

successfully in the delivery of drugs such as valdecoxib, 5-FU, curcumin, icariin, and vancomycin [19-24].

Ed, a pH-sensitive polymer protects the loss of drug in the upper gastrointestinal tract and subsequently results in the successful delivery of drugs in the colon only. Ed coating is already been used by formulation scientist for the successful delivery of 5-FU and valdecoxib [19,20].

Therefore, in the present investigation, a combination of 5-FU and Cel was loaded in Ed coated CH-microspheres (MSs) as a novel approach to deliver the "cocktail of therapeutic moieties" for the management of colon cancer. 5-FU and Cel combination loaded Ed coated CH-MSs were prepared by two methods, namely emulsion polymerization and nonaqueous solvent evaporation methods for investigating the effect on particle size and entrapment efficiency (EE). Both types of MSs were characterized with particle size analyzer, transmission electron microscope (TEM), EE, drug loading capacity, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and differential scanning calorimetry (DSC). The optimized formulation was further characterized for *in vitro* drug release and cytotoxicity analysis against human colon cancer cell line, HT-29 following standard cell proliferation assay.

MATERIALS AND METHODS

Materials

5-FU was purchased from CDH-Laboratory Chemicals India, whereas Cel was obtained as a gift sample from Cadila Pharmaceutical, Ahmadabad, India. HT-29 human colon cancer cell lines were purchased from NCS, Pune, India. CH and Ed were procured from HiMedia Laboratories, Mumbai, India. Glutaraldehyde (GA) and span 80 were purchased from Loba Chemie, Mumbai, India. Acetonitrile and water (high-performance liquid chromatography [HPLC] grade) were purchased from Fisher Scientific, Mumbai, India. All other reagents and chemicals were of analytical grade.

Cell culture and reagents

Human colon cancer cell lines (HT-29) were maintained at 37°C in CO₂ (5%), and air (95%) with Dulbecco's modified eagle medium (DMEM) enriched with fetal bovine serum (10%).

Methods

Preparation of drug loaded CH-MSs by an emulsion polymerization method

Emulsion polymerization method was employed for the development of CH-MSs loaded with 5-FU and Cel. In brief, a weighed quantity of CH (200 mg) was dissolved in 15 ml of 5% acetic acid, and 475 mg of 5-FU and 200 mg of Cel was added to it. This drug-polymer dispersion was added dropwise with the help of hypodermic syringe in a 150 ml liquid paraffin (75 ml light liquid paraffin +75 ml heavy liquid paraffin) containing 1% w/w span 80 and it was stirred with the help of mechanical stirrer for 1 h at 2500 revolutions per minute (RPM) and a temperature of 70°C. After 1 h a saturated solution of cross-linking agent (10 ml GA: 30 ml toluene) was added and stirred continuously until 2 h at 55°C. Suspension of cross-linked CH-MSs in paraffin oil thus obtained was allowed to stand for 20 min to let the MSs settle down under gravity. MSs were separated by filtration and washed several times with n-hexane to remove traces of the oil and finally with water to remove excess of GA. The MSs were dried at room temperature for 24 h and stored in desiccators [25].

Preparation of drug loaded CH-MSs by nonaqueous solvent evaporation method

Nonaqueous solvent evaporation method was also used to prepare CH-MSs. In a nutshell, a weighed quantity of CH (250 mg) was dissolved in 10 ml of acetone, and the combination of 5-FU (475 mg) and Cel (200 mg) was added to it. The prepared slurry was added into 30 ml of liquid paraffin. The mixture was stirred with the help of mechanical

stirrer at the speed of 1200 RPM for 4 h at room temperature. MSs were obtained after evaporation of the solvent and further washed repeatedly with petroleum ether to remove traces of oil and acetone. The washed MSs were stored at room temperature for 3 h and subsequently stored in desiccators [26].

Coating of drug loaded CH-MSs by an emulsification-solvent evaporation method

Emulsification-solvent evaporation method was employed for the coating of 5-FU and Cel loaded CH-MSs with Ed. The prepared drug-loaded CH-MSs (100 mg) were coated with 10 ml of 10% w/v ethanolic solution of Ed. Light liquid paraffin (70 ml) and span 80 (1% v/v) was added for emulsification followed by stirring for 3 h at 1000 RPM at room temperature for removal of the solvent by evaporation. The prepared drug-loaded Ed-CH-5-FU-Cel-MSs were filtered and washed with n-hexane and dried at room temperature and stored in desiccators [21,27].

Characterization of drug loaded MSs

FTIR spectroscopy

FTIR spectrum of 5-FU, Cel, physical mixture of 5-FU and Cel (5-FU-Cel-PM), Physical mixture of CH and Ed with both the drug molecules under investigation (Ed-CH-5-FU-Cel-PM) and Ed-CH-5-FU-Cel-MSs was recorded using infrared spectrophotometer (Alpha-E) utilizing KBr disc method (2 mg sample in 200 mg KBr). The scanning range selected for the study was 4000–400 cm⁻¹ at a resolution of 4 cm⁻¹.

DSC

DSC thermograms were recorded for 5-FU-Cel-PM and Ed-CH-5-FU-Cel-MSs using DSC (Mettler Toledo, 822e, Griefensee, Switzerland). All samples were hermetically sealed in crimped aluminum pan and subsequently heated at a temperature range of 30–300°C and cooling rate of 10°C/min in an inert environment of nitrogen gas.

Powder XRD (PXRD)

X-ray pattern of the selected samples was recorded using X-ray diffractometer (X'Pert PRO, Panalytical Company, Netherlands). Nickel filtered, CuK α radiations generated at a voltage of 60 kV and 50 mA current were employed for study at a scanning rate of 1°/min over the diffraction angle (2 θ) range of 10–60°. PXRD diffractograms of 5-FU, Cel, and Ed-CH-5-FU-Cel-MSs were recorded and analyzed.

Particle size analysis

The mean particle size distribution of prepared Ed-Ch-5FU-Cel-MSs was calculated by laser diffractometry using Mastersizer 2000, Malvern Instruments Ltd. for dry powder measurement. The accurately weighed MSs (20 mg) were dispersed in HPLC water and visualized for particle size distribution [28,29].

TEM

The shape and surface morphology of the developed Ed coated CH-MSs was investigated using TEM (FTI Tecnai F20). An aqueous suspension of MSs was drop cast onto a carbon coated grid which was then air dried at room temperature before loading into a microscope, maintained at a voltage of 80 kV.

Drug EE and drug loading capacity

Drug EE% of 5-FU and Cel in the developed MSs was determined using an HPLC method. For this purpose, precisely weighed (10 mg) of coated MSs were added into 10 ml of acetonitrile:water (50:50) mixture and subjected to centrifugation at 1500 RPM for 10 min. The resulting clear centrifuge (2 ml) was pipetted out and filtered through 0.45 μ m membrane filter. 20 μ L of this filtered solution was then injected into HPLC column and analyzed at 260 nm [28,29].

The percent drug EE and drug loading capacity were calculated using the following equations:

EE (%) = Actual drug loading / Theoretical drug loading × 100
 DLC = Drug weight within the microspheres / weight of microspheres

In vitro drug release studies

Ed-CH-5FU-Cel-MSs were evaluated for *in vitro* drug release in pH progression medium at 37°C ± 0.5°C. The dissolution study was performed using the paddle method. Accurately weighed coated MSs (100 mg) were deliberately spread over the surface of 900 ml of dissolution medium. The content was rotated at 50 RPM. To compare the release of 5-FU and Cel under different GI transit condition, the pH of the dissolution medium was altered at different time intervals. The experiments were performed in an acidic buffer (pH 2.0) for 2 h, which simulates the condition of the fasted stomach. After that, the study was conducted in the phosphate buffer (pH 4.5) to simulate duodenum for 2 h followed by in phosphate buffer (pH 6.8), which simulates mid jejunum. Finally, drug release study was continued in phosphate buffer (pH 7.4) to simulate ileo-colonic conditions for the next 2 h. Sample (5 ml) was withdrawn at specific time intervals (after 5, 15, 30, and 60 min for 1st h, afterward every hour till the end of the study) and replaced with fresh dissolution medium to mimic finite sink conditions. The samples were passed through a microfilter and analyzed by HPLC for estimation of drug release. The dissolution study was performed in triplicate [20,21,29,30].

In vitro cytotoxicity assay

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay, a standard colorimetry based assay was selected for the determination of cytotoxicity. HT-29 human colon cancer cell line was cultured in a 96 well microtiter plate. Briefly, HT-29 cells were implanted in 200 µL of the serum DMEM medium at a density of 5 × 10³ cells per well. After the incubation period of 24 h, the medium was replaced with serum-free-DMEM. After 24 h, the HT 29 cells were exposed to 5-FU, Cel, 5-FU-Cel, Ed-CH-5-FU-Cel-MSs and blank MSs (Bl-MSs) equivalent to 10–70 µM of 5-FU and Cel for 72 h. At the end of the treatment, the medium was removed and the cells were treated with 0.5 mg/ml of MTT dye for 4 h at 37°C. The HT 29 cells were lysed and thus formed formazan crystals were dissolved in 100 µL of DMSO and quantified by ELISA reader at 570 nm and 630 nm. The experiments were performed in triplicate (n=3) [31-33].

RESULTS AND DISCUSSION

Preparation and characterization of MSs

Ed-CH-5-FU-Cel-MSs were prepared by emulsion polymerization method and characterized under a set of stringent parameters [25].

Characterization of MSs

FTIR spectroscopy

The results of the FTIR spectrum of 5-FU, Cel, 5-FU-Cel-PM, Ed-CH-5-FU-Cel-PM and Ed-CH-5-FU-Cel-MSs are shown in Table 1. FTIR spectrum showed characteristic peak due to pure 5-FU at 1641 and 1229 cm⁻¹ correspond to the C=O and C-X, respectively. The spectrum of Cel showed a characteristic peak at 3331, 1345, and 1138 cm⁻¹ due to N-H stretching and S=O stretching vibrations of sulfonamide. In 5-FU-Cel-PM a slight shift was observed at 1226 and 1138 cm⁻¹ from the original peaks of 5-FU and Cel, respectively. The spectrum of Ed-CH-5-FU-Cel-MSs indicated that characteristic peaks of 5-FU, Cel, and Ed are present in the PM of drugs and excipients as well as in the drug-loaded MSs. However, broadening and decrease in peak intensity were observed in the spectrum of drug loaded MSs which indicated no chemical interaction between drug and polymer [21].

DSC

DSC thermograms of 5-FU-Cel-PM along with Ed-CH-5-FU-Cel-MSs are presented in Fig. 1. A sharp endothermic peak at 162.02°C was observed for 5-FU, close to its investigated melting point (158°C). On

Table 1: FTIR assignment of 5-FU, Cel, 5-FU-Cel-PM, Ed-CH-5-FU-Cel-PM and Ed-CH-5-FU-Cel-MSs

Sample	Peak	Assignment
5-FU	1641	C=O
	1229	C-X
Cel	3331	N-H stretching
	1345 and 1138	S=O stretching
	1226	C-X
5-FU-Cel-PM	1345 and 1147	S=O stretching
	1226	C-X
Ed-CH-5-FU-Cel-PM	1345	S=O stretching
	1227	C-X
	1148	Eudragit S 100
Ed-CH-5-FU-Cel-MSs	2912	OCH ₃ /CH ₃
	1647	C=O
	1241	C-X

FTIR: Fourier transform infrared spectroscopy, 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, PM: Physical mixture

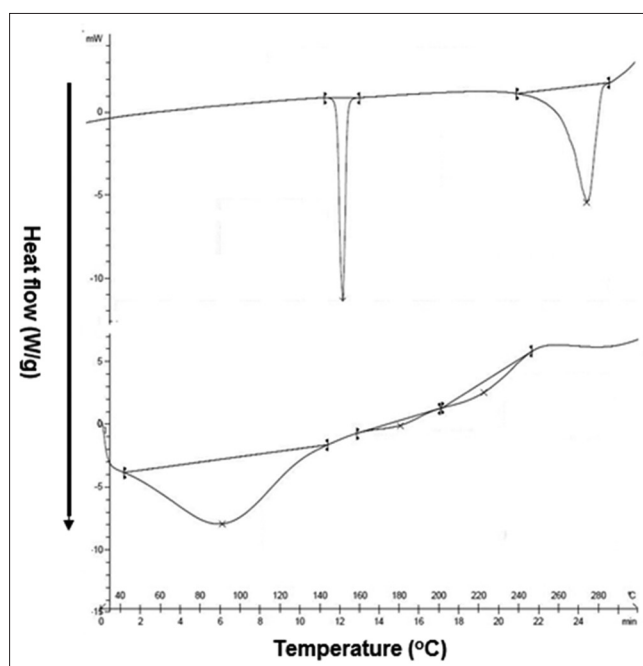


Fig. 1: Differential scanning calorimetry of (a) 5-FU-Cel-PM and (b) Ed-CH-5-FU-Cel-MSs estimated between 30°C and 300°C. Each sample under investigation was scanned thrice, and the average of it was considered. 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, PM: Physical mixture, Ed: Eudragit S 100

the other side, Cel exhibited an endothermic peak at 275.87°C which is well matched with its reported melting point range of 280–282°C. Ed-CH-5-FU-Cel-MSs presented a depressed and relatively broad endothermic peak at 90.90°C which corroborated amorphization of the crystal structure of the drugs [34].

PXRD studies

PXRD studies were carried out to determine the crystalline nature of the drugs under investigation. The XRD diffractograms (Fig. 2) of 5-FU and Cel exhibited the sharp and intense peaks, indicating the crystalline structure. While the diffractograms of Ed-CH-5-FU-Cel-MSs demonstrated, low intensity peaks designated to the amorphous lattice. This was consistent with the previous reports [33].

Particle size analysis and surface morphology

It is apparent from the results obtained from laser diffractometry, the mean particle size of Ed-CH-5-FU-Cel-MSs was found to be 4.8 ± 1.1 µm and 2.7 ± 0.9 µm, respectively, for MSs prepared by emulsion

polymerization method and nonaqueous solvent evaporation method with an insignificant difference (Unpaired t-test, $p > 0.05$). The photomicrographs obtained from TEM indicated the spherical and uniform shape of the coated MSs without any distortion of surface texture (Fig. 3).

Drug EE and drug loading capacity

The results of drug EE and drug loading capacity of the Ed-CH-5-FU-Cel-MSs are expressed in Table 2. The drug EE for MSs developed by emulsion polymerization method was calculated to be $71.30 \pm 2.3\%$ and $35.20 \pm 1.9\%$ with significant difference (Unpaired t-test, $p > 0.05$) whereas the drug loading capacity was found to be in the range of 6.5 mg/10 mg and 2.3 mg/10 mg for 5-FU and Cel, respectively. On the other hand, in case of MSs developed by nonaqueous solvent evaporation method, slight decrease in drug EE as well as in drug loading capacity was observed.

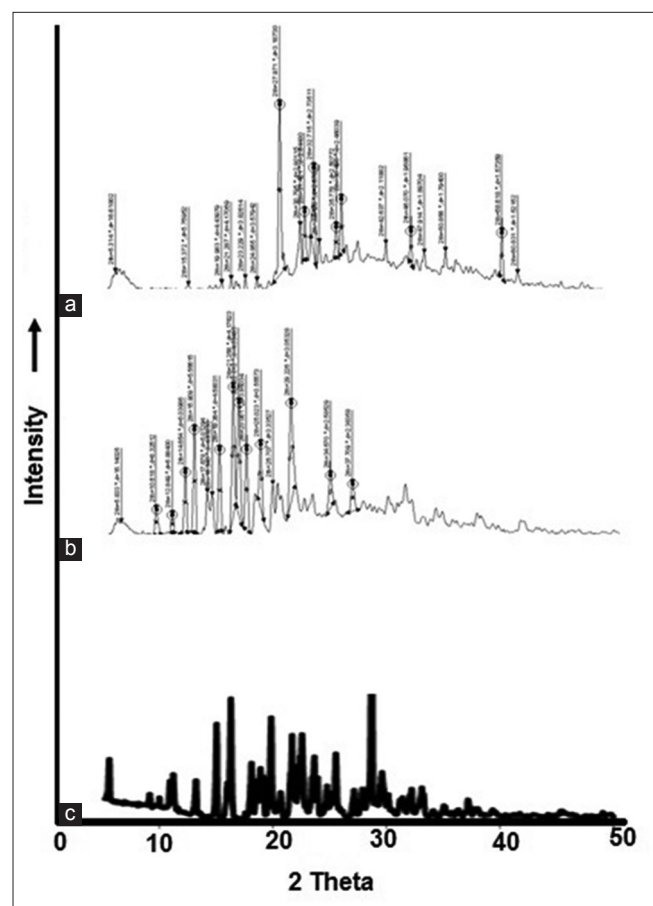


Fig. 2: Powder X-ray diffraction pattern of (a) 5-FU; (b) Cel, and (c) Ed-CH-5-FU-Cel-MSs measured between 10° and 60° at 2θ angle. Each sample was scanned thrice, and the average of that sample was considered. 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, Ed: Eudragit S 100

Table 2: Particle size, EE, and drug loading capacity of the drug-loaded MSs (Ed-CH-5-FU-Cel-MSs)

Parameter	Emulsion polymerization method	Nonaqueous solvent evaporation method
Particle size	$4.8 \pm 1.1 \mu\text{m}$	$2.7 \pm 0.9 \mu\text{m}$
EE	$71.30 \pm 2.3\%$ (5-FU) $35.20 \pm 1.9\%$ (Cel)	$70 \pm 1.7\%$ (5-FU) $34.64 \pm 1.5\%$ (Cel)
Drug loading capacity	6.5 mg/10 mg (5-FU) 2.3 mg/10 mg (Cel)	6.4 mg/10 mg (5-FU) 2.32 mg/10 mg (Cel)

EE: Entrapment efficiency, 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres

Each experiment was carried out in triplicate (n=3)

In vitro drug release studies

The formulation approach used in the development of Ed-CH-5-FU-Cel-MSs was to delay the release of the combination of drugs in the stomach and to get a controlled drug release for a relatively long duration, especially at the site of action, i.e., colon. Results obtained from *in vitro* drug release study met with the expected outcome. Dissolution study was carried out in pH progression medium at $37 \pm 0.5^\circ\text{C}$. It is evident from the drug release study that at 0.5 h, $97.5 \pm 1.1\%$ 5-FU was released as compared (two-way ANOVA test, $p < 0.01$) to $15.3 \pm 0.8\%$ release of Cel from 5-FU-Cel-PM. Ed-CH-5-FU-Cel-MSs exhibited the release of $92.1 \pm 2.9\%$ of 5-FU in 8 h as compared (two-way ANOVA test, $p < 0.01$) to 18.9 ± 0.7 release of Cel after the same duration of time (Fig. 4).

Ed-CH-5-FU-Cel-MSs showed that there was no substantial amount of drug release (5-FU and Cel) in the initial 2 h in simulated gastric fluid (pH 1.2). The release of both drugs under investigation started in intestinal pH and the maximum release observed in the colonic milieu, the site of action.

In vitro cytotoxicity assay

To investigate the synergistic effect of 5-FU and Cel combination in CRC, the standard cell proliferation assay was performed on pure drugs as well as on the developed formulations. Cytotoxicity of the 5-FU, Cel, 5-FU-Cel-PM, Ed-CH-5-FU-Cel-MSs, and BI-MSs was studied against HT-29 cell line and expressed in terms of percentage cell viability (Fig. 5). The drug-loaded MSs exhibited appreciable potency against HT-29 cells with an IC_{50} value of $35.9 \pm 4.2 \mu\text{M}$, which is notably lower than (One-way ANOVA test, $p < 0.05$) IC_{50} value of pure Cel ($48.7 \pm 2.1 \mu\text{M}$), 5-FU ($45.4 \pm 3.6 \mu\text{M}$), and the combination of both drugs ($47.3 \pm 2.5 \mu\text{M}$). BI-MSs did not produce any cytotoxicity against HT-29 cells. The results exhibited the possible synergistic effect when Cel and 5-FU were used in combination. This strategy could be used as a potential approach for the management of CRC due to controlled release phenomena [35,36].

CONCLUSION

In the present study, we have offered the oral drug delivery system in the form of MSs of 5-FU and Cel combination for the management of CRC. Carbohydrate polymer, CH was employed for the fabrication of MSs to enable the drug release in the colonic milieu. In addition, Ed coating has supported the tailored system to release the drug at the target site.

Our Eudragit coated carbohydrate based polymeric MSs of 5-FU and Cel combination will potentially improve the drug delivery in the colon while maintaining the high stability in the stomach and small intestine.

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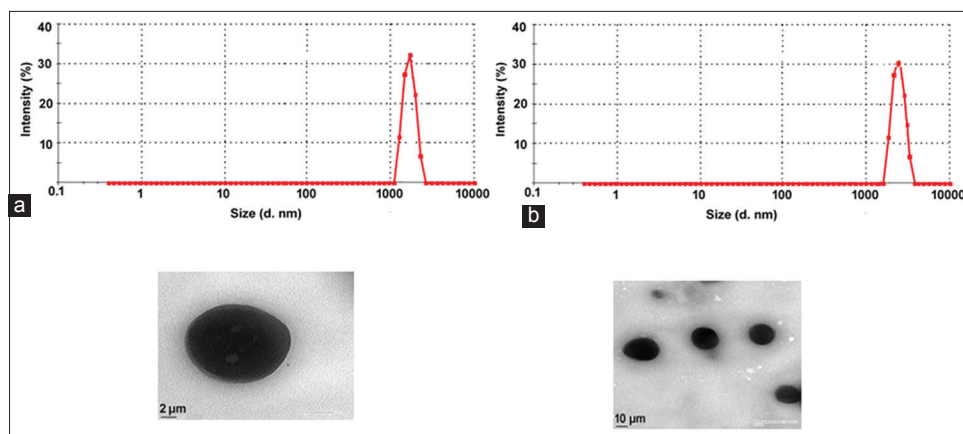


Fig. 3: Particle size distribution and transmission electron microscope of Ed-CH-5-FU-Cel-MSs prepared by (a) nonaqueous solvent evaporation method and (b) emulsion polymerization method. Each Experiment was carried out in triplicate (n=3). 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, Ed: Eudragit S 100

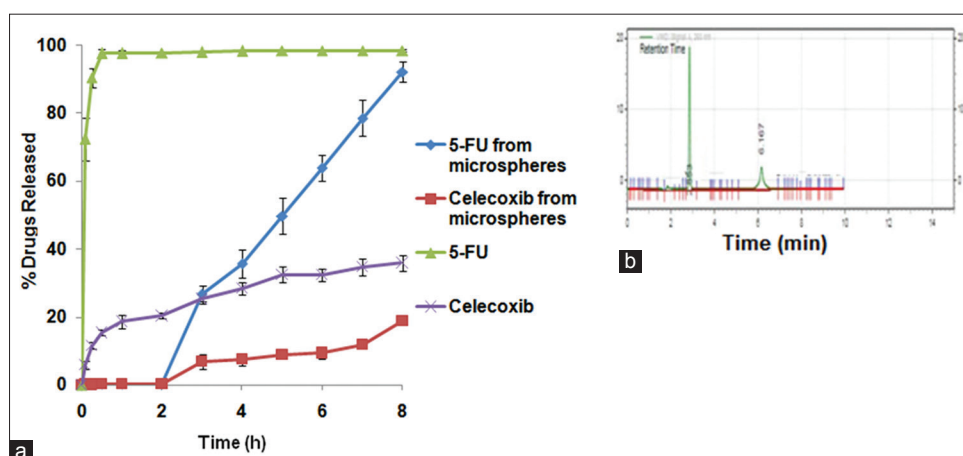


Fig. 4: (a) *In vitro* release profile of Ed-CH-5-FU-Cel-MSs in simulated gastric fluid followed by simulated intestinal fluid and finally in the simulated colonic fluid. The study was performed in triplicate (mean±standard deviation, n=3). (b) Representative chromatogram of drug release studied by high-performance liquid chromatography. 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, Ed: Eudragit S 100

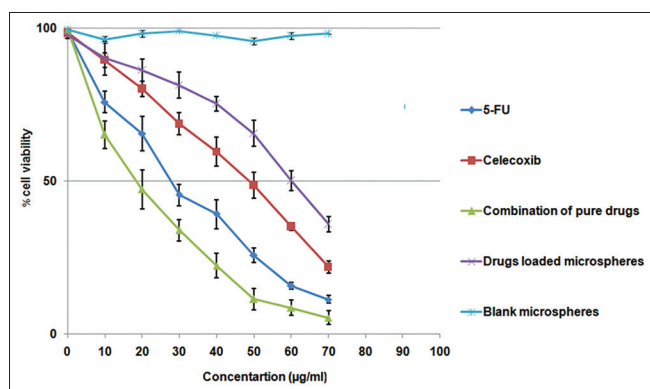


Fig. 5: Therapeutic efficacy testing of Ed-CH-5-FU-Cel-MSs against human colon cancer cells, HT-29. Each experiment was carried out in triplicate (n=3). 5-FU: 5-Fluorouracil, Cel: Celecoxib, CH: Chitosan, MSs: Microspheres, Ed: Eudragit S 100

AUTHORS' CONTRIBUTIONS

Vikas Bansal: Conceptualized the article, compiled full literature search and drafted the manuscript. Anjoo Kamboj: Supervisor of the research work, provided guidance in the preparation of a standard paper. Jitender Madan: Evaluated the manuscript and helped in statistical analysis.

CONFLICTS OF INTEREST

There are no conflicts of interest among authors.

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