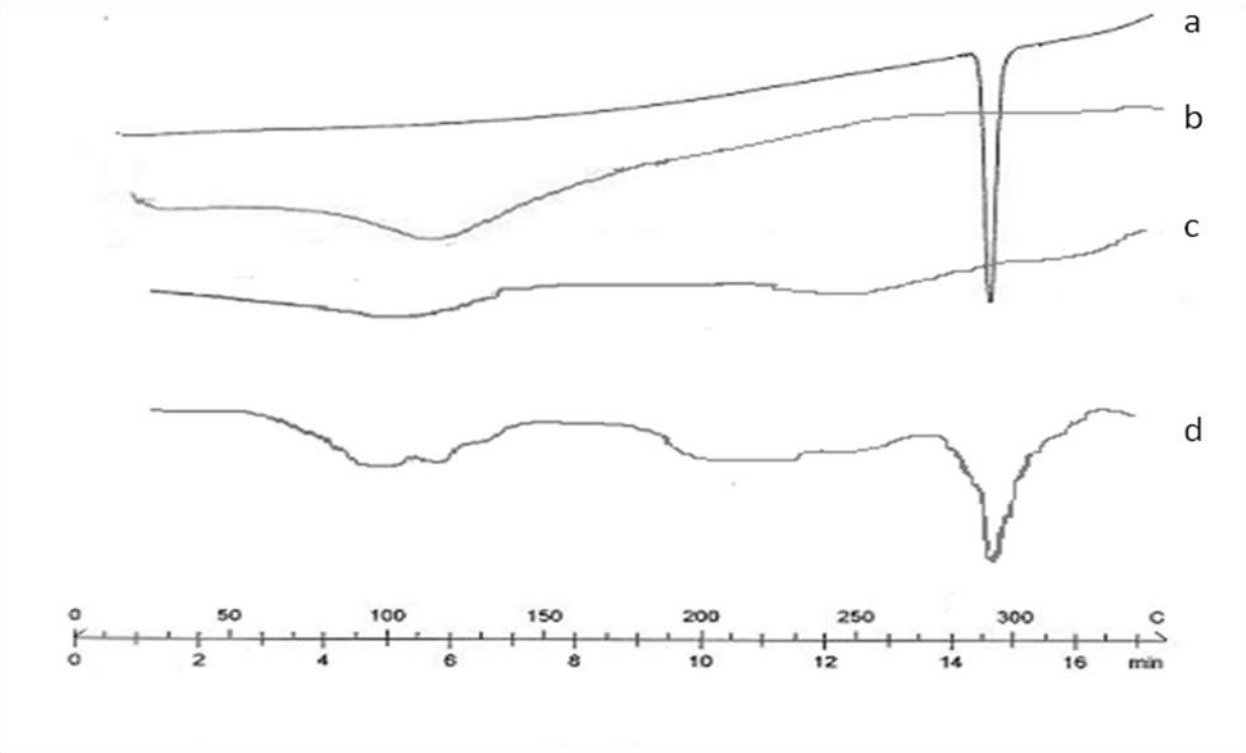

Fig.3: DSC thrmogram of (a) 5-FU, (b) Gaur gum, (c) chitosan, (d) 5-FU loaded GCNPs

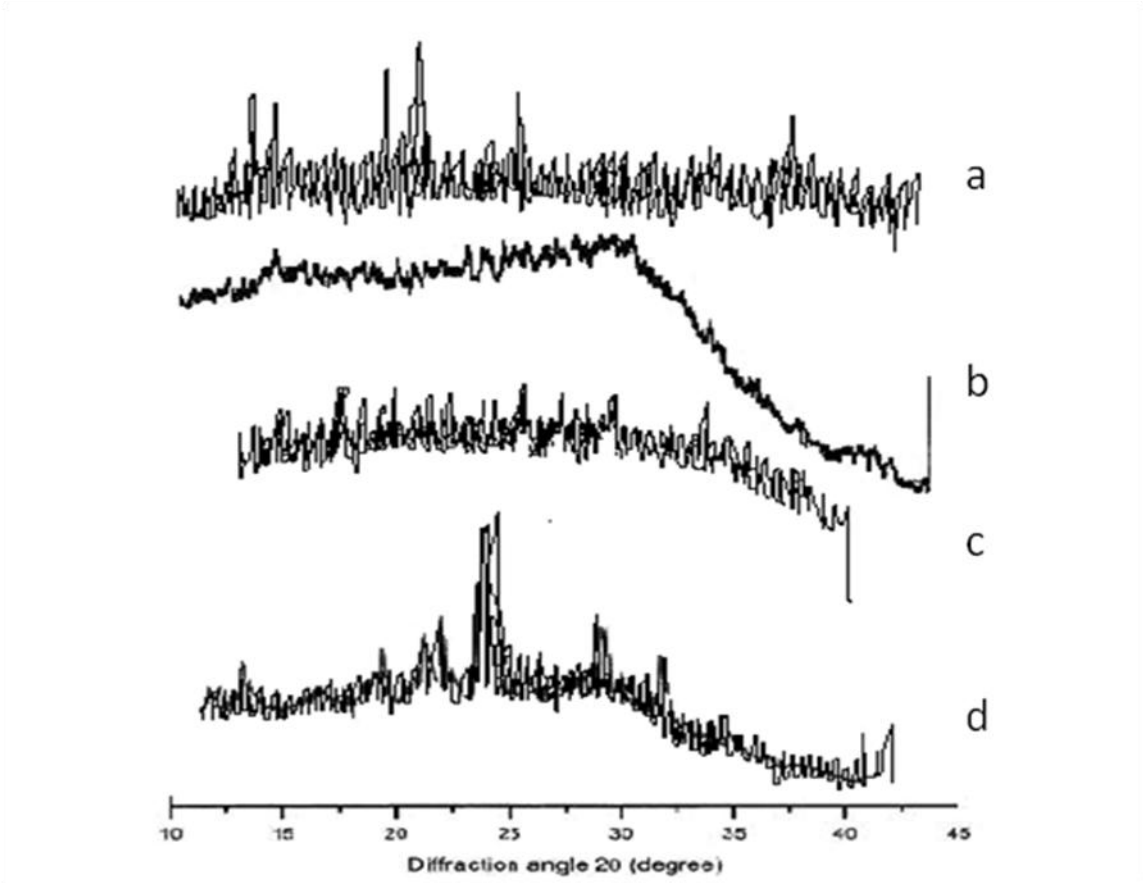

Fig.4:XRD of (a) 5-FU loaded GCNPs, (b) Gaur gum, (c) Chitosan, (d) 5-FU

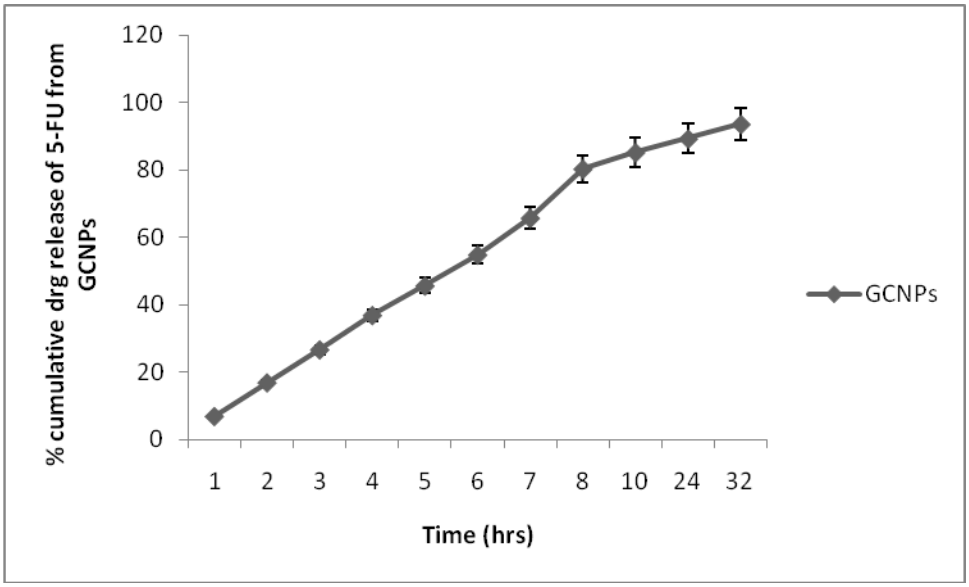

Fig.5: Percentage Cumulative Drug Release of GCNPs

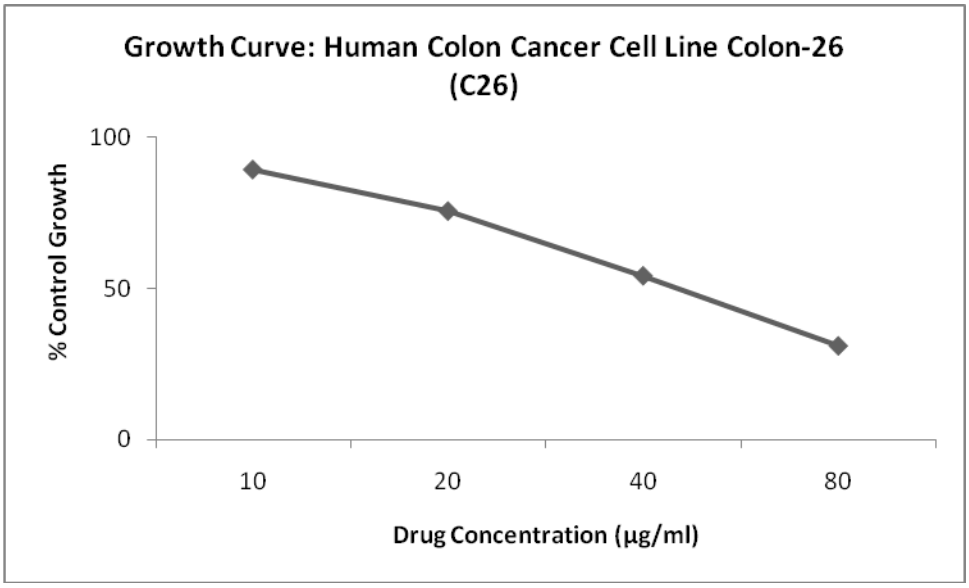

Fig.6: *In-vitro* cellline study of GCNPs on C26 cellline

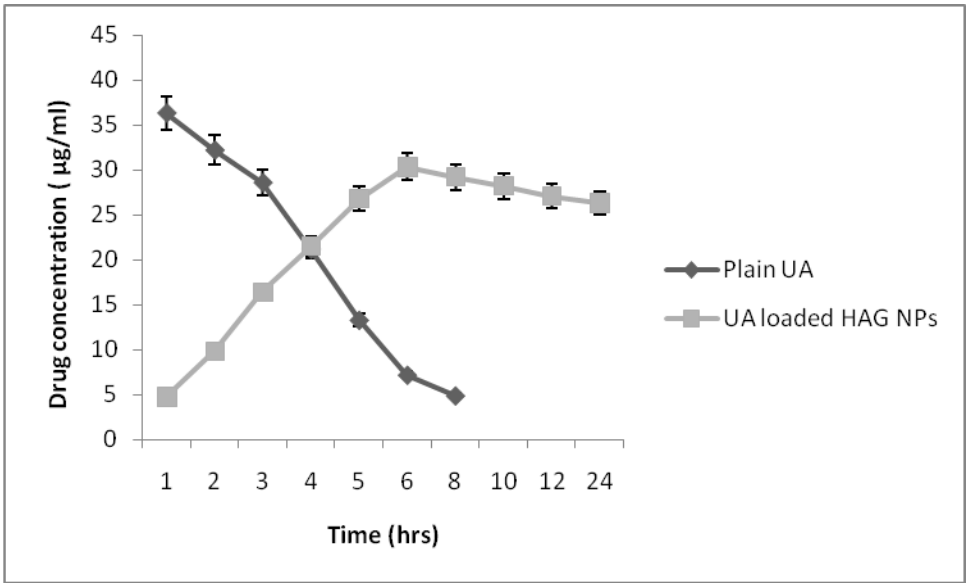

Fig.7: Blood Level Stdy of GCNPs