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Research Article

PREPARATION AND CHARACTERISATION OF 5-FLUOROURACIL LOADED PLGA NANOPARTICLES FOR COLORECTAL CANCER THERAPY

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ABSTRACT

The study is planned to develop novel PLGA based nanoparticle delivery system of 5-Fluorouracil to treat colorectal cancer. pH sensitive polymer, Eudragit S 100 and PLGA were used to design the dosage form to target colonic region. Emulsion droplet coalescence method was employed to formulate nanoparticles. These nanoparticles were further characterized for their size, morphology, zeta potential, drug entrapment, Particle Size analysis, cytotoxicity study, in vitro release study and kinetic analysis. Formulation F2 proved to be better and showed 72.89 ± 1.41 drug entrapment with ideal particle size of 132.57nm ± 12.62. In vitro release of F2 formulation showed a lag phase for 4 hrs (pH 1.2 and pH 6.8). Initially, there was a burst release of 17.22% at 7th hr and further there was a controlled release up to 78.23% for 24 hrs. In vitro kinetic study indicated that the formulations followed first order release pattern and anomalous transport kinetics, i.e. a combined mechanism of pure diffusion and Case II transport. For biological evaluation, HT29 (Human colorectal Adenocarcinoma) cell lines were selected and anti-tumoral efficacy was analyzed using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Results showed that 80% of cell lysis took place. Formulation F2 was a promising formulation to target colon cancer cells.

Keywords: Nanoparticles, 5-flurouracil, Colorectal cancer, Eudragit S 100, PLGA, Cytotoxicity study.

INTRODUCTION

Colon cancer is the second most cause of death after lung cancer by cancer diseases. Many different drugs and drug combinations have been tested for a successful therapy[1]. In recent years, chemotherapy is more and more popular, as the traditional treatments like surgery and radiation therapy hurt the patients a lot mentally and physically. Chemotherapy is convenient to be administered and painless to the patients. Among the therapeutic drugs which have been into practical use, 5-Fluorouracil is one of the most potent chemotherapeutic agents for treating colorectal cancer[2]. 5-Fluorouracil (5-FU or 5-fluoro-2, 4-pyrimidinedione) is an anti-metabolite of the pyrimidine analogue type, with a broad spectrum of activity against solid tumors (of the gastrointestinal tract, pancreas, ovary, liver, brain, breast, etc.), alone or in combination with chemotherapy regimes. 5-FU acts by interfering with nucleoside metabolism incorporated in RNA and DNA, leading to cytotoxicity and cell death[3]. In addition, because of its high rate of metabolism in the body, the maintenance of a therapeutic serum concentration requires the continuous administration of high doses, which when administered above a certain limit produces a severe toxic effect[4-5]. Nanoparticles have a large specific surface which is indicative of a high interactive potential with biological surfaces. Such multi-particulate systems tend to be more uniformly dispersed in the GI tract and ensure uniform absorption[6]. A number of studies has been carried out on sustained drug delivery systems for 5-Fu by using polymers like poly (D, L-lactic-co-glycolic acid) PLGA. Although 5-FU-loaded nanoparticles are envisaged as having great potential for drug delivery, and their capability of permeation through cell and connective tissues without blocking the capillaries[7-10]. Furthermore, due to leaky tissues that surround the tumor, these nanoparticles can pass through the vasculature and accumulate in the solid tumors. Nanoparticles generally end their life intracellularly in endosomes or lysosomes followed by degradation. For activity of the encapsulated drugs, release into the cytosol is needed. However, nanoparticles of about 20-180 nm are better accumulated and also cellular uptake without contribution by endocytic mechanisms was demonstrated[11]. Studies have shown that nanoparticles carrying anticancer agents show prolonged drug retention in the tumors, which diminishes

tumor growth and thereby increases the life-span of tumor-bearing animals[12-13].

MATERIALS AND METHODS

Materials:

5-FU was a gift sample provided by Naprod Life Sciences Pvt. Ltd., (Mumbai, India). PLGA polymer with ratio 50-50 was purchased from Sigma-Aldrich (Steinheim, Germany), Eudragit S 100 was obtained as a gift sample from Evonik Pvt. Ltd., (Mumbai, India). PVA was procured from Sigma Aldrich (St. Louis, MO, USA) and all organic solvents were of analytical grade.

Modified emulsification solvent evaporation technique

5-FU-loaded PLGA polymer nanoparticles were prepared by Modified emulsification solvent evaporation technique. An attempt has been made to optimize the nanoparticle formulation using PLGA 50:50. Different drug polymer ratios were used. Dichloromethane was used as organic solvent and PVA as surfactant in a fixed concentration of (0.5%, w/v). Weighed quantity of Drug (20mg) was dissolved in 40ml water. Varying polymer (PLGA50:50) concentration, was dissolved in 20 ml of dichloromethane. Specific quantity of Eudragit S 100 (50mg) was added to 20ml acetone. Firstly PLGA solution and Eudragit solution was mixed together with slow stirring and drug solution was added to it slowly and stirred for ten minutes. This homogeneous solution was added slowly to 100 ml of 0.5% (w/v) aqueous surfactant (PVA) solution using high speed emulsifier (ULTRA-TURRAX, Germany) and stirred continuously for 4 hrs to prepare the emulsion. The emulsion formed was stirred on laboratory magnetic stirrer for 2 hrs at 25°C followed by centrifugation (SIGMA, Germany) for 30 minutes at 15,000 x g. After centrifugation the supernatant was discharged and the pallets obtained were washed by using the same volume of distilled water as of the supernatant and again centrifuged at 15,000 x g for 10 minutes. The washing process was repeated three times and the washed nanoparticles were subjected to freeze drying using 5% of sucrose as a cryoprotectant[14]. (Christ Alpha 2-4 LD, Freeze Drying Solutions, UK).

Table1: Composition of 5- Fluorouracil nanoparticles

Ingredients	F1	F2	F3	F4	F5
5- Fluorouracil	20 mg	20 mg	20 mg	20 mg	20 mg
PLGA	20 mg	40 mg	60 mg	80 mg	100 mg
Eudragit S-100	50 mg	50 mg	50 mg	50 mg	50 mg
PVA solution	0.5%w/v	0.5%w/v	0.5%w/v	0.5%w/v	0.5%w/v
Dichloromethane	20 ml	20 ml	20 ml	20 ml	20 ml
Acetone	20 ml	20 ml	20 ml	20 ml	20 ml

Characterization of 5-FU-loaded PLGA polymer nanoparticles:

FT- IR Studies:

FTIR studies between 5-FU and the excipients were carried out to find interactions among the drug and polymers. Peaks of pure drug and combination with the polymer were obtained and were further checked for compatibility between them. FTIR spectra of pure drug and the drug-polymer mixture were obtained in KBR pellets using IR- affinity-I Shimadzu Auto 00518 spectrometer.

Particle size:

The size distributions along the volume mean diameter of the nanoparticles were measured by Dynamic Light Scattering Particle Size Analyzer (Nanotrac Particle Size Analyzer). The range of the analyzer is 0.8 nm to 6.54 µm. Particles were suspended in a dispersing fluid and subjected to random collisions with the thermally excited molecules of the dispersing fluid.

Shape and Surface Morphology:

The particle shape and morphology of the prepared 5-Fluorouracil nanoparticles were determined by SEM analysis. The nanoparticles were viewed using a Jeol-5610 LV (Tokyo, Japan) for morphological examination. Powder samples of dried nanoparticles were mounted on to aluminum stubs using double side adhesive tape and then sputter coated with a thin layer of gold at 10 Torr for vacuum before examination. The specimens were scanned with an electron beam of 1.2 kv acceleration potential and images were corrected in secondary electron mode.

Zeta potential:

Zeta potential was measured by using zeta potentiometer (Zeta 3.0+ meter, USA). Sample was filled into the cell; electrodes were inserted and placed under the microscope, and were connected to the Zeta-Meter 3.0+ unit. Electrodes were energized and the colloids were watched to move across a grid in the microscope eyepiece. Each sample was tracked by simply pressing a "track" button and holding it down while the colloid traverses the grid. When the "track" button is released, the Zeta-Meter 3.0+ instantly calculates and displays the colloid's zeta potential (or electrophoretic mobility). All data were taken in triplicate.

Determination of encapsulation efficiency (%)

The amount of the drug entrapped within the nanoparticle was determined by measuring the non entrapped (un-encapsulated) drug amount in the supernatant. The supernatant was collected after centrifugation and recovery of nanoparticles. The encapsulation was analyzed by UV-Vis spectrophotometric analysis (Shimadzu, Japan) at 265 nm. Encapsulation efficiency was determined by using following expression[15].

$$E.E.(\%) = \frac{W_t - W_u}{W_t} \times 100$$

Where, Wt, weight of initial drug; Wu, weight of un-encapsulated drug.

In vitro Drug Release:

The in-vitro release characterization of 5-FU from the prepared nanoparticles were evaluated in pH medium gradually changing from (pH 1.2, 6.8 and 7.4). The pH of the dissolution medium was kept 1.2 for 2 hour using 0.1 HCl. Then KH_2PO_4 and $Na_2HPO_4.2H_2O$ were added to the dissolution medium, adjusting the pH to 6.8 with 1.0M NaOH, and then the release study was continued for an additional 4 hours. After 4 hours, the pH of the dissolution medium was adjusted to 7.4 with 0.1 N NaOH and maintained up to 24 hours. 5-fluorouracil loaded PLGA, Eudragit S-100 nanoparticles (equivalent to 50 mg of 5-fluorouracil) were suspended in the dissolution medium. The dissolution studies were carried out in 900 ml of pH medium with 37ºC ±0.5ºC at 60 rpm. The simulation of GI transit condition was achieved by altering the pH of dissolution medium at different time interval. At selected time interval 2.0 ml of the samples were withdrawn and replaced with fresh buffer. The sample was filtered & analyzed by UV-spectrophotometer at 265 nm [16-17].

In vitro kinetic studies of 5-Fluorouracil nanoparticles:

The results of In vitro release profile obtained from all the formulations were plotted to know the mechanism of drug release. The data were treated according to zero order release, first order release, higuchi's and korsemeye peppa's model equation. The release rate kinetics data for all the other equation can be seen in table no.3.

In vitro cytotoxicity assay:

Preparation of Cell culture: The cell lines were maintained in 96 wells micro titer plate containing MEM media supplemented with 10% heat inactivated fetal calf serum (FCS), containing 5% of mixture of Gentamycin, Penicillin (100 Units/ ml) and Streptomycin (100µg/ml) in presence of 5% CO_2 at 37ºC for 3-4 days. After 3-4 days, the supernatant was removed and MEM media was replaced with Hank's balanced solution supplemented with Gentamycin, Penicillin and Streptomycin. Incubate this preparation overnight. HT-29 - Human colorectal adenocarcinoma cell lines were used for the study.

Cytotoxicity Assay: In-vitro growth inhibition effect of test compound was assessed by calorimetric or spectrophotometric determination of conversion of MTT into "Formazan blue" by living cells. The supernatant was removed from the plate and fresh Hank's balanced salt solution was added and finally treated with different concentration of extract or compound appropriately diluted with DMSO. Control group contains only DMSO. After 24 hrs incubation at 37ºC in a humidified atmosphere of 5% CO_2, the medium was replaced with MTT solution (100µl, 1mg per ml in sterile Hank's balanced solution) for further 4 hr incubation. The supernatant was carefully aspirated, the precipitated crystals of "Formazan blue' were solublised by adding DMSO (200µl) and optical density was measured at wavelength of 570nm. The results were represented as the mean of three readings.

Principle of assay: This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol- 2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilised with an organic solvent (e.g. DMSO, Isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells[18].

Formula:

$$\text{Surviving cells (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control}} \text{X } 100$$

RESULTS AND DISCUSSION

Drug polymer compatibility study was carried out by FTIR spectroscopy and the results are depicted in Figure no 1 and 2. The mean diameter of all the nanoparticles formulations were determined and are listed in Table no 2.

The results showed that, an increase in the concentration of PLGA lead to an increase in drug entrapment, drug loading and mean particle size. This was probably caused by the increasing viscosity of organic phase (polymer solution), which increases the diffusional resistance to drug molecules from organic phase to the aqueous phase, thereby entrapping more drug in the polymer nanoparticles. Increasing polymer concentration also increases particle size due to poorer dispersability of the PLGA solution into the aqueous phase. Moreover, an increase in the viscous forces resist the droplet breakdown by opposing the shear stresses in the organic phase and the final size and size distribution of particles depends on the net shear stress available for droplet breakdown. The formulation F2 had ideal 132.57nm ± 12.62 sizes with optimum entrapment efficiency was considered as the better optimized nanoparticle formulation.

The surface morphology of the nanoparticles encapsulating 5-Fluorouracil was determined by SEM scan (Figure 3) and it revealed that the particles are smooth, spherical and have uniform size distribution.

Zeta potential influences the stability of nanoparticles. Extremely positive or negative zeta potential values cause larger repulsive forces, whereas repulsion between particles with similar electric charge prevents aggregation of the particles and thus ensures easy re-dispersion. As tabulated in table no 3 zeta potential varies from -16.5±4.3 to -28.5±2.3, formulation F2 ranges around -20.4 ± 5.6 which is more desirable for combined electrostatic and steric stabilization and reveals good physical stability of nanoparticles.

The in vitro release profiles of 5 FU (figure 5) from the prepared particulate system were studied in gradually pH changing buffers. All the formulations showed a lag phase of 4 hrs. The nanoparticles show a biphasic release pattern: one initial burst release followed by a second slow-release phase. Formulation F2 showed a first burst release of 20.56%, which may be probably due to the drug that was adsorbed or close to the surface of the nanoparticles. Thereafter, the release rate decreased that reflects the release of drug entrapped in the polymer and would mainly depend on the drug diffusion and the degradation of the bulk polymer. The initial burst release can be helpful to improve the penetration of drug whereas controlled release delivers the drug over a prolonged period of time. 5FU-loaded PLGA nanoparticles released 78% of the

drug in 24 hours. Lag phase is achieved due the enteric polymer Eudragit S 100 which is insoluble in acidic pH and starts dissolving at pH 7 and above. Once the formulation reaches ileocecal region of the GI tract, drug was released from the PLGA matrix by slow diffusion in the sustained manner. The in vitro release data was applied to various kinetic models to predict the drug release kinetic mechanism. The release constant was calculated from the slope (n) of appropriate plots and the regression coefficient (R^2). Based on regression values (r), all the formulations followed first order drug release kinetics. From Peppas model, it was found that all the formulations showed anomalous transport kinetics i.e., a combined mechanism of pure diffusion and Case II transport.

Cytotoxicity study of pure drug showed that at 30 µl dilution of stock solution (10 mg/ml) cell lysis was observed. 70% of the cell lysis took place after subjecting formulation F2 to the cell lines.

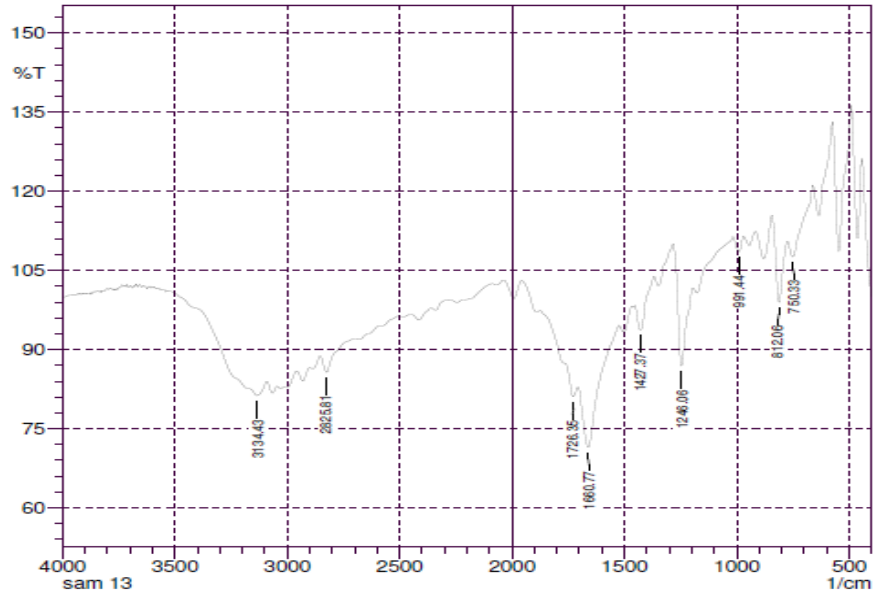

Figure 1: IR spectrum of 5-Fluorouracil and PLGA

Figure.2 IR spectrum of 5-Fluorouracil+ PLGA

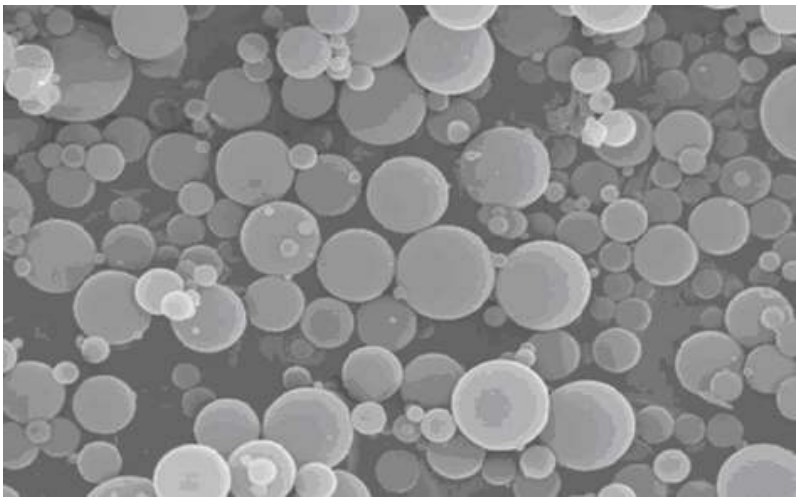

Figure 3: SEM image of 5-fluorouracil loaded PLGA nanoparticles

Table 2: Characterization of 5- Fluorouracil nanoparticles

Formulation	Drug Entrapment (%)	Particle size (nm)	5 FU content (%)	Zeta potential (mV)
F1	64.54 ± 0.88	120.67 ± 17.75	22.55 ± 1.96	-16.5 ± 4.3
F2	72.89 ± 1.41	132.57 ± 12.62	28.93 ± 1.33	-20.4 ± 5.6
F3	76.24± 0.46	138.72 ± 18.45	32.46 ± 1.67	-24.5 ± 4.5
F4	80.45± 1.34	146.43± 12.76	37.39 ± 1.55	-26.5 ± 5.2
F5	85.03 ± 0.99	150.20 ± 16.48	48.45 ± 1.40	-28.5 ± 2.3

Table 3: in vitro kinetic data analysis of 5- Fluorouracil nanoparticles

Formulation code	Zero order plot (R^2)	First order plot (R^2)	Higuchi plot (R^2)	Korsmeyer Peppas plot (n)
F1	0.990	0.986	0.894	1.402
F2	0.991	0.993	0.901	1.415
F3	0.976	0.987	0.938	1.464
F4	0.982	0.988	0.939	1.472
F5	0.979	0.985	0.936	1.481

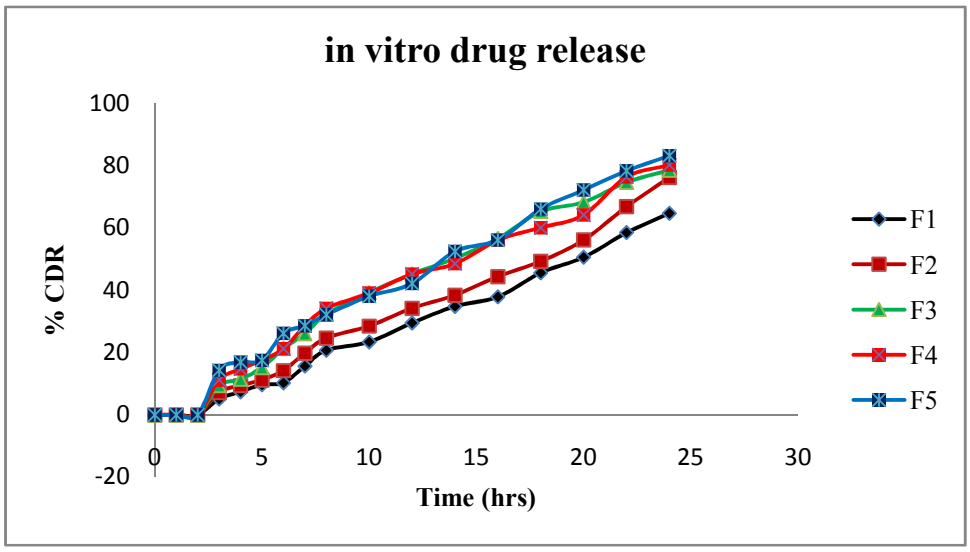

Figure 4: in vitro drug release of 5-fluorouracil loaded PLGA nanoparticles

Table 4: Cytotoxicity studies of 5FU on HT29 (Human colorectal Adenocarcinoma) Cell lines

S.No.	Compound	Concentration (10mg/ml) Cell line used- HT29 (Human colorectal Adenocarcinoma) Control OD- 2.35		
		10µl	20 µl	30 µl
1.	**5-Fluorouracil**	**O.D**-1.96 **MTT**-83.40%	**O.D**-1.93 **MTT**-82.12%	**O.D**-0.98 **MTT**-41.70%

Cytotoxicity Assay

S.No.	Compound	Concentrations(10mg/ml)		
		10µl	20 µl	30 µl
1.	5-Fluorouracil	No Lysis	No Lysis	Lysis

Table 5: Results of 5FU Nanoparticles on HT29 (Human colorectal Adenocarcinoma) Cell lines

S.No.	Compound	Concentration (µG)	O.D. at 492nm	% of cell lysis	IC50
1.	F2	10	0.337	No lysis	30 µG
2.	F2	20	0.480	35%	
3.	F2	30	0.491	70%	
4.	Control	-	0.253	No lysis	-

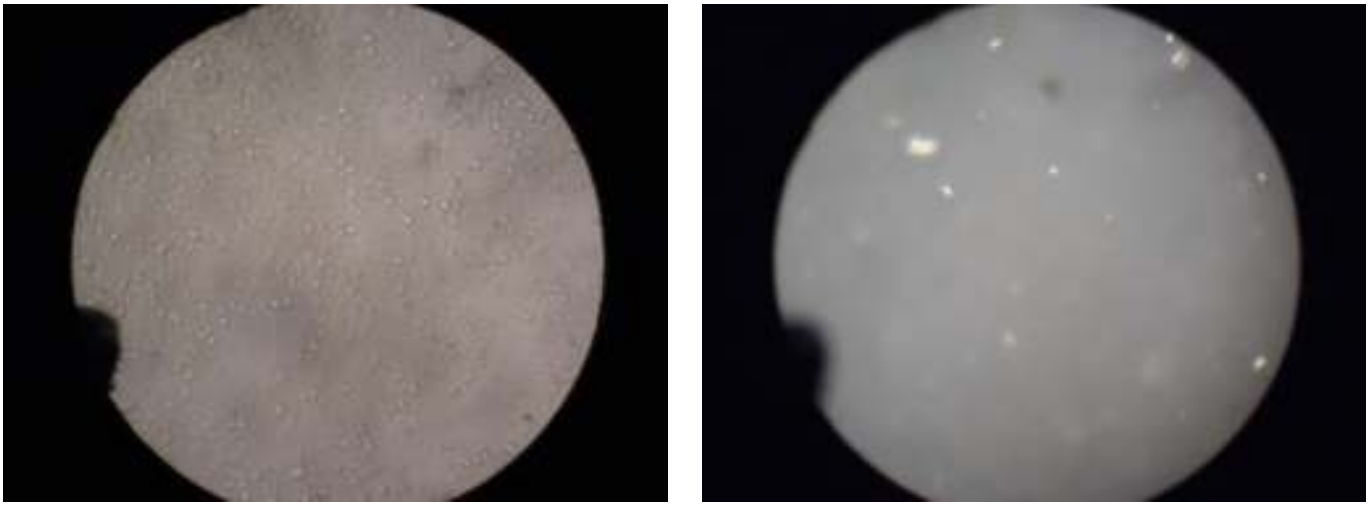

Figure 5:

i) Control HT-29 **ii) F2 nanoparticle formulation**

CONCLUSION

Colon targeted pH dependent, PLGA- 5 fluorouracil nanoparticles were formulated by modified emulsification solvent evaporation technique. The results of our study indicated that colon delivery is based on the combination of pH dependent and controlled-release properties. This combination of two types of polymers in single matrix nano-particulate delivery system could decrease the early drug loss before it reaching the site of action, a problem commonly encountered with pH dependent system. Tumor targeting of nanoparticles was carried out in HT-29 cells since they are known to over-express on colon cells. The cytotoxicity results suggest that the 5FU loaded nanoparticles exhibit increased uptake in HT-29 cells and showed cytotoxic effect. The results concluded that 5FU loaded nanoparticles developed in the study might be employed as a potential approach for targeting colorectal cancer.

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