



Evaluation of Anticancer Activity and *In-Vitro* Release Assay of a Polymeric Microsphere Formulation for Colon Targeted Delivery of 5-Fluorouracil using Biocompatible Natural Gum Katira.

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Abstract

Development of anticancer drugs is fraught with challenges which go beyond the typical problems and issues of pharmaceutical research. The chemotherapeutic agent must be able to selectively kill or restrain the growth of neoplastic cells leaving healthy cells unharmed. These drugs are almost always highly toxic, especially to rapidly dividing normally. The present aim was to develop oral site-specific rate-controlled anticancer drug delivery to subdue systemic side-effects, and provide an effective and safe therapy for colon cancer with compressed dose and duration of therapy. The double emulsion, solvent evaporation (or solvent extraction) technique also called the water-in-oil-in-water (W/O/W) emulsion was used to encapsulate hydrophilic compounds, overcoming the limitations of a single oil-in-water emulsion. Drug release-retarding polymers are the key performers in designing a microsphere dosage form for oral sustained release formulation. The microspheres were then characterized for drug loading, entrapment efficiency, SEM (Scanning Electron Microscopy), FTIR (Fourier transform infrared spectroscopy). To verify the functionality of 5-FU loaded gum katira microsphere for sustained drug release systems, cell cytotoxicity and in-vivo anticancer activity were also tested. The drug release was checked in the physiological media of the gastrointestinal tract, i.e., simulated gastric fluid (SGF) and simulated colon fluid (SCF). Formulated and optimized polymeric microsphere of 5-FU using gum katira polymer own optimal physicochemical characteristics with a fine spherical particle, with a size of $320.75 \pm 5.73 \mu\text{M}$, high drug entrapment efficiency ($74.87 \pm 1.76 \%$), and satisfactory release pattern of the drug within a time range of 12 h. According to the MTT results, targeted microsphere exhibited good cytotoxicity and have productive anticancer activity on sarcoma180 solid tumor mice. Finally, we foretell that polymeric microsphere of 5-FU using natural gum katira could be a promising micro-carrier for efficient colon targeting delivery tool with improved chemotherapeutic efficacy against colon cancer.

Keywords

Control release delivery; Gum katira; Anti-cancer; 5 fluorouracil; MTT assay; Sarcoma 180; Microsphere.

INTRODUCTION

Cancer is a class of diseases where a group of cells displays *uncontrolled growth* (division beyond the reasonable limits), *invasion* (intrusion on and disruption of adjacent tissues), and sometimes *metastasis* (expanded to other sites in the body via lymph or blood)[1]. Colorectal cancer is the second leading cause of cancer-related death for men and women[2]. Colon cancer is a disease of colon tissue where abnormal cells creating tumors are characterized by unregulated replication, and they may spread to other parts which may lead to the death of the patient[3].

Development of anticancer drugs is fraught with challenges which go beyond the usual problems and issues of pharmaceutical research. The chemotherapeutic agent must be able to selectively kill or inhibit the growth of neoplastic cells leaving healthy cells unharmed[4]. Besides, all approaches to cancer chemotherapy are ideally required to eradicate all a tumor (cancer) cells, and this rarely happens. The available chemotherapy kills only a proportion of exposed or penetrated cells rather than a fixed number of cells[5].

Antimetabolite drugs act by hindering essential biosynthetic processes, or by being incorporated into macromolecules, such as DNA and RNA, and inhibiting their regular function[6]. The 5-fluorouracil (5-FU) does both. 5-FU give cytotoxicity by ascribing to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS)[7]. 5-Fluorouracil (5-FU) also stimulate p53[8].

5-FU causes several side effect including severe gastrointestinal toxicity, hematologic disturbance, severe bone marrow deficiency, skin reactions, hair thinning, hand-foot syndrome and heart toxicosis[9][10]. Also, it is rapidly metabolized and has a short plasma half-life[11].

Sustained release systems incorporate any drug delivery system that achieves slow release of drug over an extended period. If the systems can ascertain some control, whether this to be spatial or temporal nature, or both, of the drug release in the body, or in other words, the system is successful at keeping constant drug levels in the target tissue or cells, it is considered as controlled release system[12].

It is well known that for a drug to be absorbed it first must dissolve in the aqueous phase encompassing

the site of administration and then portion into an absorbing membrane. Two of the most critical physiochemical properties of a drug that influence its absorption behavior are its aqueous solubility and, if it is a weak acid or base, it is pka value[13]. A drug with low aqueous solubility has low dissolution rates and have oral bioavailability problems. The pH partition hypothesis states that the un-ionized form of a drug will be absorbed preferentially, in a passive manner through the membrane. Since weakly acidic drugs will exist in the stomach (pH=1 to 2) primarily in the unionized form, their absorption will be favored from this acidic environment. On the other hand, weakly basic drugs will exist fundamentally in the ionized form (conjugate acid) at a similar site, and their absorption will be reduced. In the upper part of the small intestine, the pH is more alkaline (pH =5 to 7), and the reverse will be suspected for weak acids and base[14][15].

The double emulsion, solvent evaporation (or solvent extraction) technique also called the water-in-oil-in-water (W/O/W) emulsion is commonly used to encapsulate hydrophilic compounds, overcoming the limitations of a single oil-in-water emulsion. Drug release-retarding polymers are the key performers in designing a dosage form for oral sustained release formulation.[16] The natural polysaccharides (excipients of natural origin) are an attractive alternative product because of their reliability, biocompatibility, biodegradability, sustainability, low toxicity, and low cost compared to a synthetic product[17]. Natural Polysaccharide such as guar gum, xanthan gum, and locust bean gum has been investigated for their use in Novel drug delivery system[18]. They remain undigested in the stomach but get degraded by different anaerobic microflora in the intestine[19]. Gum Katira was chosen as the matrix forming material because of its well-established biocompatibility, nontoxic and safe equipment for the use in food and pharmaceutical industries[20]. Gum Katira is a novel heteropolysaccharide isolated from *Cochlospermum religiosum* and composes of D-galactose, D-galactouronic acid, and L-rhamnose in a molecular ratio 2:1:3 sequentially together with traces of a ketohexose. This gum is pale, semi-transparent and swells to produce a pasty transparent mass with water[21][22].

The present study aspired to design microspheres using gum katra through the double emulsion, solvent evaporation (or solvent extraction) technique for sustained release of 5 fluorouracil. The microspheres were then characterized for drug loading, entrapment efficiency, SEM (Scanning electron microscope), FTIR (Fourier transform infrared spectroscopy). To verify the functionality of 5-FU loaded gum katira microsphere for sustained drug release systems, cell cytotoxicity and in-vivo anticancer activity were tested. The drug release was checked in the physiological media of the gastrointestinal tract, i.e., simulated gastric fluid (SGF) and simulated colon fluid (SCF).

MATERIALS AND METHODS

Materials

The active pharmaceutical ingredient (API), 5-FU, was purchased from Sigma Aldrich. Ethyl cellulose was obtained from Quest Chemicals, Kolkata, India, whereas, dichloromethane, Tween-80, Span 80, Hydrochloric Acid (35%), dichloromethane, potassium dihydrogen phosphate was purchased from Merck India. Evonik Rohm, Pharma Polymers, Germany gifted Eudragit®RS100 and Eudragit®RL100 polymer granules to conduct our research. Tri-sodium orthophosphate was obtained from Loba Chemie Pvt. Ltd. India, whereas, crude gum katira was obtained from Seoni District of Madhya Pradesh, India. All other reagents were of analytical grade.

Preparation of 5-fluorouracil loaded gum katira microsphere(5-FLGKM)

The double emulsion solvent evaporation technique (W1/O/W2emulsion) is usually used to encapsulate hydrophilic drug, where, the limitations of a single O/W emulsion, to entrap only lipophilic drugs, can be bypassed[23].

5-FU loaded gum katira microspheres were prepared by W1/O/W2 emulsion solvent evaporation technique. Gum katira (75 mg) was mixed with 4 mL of water as an aqueous solvent with continuous stirring for 30 min using magnetic stirrer at 35°C to form a homogeneous solution. A measured quantity of 5-FU was added to the solution of gum katira and stirring for another an hour. Subsequently, a solution of Eudragit®RS100 and Eudragit®RL100 (7:1), dichloromethane (DCM), acry flow and span 80 was prepared and concurrently transferred to the homogenizer tube. The homogenizer was subjected to rotation at 4500 rpm, while the drug solution was spilled drop by drop into it through a 20-gauge needle. The homogenization process was continued for a period of 10 min to prepare the primary W1/O emulsion. Later, the prepared emulsion was added

dropwise to 100 mL of acidic aqueous solution (pH-4.0) using a 16-gauge syringe to form the W1/O/W2 emulsion with continuous stirring at 700 rpm for 2.5 h. Then the resultant microspheres formed were washed with distilled water followed by filtration, air-drying for 24 h, and finally stored in desiccators for further uses.

Physicochemical characterization of 5-FU loaded microsphere

Preparation of 5-fluorouracil stock solution

The stock solution of 5-FU (100 µg/ml) was prepared by dissolving 10 mg of 5-FU in 50 ml of distilled water, further, volume was made up to 100 ml in a volumetric flask.

Determination of percentage of yield of the microsphere

The percentage yield of the microsphere was calculated to evaluate how much microsphere formed from its actual value. The percentage yield of the formulated microsphere was calculated using the ratio of practical yield and theoretical yield. Theoretical yield is the total weight of the raw materials used for preparation. Practical yield is the weight of the microsphere which was practically obtained[24].

$$\text{Percentage of yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100$$

Estimation of drug entrapment efficiency

The loading efficiency of 5-FU in the microspheres was determined spectrophotometrically. 10 mg of the drug-loaded core-shell microspheres were crushed and powdered. Then it was dissolved in 5ml of Dichloromethane (DCM) and stirred for 10 minutes using a magnetic stirrer to complete dissolution of the polymer in DCM. 10 ml of methanol was added to this solution. This solution was magnetically stirred for a few minutes at 40-45°C and then filtered. 1 ml of filtered solution was taken in a 10 ml volumetric flask, and volume made up to the mark to make a 10ml of the aliquot. The absorbance of the final solution was measured at 270 nm using double beam UV-Visible spectrophotometer (UV1, ThermoSpectronic, Great Britain) against methanol as blank and calculations were done for the percentage of drug loading in the sample.

The % EE were calculated using the following equations:

$$\text{Percentage entrapment efficiency} = \frac{W_t - W_f}{W_t} \times 100$$

Where, W_t is the total initial amount of 5-FU incorporated during formulation development and W_f is the amount of free drug in the solution after formulating the drug entrapped microspheres. All

measurements were performed in triplicate and were reported as mean \pm SD ($n = 3$) [25].

Morphological analysis using Field Emission Scanning Electron Microscopy (FE-SEM) Study

The texture of microspheres was planned by using Scanning Electron Microscope (SEM) and also examined the morphology of the cross-sectioned surface of the prepared microsphere. FE-SEM analysis was performed by CARL ZEISS EVO 18 special edition machine with the platinum coating. The platinum coating was performed by using QUORUM Q150 TES. Diameter of the microsphere also evaluated by FE-SEM analysis.

Fourier transforms infrared (FTIR) spectroscopy:

FTIR analysis was done to find out if there were any chemical interactions between the drug molecule and other ingredients used in the microsphere preparation. It was done on 2 gm of microsphere sample was crushed and then mixed well with 10 gm of fine alkali halide (KBr) powder to form a transparent pellet using a hydraulic press. The formed pellets were then individually scanned using FTIR spectrophotometer (IR-Prestige-21, Shimadzu, Japan) at a spectral region of 4000–400 cm^{-1} . A resolution of 4 cm^{-1} and 16 scans were obtained for each spectrum.

In vitro drug release of microsphere

In vitro release of 5-FU from the 5-FU loaded microspheres was carried out using a digital USP type II dissolution test apparatus (Lab India DS 8000 USP). Dried microspheres (50 mg) were suspended in 500 mL of either pH 1.2 hydrochloric acid solutions followed by pH 7.4 phosphate buffer saline solution. The dissolution media was stirring at 50 rpm and kept under at $37 \pm 0.5^\circ\text{C}$ temperature. At predetermined intervals, 5 mL sample was withdrawn and replaced with fresh buffer saline. The concentration of 5-FU in released media was determined by analyzing the withdrawn aliquot at 270 nm using a double beam spectrophotometer (UV1, ThermoSpectronic, Great Britain). The cumulative release of 5-FU was determined using the standard calibration curves of the drug in several dissolution media (0.1 N HCl - pH 1.2; phosphate buffer saline - pH 7.4), respectively [26].

Cell cytotoxicity assay of the microsphere

Cell line and cell culture

HCT-116 (human colon cancer cell line) cancer cell was obtained from Chittaranjan National Cancer Research Institute (CNCRI), Kolkata, India. All the cells were incubated with 5% fetal bovine serum (FBS) and 100 $\mu\text{g}/\text{ml}$ each of penicillin and streptomycin; cells were incubated at 37°C in a humid atmosphere (5% CO_2 ; 95% air). Cells were

harvested by brief incubation in 0.02% (w/v) EDTA in PBS. The cells were maintained routinely in subcultures in tissue culture flasks.

Cell Cytotoxicity assay

The cytotoxicity of free 5-FU, 5-FU loaded microsphere, and blank microspheres were ascertained by MTT assay. Investigations were performed in 96-well flat-bottomed culture plates (BD Biosciences, USA). MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml. Next, to this, different concentrations of the sample (0.5, 5 and 10 $\mu\text{g}/\text{ml}$) were added, and the plate was incubated for a period of 24 h. Following this, 20 μL of MTT solution, prepared as above, was added to each well and incubated for four h at 37°C , the culture medium was removed, and the formazan crystals were dissolved in 200 μL DMSO. The absorbance of formazan dye was measured at 570 nm using a microplate reader (Tarsons, India, Cat. No: 980040). The negative control was the cells without treatment (MTT group) as well as cell incubated with blank microsphere. No positive control was used in this experiment; the cytotoxicity of 5 FU loaded microspheres was compared with free 5-FU, a standard antitumor drug. The amount of formazan produced resembles with the number of live cells. Cell viability was calculated using the following equation:

$$\text{Cell viability \%} = \frac{\text{Absa}}{\text{Absc}} \times 100$$

Where Absa is the absorbance of the sample treated with 5-FU (free or microencapsulated) and Absc is the absorbance of the control group cell [27].

In Vivo study of the microsphere

The anticancer activity of 5-FU loaded microspheres was assessed in vivo on Sarcoma-180 bearing mice. Sarcoma-180 was obtained from Chittaranjan National Cancer Research Institute (CNCRI), Kolkata, India. The cancer cell viability was assessed at 98%, as inferred by the trypan blue exclusion method. A xenograft model of solid sarcoma 180 induced in female Swiss albino mice by implanting 2×10^6 viable sarcoma 180 cells suspension in 0.2 ml isotonic saline solution. Sarcoma 180 cells were aspirated from the peritoneal cavity of the mice, rinsed with saline and implanted subcutaneously in the left hind of each mouse. The tumor grew in 100 % of mice with palpable solid tumor mass achieved within ten days post-implantation [28].

Animals

The animal study was conveyed in agreement with the ethical rules on animal experimentation approved by the Ethical Committee, Department of Pharmaceutical Technology, Jadavpur University

(Approval No: 147/1999/CPCSEA). CPCSEA guideline (Committee for the Purpose of Control and Supervision of Experiments on Animal) was also followed for the animal handling and care throughout the trial period. All the animals were obtained from a registered breeder, and these were then acclimatized at $25\pm 2^{\circ}\text{C}$ and $50\pm 5\%$ relative humidity with 12 h light/12 h dark cycle. Before the start of the experimentation, a period of 10 days was provided to the animals to acclimatize under particularized laboratory conditions. Throughout the investigational period, animals were housed in polypropylene cages provided by standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water ad libitum [29]. All mice were furnished tumor-bearing and distributed randomly into three equal groups, each comprising six animals. The control group received isotonic saline. The second groups received 5-FU loaded gum katira microsphere by orally at a dose of 10mg 5-FU/kg b.w and the third group received an intraperitoneal administration of marketed 5-FU injection of a dose of 10mg/kg.b.w[30]. The Treatment protocol for all groups was commenced on day 10 and lengthened to day 31 post-implantation.

Animals groups and treatment protocol

Sarcoma-180 solid tumor model

Sarcoma-180 ascites tumor cells (2×10^6 cells) were implanted subcutaneously into the left hind of the trial mice. On the 10th day of the experiment after inoculation, the 5-FU loaded gum katira microsphere (10 mg/kg b.w. by oral) and standard 5-fluorouracil injection (5-FU, 10 mg/kg.b.w. through i.p.) was administered for 21 consecutive days. After 21 days of drug administration, the animals were sacrificed by cervical dislocation and blood, and the tumors were collected for evaluation of various exercises.

Tumor volume and tumor weight

Tumour volumes were reported from the 10th day of post implantation and after that every two days till the last measurement taken at day 21 post-implantation and just before the sacrifice of surviving animals. A vernier caliper was used to record dimensions (mm), and the ratio of the developing tumors was measured, and the tumor volume calculated using the formula:

$$V = \frac{\pi \times D_1 \times D_2 \times D_2}{6} \quad [31]$$

Where D_1 is the longer diameter and D_2 is the shorter diameter.

At the end of 21 days of the drug treatment, animals were sacrificed by cervical dislocation, and all tumors were punched out, washed with normal saline, weighed immediately, and stored in a 10%

formaldehyde solution for further use. All tumors were excised and divided into two portions. One used for SEM study and the other for histopathological examination. The drug efficacy expressed as the percentage of tumor growth inhibition. The percentage of inhibition calculated using the following formula:

$$\% \text{ Inhibition} = \left(1 - \frac{B}{A}\right) \times 100 \quad [31]$$

Where A is the average weight of the control group and B is the average tumor weight of the treated group.

Mean survival time (MST) and Percentage increase in lifespan (%ILS)

After sacrifice, three mice from each group were kept observing for mean survival time (MST). The effect of drug on the percentage increase in lifespan (%ILS) calculated from the mortality of the experimental mice. Equations are as follows

$$\begin{aligned} \text{Mean Survival Time (MST)} &= \frac{\sum \text{Survival time (days) of each mouse}}{\text{total number of mice in a group}} \\ \% \text{ ILS} &= \left[\frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right] \times 100 \quad [32] \end{aligned}$$

H&E staining of sarcoma-180 tumor section

At the end of the trial (after 21 days of drug treatment), sacrificed animal's tumors were excised, washed immediately with cold water the specimen preserved in 10% formalin solution. After treatment with xylene, the specimens were embedded in paraffin blocks. Sections (5 μm) cut and stained with hematoxylin and eosin (H & E). The slides were scanned for histopathological changes such as necrosis, mitotic figures and inflammatory reactions, using light microscopy (Eclipse TS100, Nikon, Japan). Three animals per group were used.

Estimation of blood hematological and biochemical parameters

An examination of specific blood hematological and biochemical parameters was used to evaluate the effects of 5-FU at the cellular level. After 21 days of the treatment, animals were sacrificed by cervical dislocation, and blood samples were collected using heparinized syringes/ non-heparinized syringes. Hematological parameters, including red and white blood cell counts and hemoglobin concentration, are widely used clinical indicators of internal infection and disease condition.

Serum samples were examined for aspartate aminotransferase (AST) and alanine aminotransferase (ALT), to estimate hepatic damage. Serum creatinine level was appraised and used as an indicator for possible renal damage. Blood biochemistry was checked in free drug-treated and

5-FU loaded gum katira microsphere treated groups and compared with data from the control group.

Immunohistochemical analysis:

Immunohistochemical examination of p53 proteins in 5 μ m thick tumor section was carried out. Briefly, the sections were hydrated in 1 X PBS for 5min. Antigen retrieval conducted by incubating the sections in 10 mM sodium citrate buffer (pH 6.0) at 80°C for 10 min. The sections were cooled to room temperature for 20 min. Following a 5 min wash with 1 X PBS, the endogenous peroxides were blocked by 1% hydrogen peroxide in PBS for 5min. The sections washed and blocked for 1h in PBS containing 1.5% normal serum. The slides have been incubated overnight with primary antibodies against p53 at 4°C in a humidified chamber. After washing with PBS, the sections were nurtured with horseradish peroxides (HRP) – conjugated secondary antibodies at 1:100 dilutions for 30 min. At 37°C The immune reactions were visualized by immersing the slides in 3, 3'-diaminobenzidine tetrahydrochloride reagent. The sections were counterstained with hematoxylin. Negative control sections were processed simultaneously with the omission of the primary antibodies. All sections were dehydrated, mounted under a light microscope (Eclipse TS100, Nikon, Japan) and photographed (10X).

Histopathology of liver and kidney tissue

Three randomly selected mice from each group sacrificed at the end of the experiment, and their liver and kidney tissues were detached. Post-isolation from adhering tissue matter, tissues were rinsed with cold normal saline and weighed, cut into small pieces, fixed in 10% buffered formalin, dehydrated in increasing concentrations of ethanol, cleared in xylene and planted in paraffin wax, Sections (5 μ m) cut, stained with hematoxylin and eosin (H & E) and examined under a light microscope (Eclipse TS100, Nikon, Japan).

Scanning electron microscopy (SEM) study of solid tumor

After completion of the experiment, the mice were sacrificed, and their tumor was isolated. After isolating a narrow tissue section, they were prepared for scanning electron microscopy (SEM) study. On fulfillment of ultrasonic cleaning, the tissue sections were fixed in 2.5% glutaraldehyde for 1 h at 4°C,

rinsed in PBS, stained in 1% osmium tetroxide for 1 h at 4°C, followed by washing in PBS, dehydrated using ethanol, displaced in isoamyl acetate, dried at the critical point, gold coated and studied using a scanning electron microscope (Quanta 200; Philips/FEI, Hillsboro, OR)[33].

Result and discussion

The solvent evaporation or extraction techniques are generally used in the field of microspheres preparation to the extent of drug release. The classical solvent evaporation /extraction method, in which a water-immiscible organic polymer solution is emulsified into a continuous aqueous phase, is limited to the encapsulation of the water-insoluble drugs. Modified methods were developed for the encapsulation of the water-soluble drugs. For example, a continuous aqueous phase saturated with the drug [34] or water-in-oil-in-water emulsion[35] can be used. Similarly, an oil-in-water-in-oil solvent evaporation/extraction process whereby an oil phase replaces the continuous aqueous phase has also been used to encapsulate water soluble drugs[36][37]. 5-FU is a water-soluble drug; the former technique was applied to prepare microsphere.

Depending upon the trial and error method, microsphere has been prepared on the basis of different processing variable like; gum katira amount, aqueous phase volume, stirring speed, processing temperature. And that microsphere was selected as final formulation which has high percentage yield, and high drug entrapment efficiency and also released behavior was followed the sustained action.

The percentage yield, drug entrapment efficiency and diameter of the microsphere

All above said parameters of the 5-FU loaded microspheres were varied upon varying of the process variables. The final formulation with the optimized parameters based on outcomes through process variable patterns, the diameter of the microsphere was found to be $320.75 \pm 5.73 \mu\text{m}$. Finally, within the limit of our experimentation, the percentage yield for the optimum formulation of gum katira microsphere was found to be 90.53 ± 1.73 % and with drug entrapment efficiency 74.87 ± 1.76 % (table 1 & Figure 1, panel B).

Table:1 Physical characteristic of 5-FU loaded microsphere

Percentage Yield	Drug entrapment efficiency	Cumulative drug released up to 12 h	Diameter of the microsphere
90.53±1.73	74.87±1.76	81.53±2.35	320.75±5.73

Values are expressed as mean ± standard deviation (n= 6).

Table: 2 Effect of 5-FU loaded microsphere on blood hematological and serum biochemical parameter in sarcoma 180 bearing mice.

Parameters	Sarcoma control mice (2×10 ⁶ cells/mouse)	5-FU injection (10mg/kgb.w./i.p.)	5-FU loaded microsphere (10mg 5FU/kg b.w./oral)
Hematological study			
Hemoglobin (gm%)	7.30±0.05	9.10±0.13	9.23±0.08
Erythrocyte (RBC) (Cells×10 ⁶ /mm ³)	5.13±0.25	7.86±0.15	7.25±0.17
Leucocytes (WBC) (Cells×10 ⁶ /mm ³)	16.27±0.35	12.97±0.20	12.63±0.08
Biochemical study			
AST(IU/L)	65.23±2.11	40.11±3.5	38.27±1.8
ALT(IU/L)	58.93±3.10	32.17±3.15	31.38±2.9
Serum alkaline phosphates (IU/L)	105.26±1.50	79.35±2.5	77.81±3.1
Cratinine(mg/dL)	1.15±0.11	0.95±0.05	0.83±0.10

Values are expressed as mean ± standard deviation (n= 3).

Table: 3 Effect of 5-FU loaded microsphere on tumor weight, mean survival time (MST) and percentage increased life span in sarcoma 180 bearing mice.

Parameters	Sarcoma control mice (2×10 ⁶ cells/mouse)	5-FU injection (10mg/kgb.w./i.p.)	5-FU loaded microsphere (10mg 5FU/kg b.w./oral)
Tumor weight(gm)	15.25±1.01	4.31±0.23	3.91±0.31
Tumor volume(mm ³)	75.15±2.3	33.17±0.51	31.27±0.23
Body weight(gm)	35.22±1.56	19.85±0.37	21.35±0.95
MST (days)	22.75±1.15	65.50±2.21	70.75±1.17
ILS (%)	-	148.40±9.23	164.83±1.73

Values are expressed as mean ± standard deviation (n= 3).

Morphological analysis of the microsphere

The FE-SEM was used to determine the particle size, surface morphology, texture and also to examine the morphology of the fracture or sectioned surface of the prepared microspheres. The inadequacy of pores in the microspheres was of vital importance for the underlying drug release mechanisms because drug releases through the water-filled cavities were much

faster than through dense polymeric matrix network. All microspheres were spherical, smooth surfaced and had tiny pores on the surface. A small hole was found. It has formed due to the evaporation of the solvent at the time of preparation. Cross section of the microsphere showed the nature of the core of the cell (figure 1, panel A).

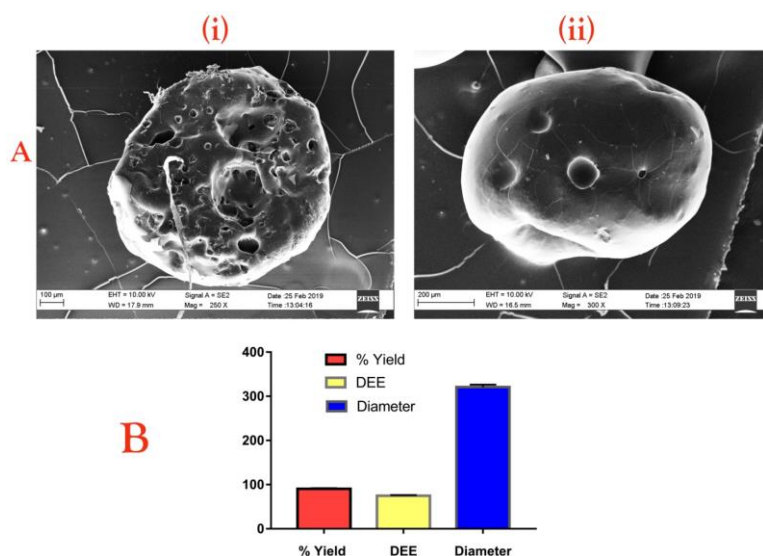


Fig.1: Panel A, subpanel (i), Drug-loaded microspheres are showing an apparently smooth surface, the existence of microscopic holes in the surface due to the rapid evaporation of the solvent in the magnetic stirrer. Subpanel (ii) shows the cross-section of the microsphere. Panel B represents the % yield, drug entrapment efficiency, and diameter of the final formulation.

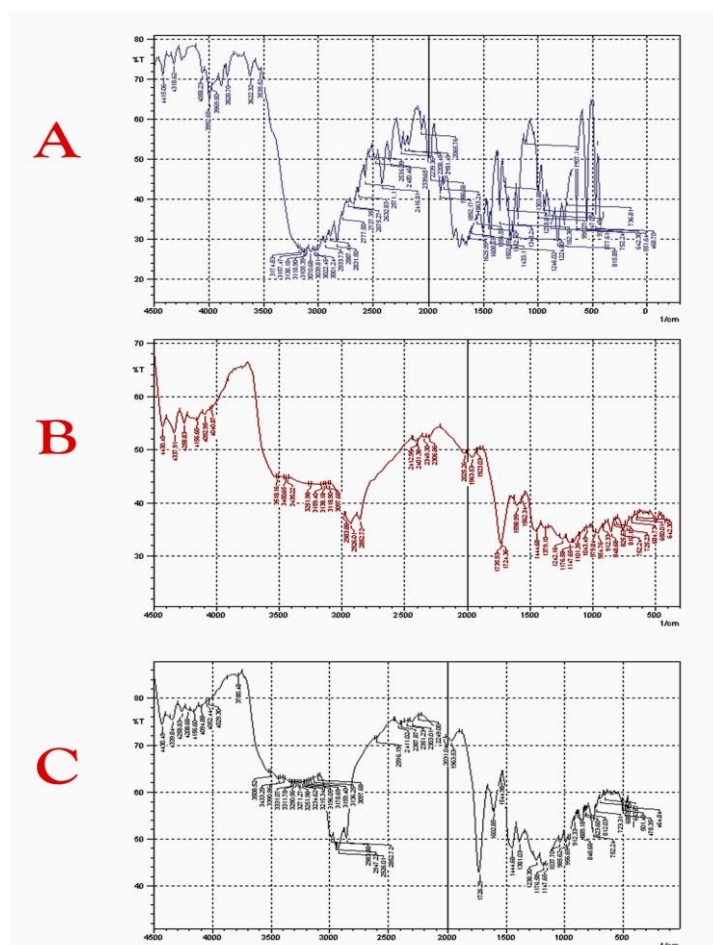


Fig.2: Panel A shows FTIR spectrum of pure 5-FU, Panel B shows blank microsphere and panel C demonstrates 5-FU loaded microsphere.

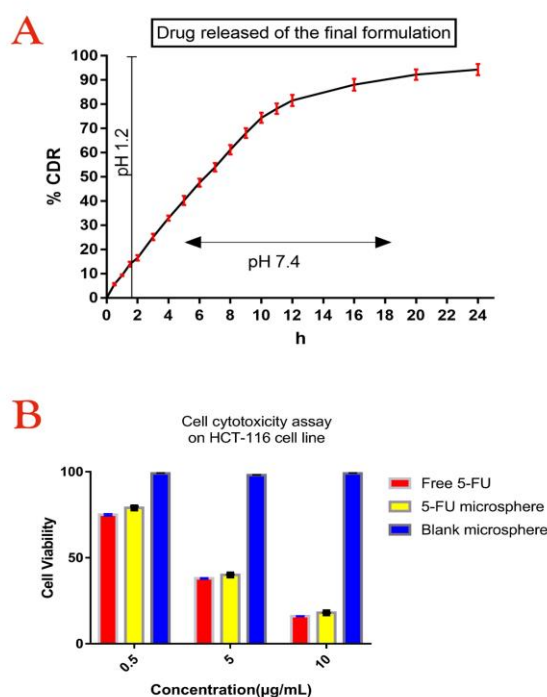


Fig.:3 Panel A shows in vitro released profile of the final formulation. Where processing parameters were gum katira(75mg), Stirring Speed (800rpm), Copolymer ratio (7:1), Processing Temp (35°C), aqueous Volume (4ml), Concentration of span 80 (0.5%). Panel B represented Cell viability percentage of free 5-FU, 5-FU loaded microsphere and blank microsphere on HCT-116 cell line, in 24 h. 5-FU-loaded microsphere compared with free 5-FU in the same concentration.

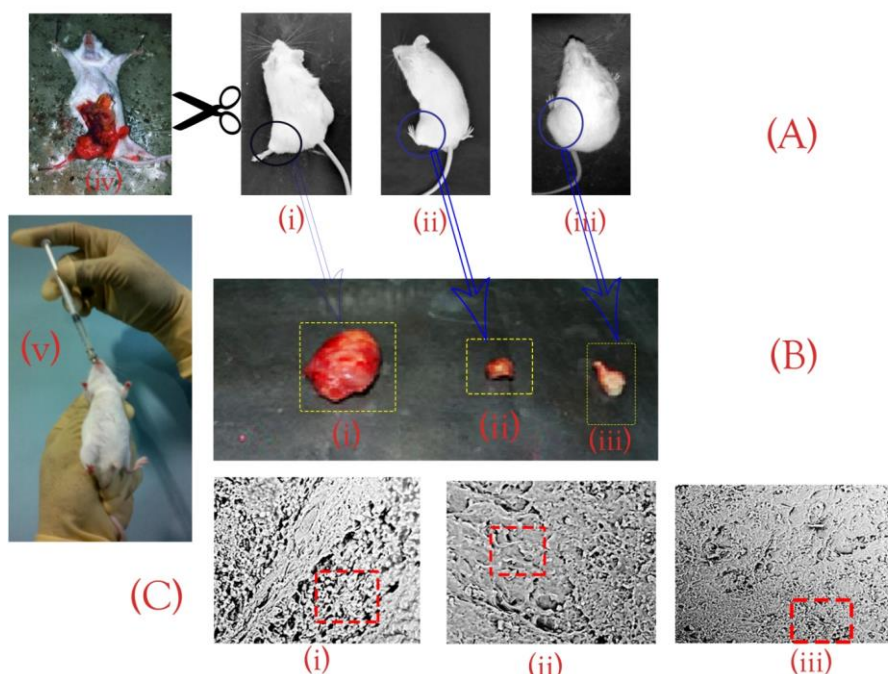


Fig.4: Effect of 5-FU Treatment on Solid Sarcoma-180 Tumor: panel (A), subpanel (i) sarcoma-180 control (ii) 5-FU injection through i.p. (iii) 5-FU loaded microsphere treated (iv) intersection of treated mice and (v) picture of oral feeding mice. Panel (B) shows the dissected tumor tissues in the same order as in (A) above. Panel (C) SEM of tumor section of the solid tumor in the order as in panel (A) above, as (i) control (ii) 5-FU injection (iii) 5-FU loaded microsphere, the cellular features are marked red rectangular box.

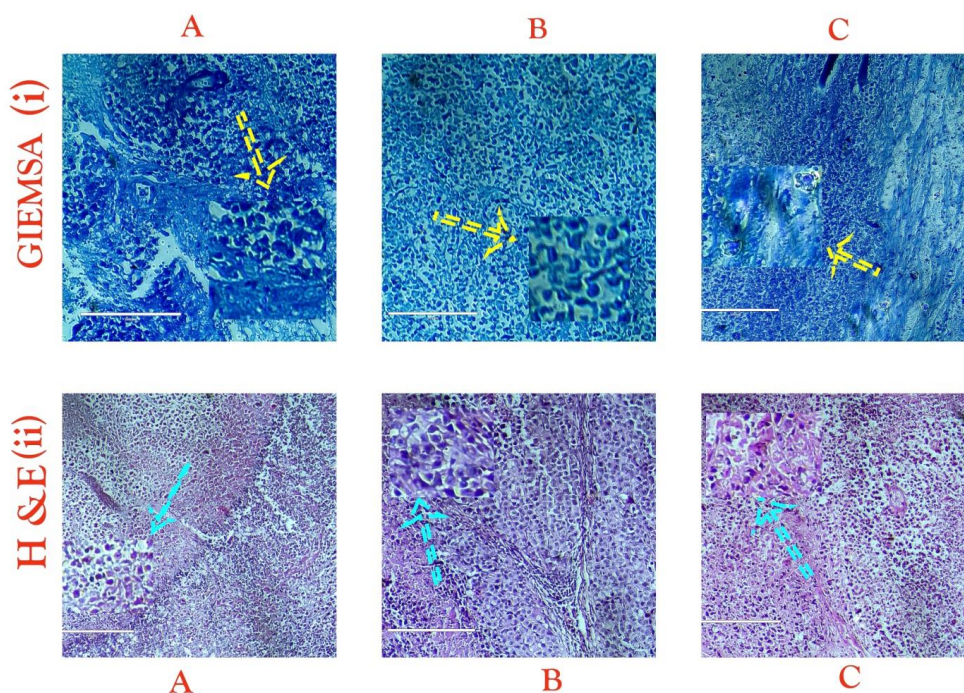


Fig.5: Panel (A) shows Giemsa stained histological section of the solid tumor in the order as (i) control (ii) 5-FU injection (iii) 5-FU loaded microsphere. Panel (B) shows H & E stained histological section of the solid tumor in the order as (i) control (ii) 5-FU injection (iii) 5-FU loaded microsphere.

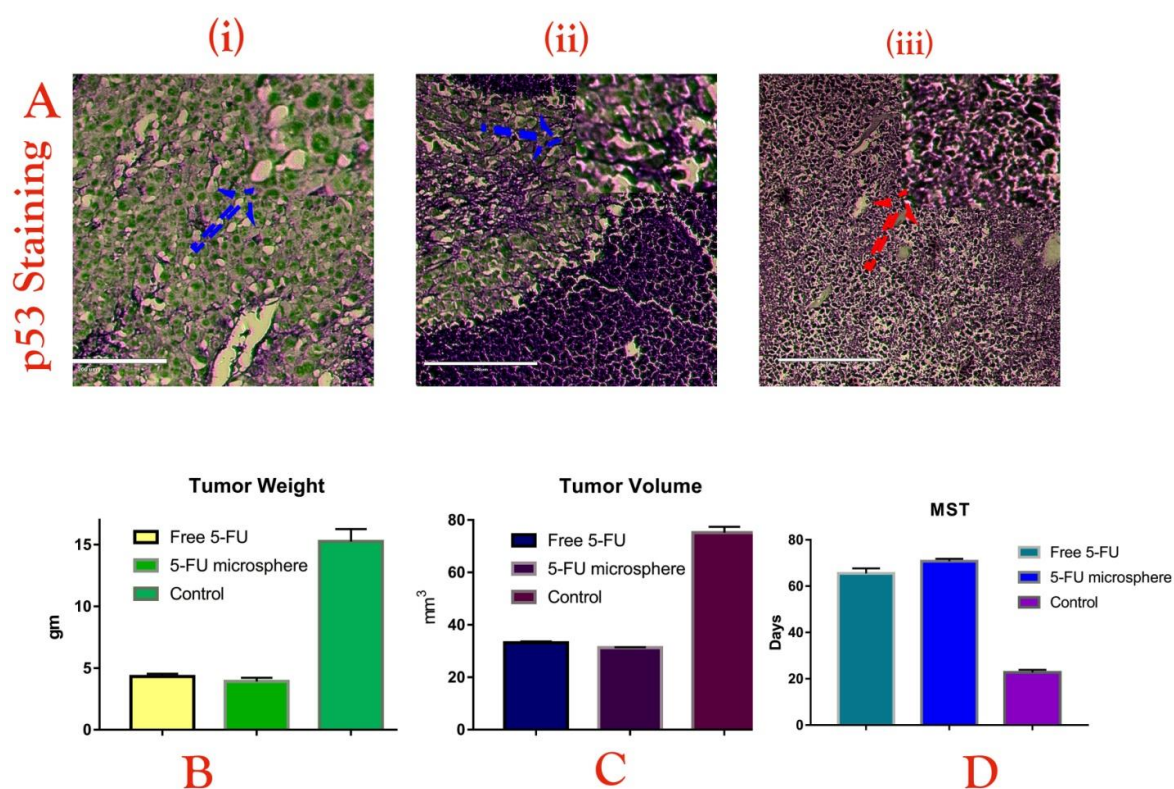


Fig.6: Panel A shows p53 immunohistochemical staining of solid tumor section in order as (i) 5-FU loaded microsphere, (ii) 5-FU injection and (iii) control. Panel B, C and D show graph of tumor weight, tumor volume and MST respectively.

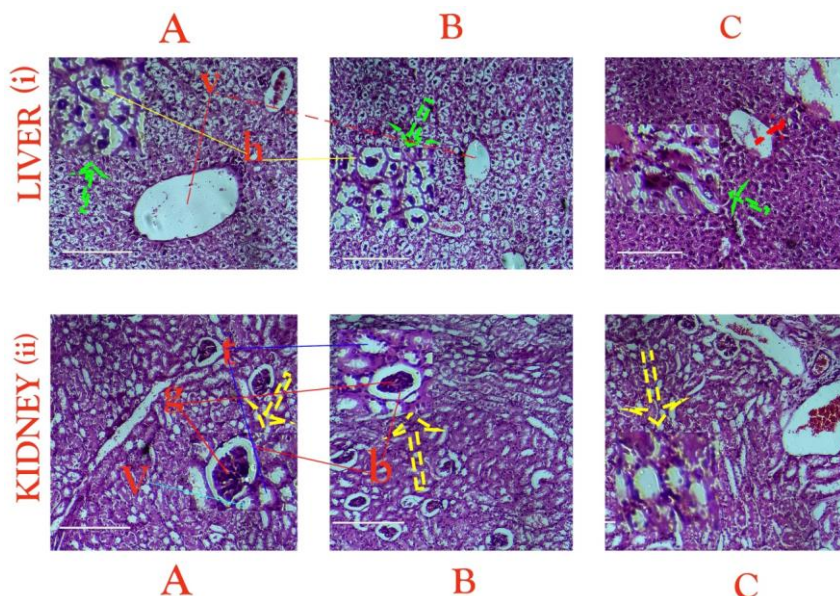


Fig.7: H& E staining of liver (panel (i)) and kidney (panel (ii)). Subpanel A shows 5-FU loaded gum katira microsphere at 5-FU 10mg/kg b.w., subpanel B for i.p injection at 10 mg/kg.b.w. of 5-FU and subpanel C for control treated. Subpanel A & B show less neoplastic focal lesion, almost normal hepatocellular architecture (Cyan arrow) whereas in sarcoma 180 control mice subpanel C shows an extensive hepatocellular lesion, as shown by the cyan arrow and canalicular irregularity as shown by the red arrow, pyknotic nuclei, exhibiting necrotic hepatocytes. h= Normal hepatocytes; v= Wall of the centrilobular vein, surrounding by liver lobe parenchyma. Panel (ii) shows Subpanel A shows 5-FU loaded gum katira microsphere at 5-FU 10mg/kg b.w., subpanel B for i.p 5-FU injection at 10 mg/kg.b.w. of 5-FU and subpanel C for control treated. g=Vascular pole of the glomerulus; b=Bowman's space; t=The glomerulus is surrounded by convoluted tubules(t); v= blood vessel.

Interaction study between polymer and drug (FTIR study)

FTIR was conducted to study the chemical interactions between the active ingredient 5-FU and all the other excipients used in the microsphere formulations. The entire observed spectrum is represented in fig.2, where the characteristic peaks of drug-loaded microspheres were correlated with that of the standard spectrum of 5 FU. From the FTIR studies, it was noticed that no chemical interaction occurred between the drug 5-FU and gum katira and other excipients. Figure (A, B and C) explained –NH bond stretching between 3000 and 3500 cm^{-1} in the spectrum of 5-FU. In the microsphere where the 5-FU drug was entrapped with gum katira, this bond was found to occur at 3500 cm^{-1} .

Further, C=O stretching bands were also found in the drug-loaded microspheres, blank microsphere, and the pure 5-FU at 1735 cm^{-1} , 1737 cm^{-1} , and 1625 cm^{-1} sequentially. 5-FU loaded gum katira microsphere, blank microsphere, and pure 5-FU produced peaks due to the presence of C-H groups at 2926 cm^{-1} , 2927 cm^{-1} , and 2933 cm^{-1} , respectively.

The C-F stretching bands of the 5-FU molecules provide peaks at almost similar wavelengths, at 1242 for the drug-loaded microsphere and 1246 for the pure drug, suggesting an interaction-free formulation between the API and polymer.

In vitro 5-FU release assay of the microsphere

Optimized gum katira containing 5-FU microsphere were subject to the in vitro drug release assay. The release of 5-FU from the final microspheres was carried out using a digital USP type II dissolution test apparatus (Lab India DS 8000 USP). The in vitro 5-FU release from microsphere was evaluated simulating physiological conditions (37 °C, pH 1.2 and pH 7.4). The in vitro release profiles of 5-FU were obtained by graphing the cumulative percentage of the drug released with respect time. The experiment was performed over 24 h, and the results of 5-FU release from gum katira microsphere were expressed in Fig.3, panel A. Results show that there was a pronounced time prolongation on drug release. A biphasic release pattern of 5-FU was observed from the microsphere, where there was an initial release followed by a sustained release. At first 2 hours,

microsphere release approximately 16% of 5-FU, and after ten h 5-FU released 82% of drug and final up to 24 h 5-FU released 95%. This released pattern proved its sustained action.

Cell Cytotoxicity assay

Cytotoxicity of free 5-FU, 5FU loaded microsphere and blank microsphere over HCT-116(human colon cancer cell line) were assessed in 24 h, but only after 24 h, both free and microencapsulated 5-FU inhibited viable cell.

In vitro MTT results showed that the blank microsphere exhibited no significant cytotoxicity after 24 h, indicating gum katira microsphere as a carrier of the drug without toxic side effect. Free drug and drug-loaded microsphere (from 0.5 to 10 µg/ml) similarly reduced viable cell, but there was a slight difference between free 5 FU and drug-loaded microsphere (Fig.3, panel B). The reason may be that the release of drug from the microsphere was incomplete (95% of the drug released from the microsphere up to 24 h in the in-vitro release study).

In-vivo study of the microsphere in the sarcoma 180 bearing mice

Tumor evaluation

The average tumor volume in Sarcoma 180 control mice progressively increased with time up to $75.15 \pm 2.30 \text{ mm}^3$ for 21 days post-tumor implantation study. In the drug-treated groups, the tumor volume was observed 33.17 ± 0.51 for 10 mg/kg b.w 5-FU injection through i.p and 31.27 ± 0.23 for 10 mg/kg b.w. 5-FU loaded microsphere respectively (table 3). Figure 4 showed the photographic view of the tumor portion.

The body weight changes were significantly higher in microsphere treated groups compared to control; indicating the effect of trapped 5-FU into the microsphere in preventing the tumor growth (table3). Tumor weight and size were also significantly low in the microsphere treated group compared to control (fig.4).

The survival rate of sarcoma-180 bearing mice in the drug-treated groups was significantly increased as compared to sarcoma-180 bearing control group mice. The %LS in the 5-FU injection and microsphere were found 148.40 ± 9.23 and 164.83 ± 1.73 respectively (table 3). That proved that microsphere of 5-FU had given anticancer activity in the tumor-bearing mice.

H & E and Giemsa staining of sarcoma-180 tumor section

After fixation in formaldehyde, tumor parts were grossly inspected for size or color changes and hemorrhage (Figure 5, panel (ii), subpanel A, B and C marked by arrow). Histopathological analysis of the

solid tumors of H & E staining from control mice showed sheets of large round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclear and binucleation. Several degrees of cellular and nuclear pleomorphism was viewed, marked by an arrow. In the tumors extirpated from animals treated with 5 FU loaded gum katira microsphere and 5-FU intraperitoneal injection, extents areas of coagulative necrosis showed less proliferation and muscle invasion was also observed. Such finding was significantly improved in mice treated with 5-FU loaded microsphere as evident by progressively increasing multiple apoptotic bodies, fibro skeletal muscle fiber and lymphoid aggregation show some malignant cell infiltration.

Giemsa's stain is frequently used to detected differentiate nuclear and cytoplasmic morphology of cells. The Giemsa stain was adapted to histology due to its unique staining of chromatin, nuclear membranes, and cytoplasmic elements. In histological sections, cell nuclei can range from deep purple to dark blue.

The inhibitory effect of 5 FU on sarcoma 180 cells of the mice model as mentioned above was observed by Giemsa staining, as shown in (Figure 5, panel (i)). Of this, (Figure 5, panel (i) A to C) corresponds to Giemsa staining that demonstrates blebbing of the plasma membrane (marked by arrow). In general, the control cells have good circular morphology, intact plasma membrane and nucleus (subpanel C), whereas, in the case treated the nuclear condensation and deformity was evident (marked by arrow).

On the contrary, mice treated with 5 FU loaded microsphere showed significantly more tumor apoptosis or necrosis (marked by yellow arrow) than control. The results showed that the loaded microsphere had increased antitumor effectiveness, leading to more cells killed at the cellular level.

Estimation of hematological and biochemical parameters

Treatment with 5-FU at dose 10mg/kg b.w significantly increased the hemoglobin counts towards the normal levels in respect to control mice. The RBC count was restored to normal range. 5-FU loaded microsphere could bring down the WBC level to the normal level. In compare to free 5-FU, 5-FU loaded microsphere has statistically significant lowering power of WBC, RBC and hemoglobin toward normal (Table 2). It can conclude that 5-FU microsphere loaded has more acceptability then free 5-FU.

In the final part of the study, an assessment of the effect of 5FU loaded microsphere on liver and kidney

functions was compared to that following exposure to free 5-FU. ALT and AST are released into the blood after extensive tissue injury. Specifically, elevated levels of ALT are associated with liver injury[38]. Biochemical analysis of ALT and AST levels in the serum samples did not show any significant change between free 5-FU and 5-FU-loaded microsphere treated group. However, significant elevations in the levels of these enzymes were observed in the animals treated with the drug when compared to controls (Table 2). With regards to kidney function, serum creatinine levels from the control group with 5-FU treat were higher. However, 5-FU loaded microsphere showed a towards normal level (Table 2). It can be inferred from these results that 5FU-loaded microsphere is better tolerated when compared to the free drug.

Immunohistochemical analysis:

Immunohistochemical staining of p53 uses as a substitute for mutational analysis in the diagnostic workup of carcinomas. Diffuse and robust immunoexpression of p53 is generally interpreted as likely indicating a TP53 gene mutation[39]. Many studies have examined their significance in diagnosis, prognosis, and treatment in tumors of different sites. For example, the ubiquity of at p53 mutation in colorectal, lung, prostate, and breast carcinomas is a minor prognostic factor, and for the tumors of other sites, a p53 mutation has been correlated with chemoresistance in some studies[40][41][42]. To detect gene mutation, nucleotide sequencing is the most reliable technique, while it is labor-intensive, time-consuming, and therefore, currently has inadequate application in clinical pathology practice. Immunohistochemical analysis of p53 expression is therefore commonly used as a surrogate for mutational analysis[43][44][45]. All tumors of positive cells demonstrated predominately weak staining (Figure 6, panel A). Block (iii) demonstrated high-grade carcinoma, where maximum cells displayed staining. Whereas (i) and (ii) have shown less carcinoma due to these mice treated with 5 FU in any formulation form. In compared with (i), (ii) has shown heavy staining means heavy carcinoma. It may be due to short-acting and high first-pass metabolism of 5-FU. In can now infer that controlled released microspheres furnished good result than i.p. 5 FU formulation.

Histopathology of liver and kidney tissue (H&E staining)

Liver

As like normal healthy mice, H&E stained sections of 5-FU loaded gum katira microsphere liver slices presence of all the features, including circular

hepatic portal vein and branch of the hepatic artery, as marked by arrows. The hepatocytes displayed prominent nuclei (marked by arrow), and the tissue section comprises hepatic sinusoids as usual. Hepatocytes are relatively large, polyhedral cells with a central, spherical nucleus, which was rather euchromatic and contained a large nucleolus. The size of the nuclei varied, because many hepatocytes were tetraploid or even polyploid, which may also result in multinuclearity. At the center of the cell narrow intercellular biliary canals present (fig.7).

On the contrary, for the control mice (Figure 7, panel B), none of the regular features as above mentioned could be found; instead, it reveals large hepatocellular lesions as pyknotic nuclei (marked by arrow), exhibiting necrotic hepatocytes. On treatment using 5-FU injection through intraperitoneally, the cellular features were ascertained to be close to normal, as shown in (panel C of Figure 7), although some irregularities, e.g., deformity in the hepatic artery and irregular bile duct could also be found. The function of the liver is to filter the blood emanating from the gastro intestinal tract, before passing it to the rest of the body and also detoxifies chemicals and metabolites[46]. 5 FU has a short half-life and high first-pass metabolism[11]. This kind of dissimilarity in the histopathology may be issued due to the control released formulation have slow release rate, and that may give less trace on liver function.

Kidney

The nephron, the structural and functional unit of the kidney is a blindly terminating tubule, the wall of which is formed by a simple epithelium[47]. In figure 7, panel(ii) A & B, glomerulus was seen. In the renal corpuscle, the glomerular endothelium was closely associated with the visceral epithelium of Bowman's capsule. At the vascular pole of the glomerulus (g), a blood vessel (v) enters the capillary network. Convoluted tubules surround the glomerulus. , Bowman's space (b) was confluent with the proximal convoluted tubule at the urinary pole of the glomerulus (g), the wall of which was a simple prismatic epithelium. The visceral layer was a specialized epithelium, made of podocytes which were epithelial cells with a cell body and a number of branched processes.

Kidney histopathology of control mice showed that pathological changes in the form of diminished and distorted glomeruli, dilated tubules, edema exudate, mild necrosis, and infiltration of inflammatory cells (marked by yellow arrow). In compared to the 5 FU treated groups these all was massively lower. That indicated that control released formulation has less

toxicity than other conventional formulation (figure7, panel (ii)).

Scanning Electron Microscopy (SEM) study of tumor cell

SEM examination revealed cell morphology of tumor cells. The control tumor cells were found with intact nuclear membrane with huge and circular nuclei, numerous long microvilli (Figure 4, panel C marked by rectangle), while, on treatment with 5-FU, these cells were less in number (Figure 4, panel C, Subpanel (ii) marked by rectangle) and exhibited a wrinkled morphology with nuclear membrane lumped, disrupted with decreased surface microvilli. A similar observation was observed in case of the 5 FU loaded microsphere treated (Figure 4 panel C, Subpanel (iii) marked by the rectangle), where nuclear membrane was disrupted, cells were shrunken, the nuclei were broken, and the dense network of microvilli was decreased. These features evaluate that, apoptosis appeared Gum katira microsphere treated mice is better in compared to injected formulation.

CONCLUSION

Formulated and optimized polymeric microsphere of 5-FU using gum katira polymer own optimal physicochemical characteristics with a fine spherical particle, with a size of $320.75 \pm 5.73 \mu\text{M}$, high drug entrapment efficiency ($74.87 \pm 1.76 \%$), and satisfactory release pattern of the drug within a time range of 12 h. FTIR results indicated that drug and excipients have compatibility and FE-SEM revealed spherical and smooth surface of the microsphere. According to the MTT results, targeted microsphere exhibited good cytotoxicity on HCT-116 cell line and have productive anticancer activity on sarcoma 180 solid tumor mice model. Histopathology of liver and kidney of sarcoma 180 bearing mice shown that formulated microsphere is better than free 5-FU. Immunohistochemistry result of p53 also displayed formulated microsphere was more susceptible to apoptosis than free 5-FU. Finally, we foretell that polymeric microsphere of 5-FU using natural gum katira could be a promising micro-carrier for efficient colon targeting delivery tool with improved chemotherapeutic efficacy against colon cancer.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interests.

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