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**Manuscript**

# Synthesis and bio-evaluation of xylan-5-fluorouracil-1-acetic acid conjugates as prodrugs for colon cancer treatment

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**Revised Highlights:**

- Xyl-5-FUAC conjugates were synthesized as prodrugs for colon specific drug delivery

- Higher amount of drug was released from the conjugates in presence of colonic contents

- Xyl-5-FUAC conjugates shows better cytotoxicity than free drug

**Abstract**

In the present study, xylan-5-fluorouracil-1-acetic acid (Xyl-5-FUAC) conjugates as colon specific prodrugs were synthesized and evaluated by *in-vitro* release study. The chemical stability of the conjugates was performed in acidic (pH 1.2) and basic buffers (pH 7.4), which showed their stability in upper gastrointestinal tract. The *in-vitro* drug release profiles of the conjugates were studied in the presence of rat's gastrointestinal contents. The results showed that the low amounts of drug 3-4% and 5-7% was released in gastric and small intestine contents respectively, while 53-61% of the drug was released in cecum and colonic contents. The cytotoxicity studies of the conjugates were also evaluated on human colorectal cancer cell line (HTC-15 and HT-29), which showed that the conjugates are more cytotoxic than the free drug. Therefore the results reveal that Xyl-5-FUAC conjugates are potential candidates for colon specific drug delivery in the treatment of colonic cancer with minimal undesirable side effects.

*Keywords:* Xylan, 5-fluorouracil-1-acetic acid (5-FUAC), drug release, gastrointestinal contents, cell cytotoxicity

## 1. Introduction

In recent years, oral drug delivery to the colon has gained much interest for the treatment of colonic diseases such as inflammatory bowel disease, Crohn's disease, ulcerative colitis, and colon cancer (R. Sharma, Rawal, Malhotra, Sharma, & Bhardwaj, 2013). Among the various cancers, colon cancer is the third most leading causes of deaths in the world (Plyduang, Lomlim, Yuenyongsawad, & Wiwattanapatapee, 2014). Although several chemotherapeutic agents such as 5-fluorouracil, oxaliplatin, methotrexate, folinic acid, bevacizumab (Avastin) and Celecoxib (Celebrex) have been used for the treatment of colorectal cancer but due to their short half life or absorption in the upper GIT tract, an effective concentration of drug cannot reach the target site and also their nonspecific selectivity adverse side effects (R. Sharma, Rawal, Malhotra, Sharma, & Bhardwaj, 2014). The use of conventional oral formulations is limited for colon cancer, as they are designed to achieve systemic delivery of chemotherapeutic agents. Since systemic delivery leads to the distribution of the drug throughout the body, it may result in systemic side effects and toxicity (Hua, Marks, Schneider, & Keely, 2015). Selective delivery of drugs to the colon provides maximum therapeutic activity by preventing degradation or inactivation of drug during transit to the target site. The most critical challenge in such a drug delivery approach is to preserve the formulation during its passage through the stomach and small intestine.

5-fluorouracil (5-FU) is one of the most prescribed anti-cancer agent, used clinically alone or in combination of other anti-cancer agents, for a long time (Daumar et al., 2011). A large number of chemical-modified derivatives of 5-FU with low sugar molecule, amino acid, short peptide, porphyrin compounds among others have been synthesized to prolong its half-life as well as antitumor curative efficacy, but their low bioavaibility and uncontrolled release profile makes them unsuitable for colon specific drug delivery (Huang et al., 2014; Li, Wang, & Yu, 2011). Extensive research has been carried out in developing various drug delivery systems for colon specific drug delivery such as pH dependent, time dependent and Polymer-drug conjugate or prodrug (Philip & Philip, 2010).

In order to improve the oral bioavaibility and versatile drug releases kinetics; numerous polymer conjugated prodrugs have been synthesized, in which drug attached directly or through the

linkers (Wang et al., 2007). Over the last several years, biopolymers extracted from agro-waste have received much interest because of their natural abundance, relative ease of isolation, sustainable and renewable materials and widely used in a variety of applications such as film packing, drug delivery, paper making, adhesive or additive in plastic, or as a food additive and stabilizer due to their excellent physiochemical and biological properties (Ebringerová & Heinze, 2000; Petzold-Welcke et al., 2014; Sharma & Varma, 2014).

Apart from cellulose, starch and lignin, hemicellulose xylan is also one of the most abundant biopolymer, consist of β-(1-4) linked backbone of xylose units which is partially associated with galactopyranosyl, glucuronosyl, arabinosyl and acetyl residues, depending upon the biological source and the method of extraction (Bosmans et al., 2014; Deutschmann & Dekker, 2012). Recently, xylan is gaining increasing interest as a new biopolymer in the field of biomedical applications because of their potential bio-application including wound dressings, hydrogels or as a carrier in drug delivery (Fonseca Silva, Habibi, Colodette, & Lucia, 2011).

The colon specific drug delivery is mainly based on the degradation of a prodrug by microorganisms of the microflora and colonic enzymes, which is not present in other regions of GIT( Chourasia & Jain, 2003). Xylan is a promising biopolymer as it remains intact in the upper GIT and subsequently degraded by xylanses and β-xylosidase enzyme present in colon (S. Kumar & Negi, 2012). In addition to its biocompatibility, non-immunogenicity and non-toxicity it also possesses antitumor properties which makes it a suitable carrier for colon specific drug delivery (Peng, Peng, Xu, & Sun, 2012).

The aim of this study was to further explore the potential of prodrugs for colon cancer treatment. In this regards, Xylan-5-fluorouracil-1-acetic acid (Xyl-5-FUAC) conjugates were synthesised and evaluated as prodrugs for colon cancer treatment. Xylan from corn cob was used as a carrier to examine the potential for the delivery of 5-fluorouracil, where the drug and carrier were bound through ester linkage. The *in-vitro* drug release of the conjugates in rat's gastrointestinal contents as well as the cytotoxic activity on human colorectal cancer cell line (HTC-15 and HT-29), was also investigated. To the best of our knowledge this is the first report on the synthesis of Xyl-5-FUAC conjugates to evaluate as colon-specific prodrugs for the delivery of anti-cancer drug.

## 2. Experimental

2.1. Materials

Corn cob was collected from agricultural field (Saharanpur, Uttar Pradesh, India) and used as the xylan source. 5-fluorouracil ($\geq$ 99%) and dialysis membrane (diameter = 35 mm, Mw cut-off 12 KDa) were purchased from Sigma-Aldrich chemicals Pvt. Ltd. (Bangalore, India). chloroacetic acid, sodium chlorite and 1, 1-carbonyldiimidedazole (CDI), potassium hydrogen carbonate ($KHCO_3$), hydrochloric acid (HCl), were obtained from Himedia Pvt. Ltd. (Mumbai, India). HCT-15 (human colorectal adenocarcinoma) and HT-29 (human colon carcinoma) cell lines were obtained from National Centre for Cell Science (Pune, India) and were subsequently cultured in RPMI 1640 medium supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin-streptomycin under 5% $CO_2$ atmosphere at $37^{\circ}C$. The cell staining dyes, acridineorange (AO) and ethidium bromide (EtBr) were obtained from Sigma-Aldrich (USA) and SRL (India) respectively. All others chemicals and solvents were of analytical grade and used without further modification.

2.2. Isolation of xylan from corn cob

The extraction of xylan was carried out by alkali treatment as previously reported (Fundador, Enomoto-Rogers, Takemura, & Iwata, 2012; S. Kumar & Negi, 2012). First, corn cob was milled into powder by Wiley mill and sieved under 40 mesh screen after drying in sunlight. The dried powder of corn cob (10g) was dewaxed in soxhlet apparatus with a 2:1(v/v) mixture of benzene and methanol for 6h and then delignified with acidified sodium chlorite solution at $70^{\circ}C$ for 2h. This process was repeated 3-4 times after that mixture was cooled to room temperature, filtered and washed with water until free of acid. The recovered product was almost white in colour and known as holocellulose (mixture of cellulose and hemi-cellulose). The holocellulose was extracted with 10% NaOH with a solid to liquor ratio of 1:20 for 24h at room temperature. Then the filtrate was neutralised with acetic acid and the mixture was allowed to stand overnight after adding the twice volume of ethanol. The precipitated xylan was collected by vacuum filtration

and washed with ethanol, and then it was dried in vacuum oven at 50 $^{\circ}$C for 24 h (yield = 1.2g, 8-10%). The schematic diagram for extraction of xylan from corn cob is shown in (Fig.S1, Supplementary data).

2.3. Synthesis of xylan-5-fluorouracil-1-acetic acid (Xyl-5-FUAC) conjugates

*2.3.1. Synthesis of 5-fluorouracil-1-acetic acid (5-FUAC)*

5-FUAC was synthesized according to previously described method (Sun et al., 2012) with some modification. 5-FU (0.5g, 2.6 mmol) was dissolved completely in 5ml aqueous solution of KOH (0.3g, 5.3 mmol), and the reaction mixture was stirred at 100$^{\circ}$C for 70 min. After that 4ml of chloroacetic acid (0.25g, 2.6 mmol) solution was added slowly and stirred in an oil bath under 60 $^{\circ}$C for 6h. The product was acidified to pH 2 with HCl, followed by cooling 4$^{\circ}$C for 12h. Then, the precipitate was filtered and re-dissolved in a saturated KHCO$_3$ solution, again acidified to pH 2 with HCl to obtain the needle-like crystals of 5-FUAC (Yield= 0.41g, 57%).

*2.3.2. Synthesis of Xyl-5-FUAC conjugates*

In a typical procedure, 5-FUAC (0.188g, 1mmol) was reacted with CDI (0.326g, 2 mmol) for 3h in 5ml DMSO, to get the 5-fluorouracil-1-acetic acid imidazolide as intermediate. Then previously dissolved xylan (0.132g, 1 mmol) in DMSO was added to the solution, followed by addition of 200 µl triethylamine (TEA). The mixture was stirred at 80$^{\circ}$C for 20 h. After cooling, the solution was poured into ethanol (50 ml), to precipitate the resulting product. Finally, the product was filtered, washed with acetone to remove the excess reactants and then dried in vacuum. The whole synthesis process and structure of the conjugates is depicted in (Fig.1).
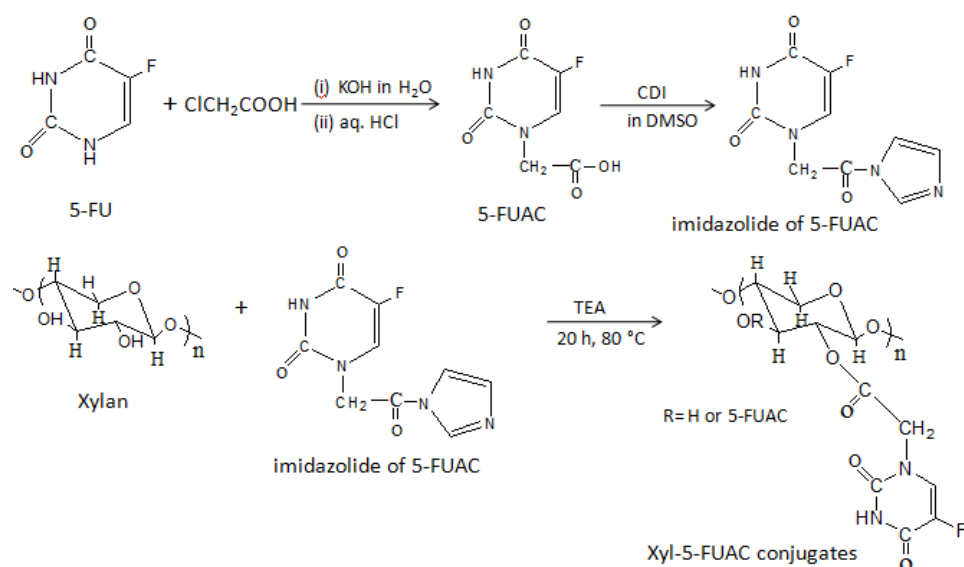
**Fig.1.** Proposed chemical reaction mechanism for the synthesis of Xyl-5-FUAC conjugates.

2.4. Physicochemical characterization of Xyl-5-FUAC conjugates

*2.4.1. Structural characterization*

The Chemical structure of isolated xylan, 5-FUAC and Xyl-5-FUAC conjugate was evaluated by FT-IR spectroscopy using PerkinElmer FT-IR C91158 spectrophotometer. A small amount of each material was mixed with KBr in a ratio of 1:100 (w/w) and compressed into tablet. All the spectra were recorded in transmittance mode with 32 scans between the ranges of 4000-400 cm$^{-1}$ at room temperature.

The $^{1}$H and $^{13}$C NMR spectra of the samples were obtained from a Bruker spectrophotometer operating at 500 MHz at room temperature with tetramethylsilane (TMS) as the internal reference. $^{1}$H NMR spectra were recorded from 20 mg of sample dissolve in 1.0 ml DMSO-d$_6$ after 16 scan whereas the $^{13}$C NMR spectra was recorded from 60 mg of sample dissolve in 1.0 ml DMSO-d$_6$ after 3,000 scan.

*2.4.2. Spectral characterization*

The Spectral characterization of the free drug and xyl-5-FUAC conjugates was analyzed by UV spectrophotometer (UV-1800 Schimadu, Japan). The absorption intensities of xyl-5-FUAC conjugates and free drug were studied at 273 nm.

The structural (physical) analysis of the isolated xylan, free drug and prodrug was analyzed by X-ray Diffraction (XRD). The X-ray diffraction patterns were obtained on a (Rigaku Ultima IV) diffractometer with a scanning rate of $5^{\circ}C$ /min using Cu Kα irradiation, over the angular range $2\theta = 5–60^{\circ}$.

*2.4.3. Thermogravimetric analysis (TGA)*

TGA thermogram of the isolated xylan was recorded on a Exstar TGA/DTG 6300 thermogravimetric analyzer (TA Instruments) under $N_2$ atmosphere. Measurements were performed on 3-5 mg samples in an aluminium pan at a heating rate of $10^{\circ}C$/min, from $30^{\circ}C$ to $600^{\circ}C$. The weight loss (TG curve) and its first derivative (DTG curve) were recorded simultaneously as a function of temperature.

2.5. Determination of drug content in conjugates

The content of 5-FUAC in conjugates was determined by the hydrolyzing the glycosidic bond between the 5-FUAC and xylan in basic condition. A series of standard solutions of 5-FUAC were prepared in 1N NaOH solution and their absorbency were measured at 273nm using UV spectrophotometer. A calibration curve (10-50μg/ml) was plotted according to the absorbency and to estimate the actual amount of drug, Xyl-5-FUAC conjugates dissolved in 1 N NaOH (1mg/ml) Solution, and the absorbance also were recorded at same wavelength (273nm). The amount of 5-FUAC in conjugates was calculated by comparison with the standard. The percent drug loading was calculated using following equation.

% of drug loading (w/w %) =         (Amount of 5-FUAC )                X 100
                              Amount of Xyl-5-FUAC conjugates

2.6**.** Chemical stability study of the conjugates

The chemical stability of the conjugates was checked in (0.1 N HCl) acidic buffer pH 1.2 and phosphate buffer pH 7.4, by using the dialysis method (Yang et al., 2016). The synthesized conjugates (10 mg) were dissolved in their respective media and transferred to a dialysis bags,

and kept in a beaker containing 90 ml buffer solution of pH 1.2 and 7.4 respectively. The solution was incubated at 37 °C under shaking. At a fixed time intervals, 3 ml of sample was taken out and replace with the same amount of fresh media to maintain the sink condition. The amount of drug released was analyzed by UV spectrophotometer.

2.7. In-vitro drug release study in presence of gastrointestinal contents of rats

*2.7.1. The drug release study in gastric and small intestine contents of rats*

To achieve the actual release behaviour of the conjugates at the target site, i.e. colon an *in-vitro* drug release study was performed in the presence of GI contents of rat. Section of stomach and small intestine were removed separately from male wistar rats (120-40g) after midline incision (Plyduang et al., 2014). The contents of stomach and small intestine were collected and suspended in acetate buffer pH 4.5 and phosphate buffer of pH 7.4. After that content were homogenized using blender and diluted with appropriate buffers to produce the 4wt% slurry. For the drug release study, (10mg) of the conjugates were dispersed in 10 ml solution of acetate buffer pH 4.5 and phosphate buffer of pH 7.4 containing 4wt% appropriate content of the GI tract and transferred into a dialysis bag, which was further immersed in 90 ml release medium at 37 °C. At a fixed time intervals, 3 ml of the release medium was taken out for further determination and replaced by fresh release medium at the same volume and temperature.

*2.7.2. The drug release study in the content of cecum and colon of rats*

The contents of the cecum and colon were collected and immediately dispersed into cold PBS (pH 6.8) previously bubbled with nitrogen to maintain the anaerobic condition. The synthesized conjugates (10 mg) were dispersed in a phosphate buffer of pH 6.8 containing 4wt% content of cecum and colon, and transferred into a dialysis bag, which was further immersed in 90 ml release medium at 37 °C. At a fixed time intervals, 3 ml of the samples were taken out and the concentration of the drug was determined by UV spectrophotometer as described previously.

*2.7.3. In-vitro cytotoxicity Study*

The cytotoxicity of the Xyl-5-FUAC conjugates, free drug (5-FU or 5-FUAC) against HCT-15 and HT-29 cells was determined by MTT assay (S. U. Kumar & Gopinath, 2015). Around $5 \times 10^3$ cells were seeded in 96-well plate and then treated with different concentrations (1-10 µM) of

Xyl-5-FUAC conjugates and free drug (5-FU or 5-FUAC) for 12, 24 and 48h. At the end of respective time points, spent media was removed from each well and subsequently replenished with fresh media (DMEM) supplemented with 10µL of MTT (concentration-500µg/mL). The cells were then incubated at 37 $^{\circ}$C for 3-4 h during which metabolically active viable cells converts MTT to insoluble formazan crystals. The resultant dark-blueformazan crystals were then solubilized in dimethyl sulfoxide (DMSO) and the corresponding absorbance at 574 nm was recorded for each well by Cytation 3 multimode plate reader (Biotek, USA).The acquired absorbance values were represented as percentage viable cells with respect to untreated control cells by the following equation

$$\text{Relative cell viability} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

All experiments were carried out in triplicates and the results were plotted as mean ± SD.

*2.7.4. Acridine Orange-Ethidium Bromide (AO-EB) staining*

AO-EB staining was performed in order to monitor the extent of apoptosis inflicted by Xyl-5-FUAC conjugates and free drug (5-FU or 5-FUAC) against HCT-15 and HT-29 cells at different time points (i.e. at 12, 24 and 48h). HCT-15 and HT-29 cells were treated with 5µg/ml and 3µg/ml 5-FU equivalents of drug-polymer conjugates. At the end of respective incubation period, growth medium was replaced with PBS containing 10 µg/ml of AO-EB mixture and subsequently incubated at 37 $^{\circ}$C for 10–15 min. The cells were then briefly rinsed with PBS in order to remove excess dyes. After staining the cells, images were captured by EVOS cell imaging system (Life technologies, USA) under blue filter and green filter.

**3. Results and discussion**

3.1 Characterization of extracted xylan

The FT-IR spectrum of the extracted xylan (Fig. 2b) shows a characteristic peaks at 3420 cm$^{-1}$ due to the -OH stretching and an intense peak at 1632 cm$^{-1}$ is due to absorbed water. The spectral bands between 1465- 1043 cm$^{-1}$ corresponds to the stretching and bending vibrations of C-O, C-

C and C-OH. A sharp peak at 885 cm$^{-1}$ represents the β-glucosidic linkages between the sugar units.

Fig. 3a represents the $^1$H NMR Spectrum of extracted xylan, signals at δ 4.3-3.0 ppm originate from (1-4)-β-D-xylopyranosyl units correspond to the H-2, H-5a, H-3, H-4, H-5e and H-1, respectively. However the signals appearing at δ 5.1 and 5.2 ppm originated from the protons of the –OH groups attached at $C_3$ and $C_2$ carbon of the D- xylopyranosyl units of xylan.

The $^{13}$C NMR spectrum of extracted xylan (Fig.S2, Supplementary data), shows the signals at δ 63.3, 72.7, 74.0, 75.5 and 101.8 ppm that are assigned to the C-5, C-2, C-3, C-4 and C-1 of the D-xylopyranosyl units in xylan. Thus, the $^1$H and $^{13}$C NMR spectrum of the extracted xylan revealed that it was composed mostly of xylose units and the hydroxyl groups of xylan were not substituted with acetyl groups or any other minors sugar residues such as arabinose or glucuronic acid. Acetyl groups present on the surface of xylan were removed during alkaline extraction and the others functional groups such as neutral sugars and lignin have been also removed during the extraction process (Grace, Fundador, Enomoto-rogers, Takemura, & Iwata, 2012). Thus, based on the above observations the isolated hemicellulose was a homoxylan.

X-ray diffraction (XRD) pattern of the isolated xylan (Fig. 4b) shows a broad diffraction peak at 2θ = 18.8$^°$ indicating the amorphous polymeric structure of xylan.

TGA and DTGA curves of the isolated xylan are shown in figure (Fig.S3, Supplementary data). The TGA thermogram of xylan shows only 2% weight loss below 200$^°$C due to the degradation of water. The weight loss between 220 $^°$C and 400 $^°$C was attributed to the degradation of xylan chains. In the DTGA curve, the typical degradation peaks of xylan were observed at 242$^°$C and 288$^°$C (Gao, Ren, Kong, & Chen, 2015).

3.2. Synthesis and characterization of Xyl-5-FUAC conjugates

Xyl-5-FUAC ester conjugates were synthesized by homogeneous reaction of the xylan with 5-fluoroural-1-acetic acid imidazolide, which is prepared in situ by conversion of 5-fluorouracil-1-acetic acid with N, N-carbonyldiimidazole (CDI) in the presence of TEA as a catalyst. CDI is a suitable carboxylic acid activating reagent which is widely used for the functionalisation of the biopolymers. In comparison to others COOH activating agents, CDI is more efficient and freely

soluble in variety of solvents and can be easily removed and avoids the side reactions ( Liebert & Heinze, 2005). The chemical reaction is performed in DMSO with different mole ratios of xylan: 5-FUAC at the same time and temperature.

The synthesis and chemical structure of the conjugates were confirmed by FT-IR and ^{1}H NMR Spectroscopy. The FT-IR spectra of 5-FUAC (Fig. 2a), shows characteristic peak at 3140 and 3060- 2830 cm^{-1} are attributed to the N-H, C-H stretching respectively. The Peaks at 1740, 1705 and 1660 cm^{-1} are related to C=O stretching in pyrimidine ring and C=O stretching of COOH group. The peaks between1495-1180 cm^{-1} is attributed to the C-N, C-C, C-F and C-O stretching mode respectively. The FT-IR spectrum of Xyl-5-FUAC conjugates (Fig. 2c), shows the characteristic peaks of both 5-FUAC and xylan, in addition presence a new peak at 1710 cm^{-1} confirms the formation of ester bond between 5-FUAC and xylan.
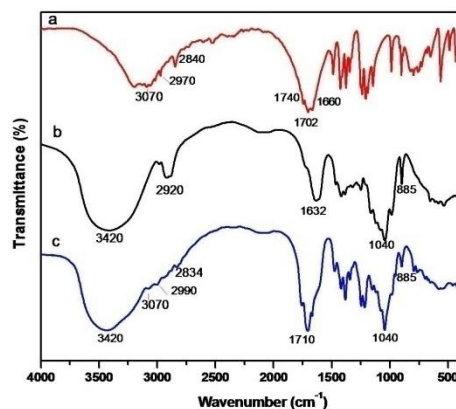


**Fig.2.** FT-IR spectra of  (a) 5-FUAC, (b) Xylan and (c) Xyl-5-FUAC conjugate (1:1).

The synthesis of the conjugates was further confirmed by ^{1}H NMR analysis. The ^{1}H NMR spectra of 5-FUAC (Fig S4, Supplementary information), clearly indicates the signals at $\delta$ 4.3 due to –CH$_2$ of the carboxymethyl group and the signals at $\delta$ 8.1 and 11.8 ppm due to the aromatic –CH and –NH proton of the 5-FU ring. Compare to the native xylan, the ^{1}H NMR spectrum of Xyl-5-FUAC conjugates (Fig. 3b), shows the three additional signals at $\delta$ 4.4 due to –CH$_2$ of the methylene group and the signals at $\delta$ 8.1 and 11.8 ppm due to the aromatic –CH and –NH proton of the 5-FU ring, confirm the successful coupling of 5-FUAC with xylan.
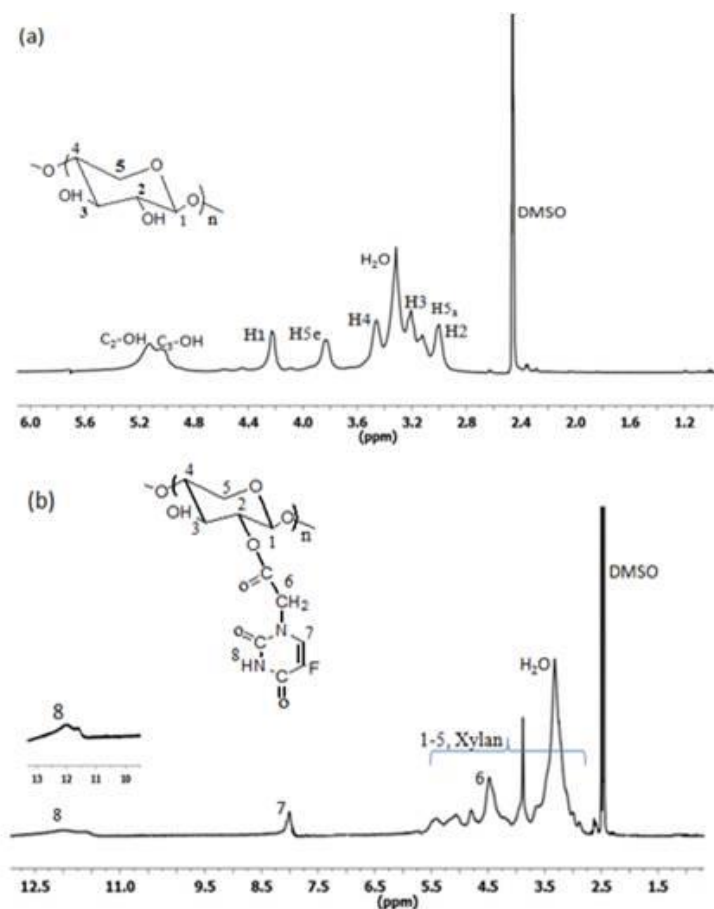
**Fig.3.** $^1$H NMR spectra of (a) Xylan and (b) Xyl-5-FUAC conjugate (1:1).

The presence of the 5-FUAC in conjugates was confirmed by UV-Vis spectroscopy. The UV absorption spectrum of the 5-FUAC, xylan and xylan-5-FUAC conjugate are shown in (Fig. 4a). The UV-Vis spectrum of xylan-5-FUAC conjugate presents a broad absorption band at 273nm, which indicates the presence of 5-FU in conjugate. The $\pi \rightarrow \pi^*$ energy level transition of 5-FUAC is responsible for the characteristic absorption peak of 5-FUAC at 273 nm, this peak also assign the stability of conjugates.

The Interaction between 5-FUAC and xylan in conjugate was studied using X-ray diffraction (XRD). The diffraction peaks at $2\theta = 14.5°$, $16.5°$, $22.5°$ and $34.7°$ (Fig. 4b), represents the crystalline nature of 5-FUAC while these characteristic peaks were not observed in the XRD patterns of Xyl-5-FUAC conjugate, which shows a new broader peak at $2\theta = 17°$. This data suggest that the intermolecular interaction between the polymer chain and drug offers the amorphous nature of drug in conjugate (Yallapu, Gupta, Jaggi, & Chauhan, 2010).
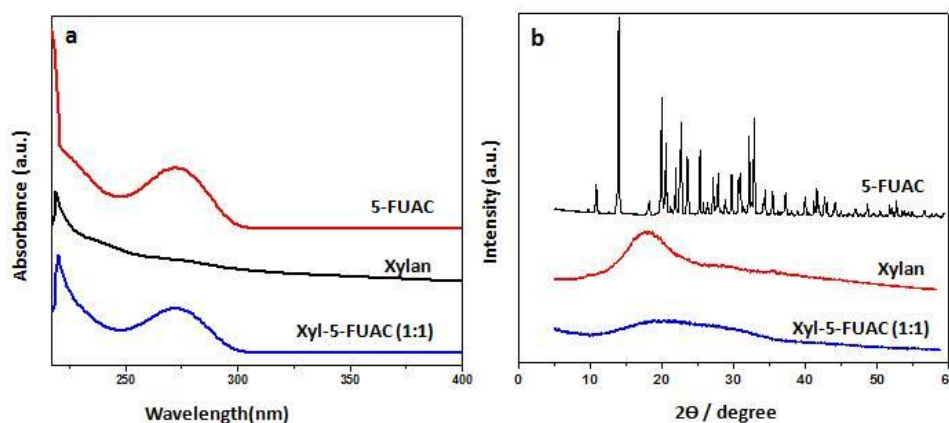
**Fig.4.** (a)UV-visible spectra of 5-FUAC, Xylan and Xyl-5-FUAC conjugate (1:1), (b) X-ray diffraction patterns of 5-FUAC, Xylan and Xyl-5FUAC conjugate (1:1).

### 3.3. The percentage of drug content in conjugates

Amount of 5-FUAC conjugated to xylan was quantified by UV-visible spectroscopy, after hydrolyzing the glycosidic bond in basic condition. As expected, the 5-FUAC content was increased with increasing the molar ratio of xylan and 5-FUAC. The 5-FUAC content in the conjugates was determined 57% and 73% when the molar ratios of xylan and 5-FUAC were, 1:1 and 1:2, respectively (Table S1, Supplementary data).

### 3.4. Chemical stability of the conjugates

Colon targeted prodrug should be stable at a wide pH range throughout the alimentary canal. Since the esteric linkage between 5-FUAC and xylan is susceptible to hydrolysis (Daus & Heinze, 2010), to explore the behaviour of the conjugates in acidic as well as basic environment of upper GI tract, the stability study were performed in buffers of pH 1.2 and 7.4 respectively. For acidic buffer of pH 1.2, stability was studied for 6h (Fig. 5a), but our concern is upto 2h because the transition time of the stomach is about 2h. Similarly, in case of pH 7.4, stability study was carried out for 12h (Fig. 5b), but our concern is upto 4h because the transition time for small intestine is about 4h. The release patterns of the conjugates show, only 3-5% release in case of acidic buffer (pH 1.2), while it was observed, 3-4% in case of PBS buffer (pH 7.4). However, the drug release was marginally higher in acidic buffer (pH 1.2) as compared to basic

buffer (pH 7.4) due to the fact that ester hydrolysis is catalyzed in acidic media. In-spite of that, less amount of drug release confirmed the stability of conjugates in the upper GI tract, during the transit through the GI tract, and this may be due to steric hindrance of the polymer.
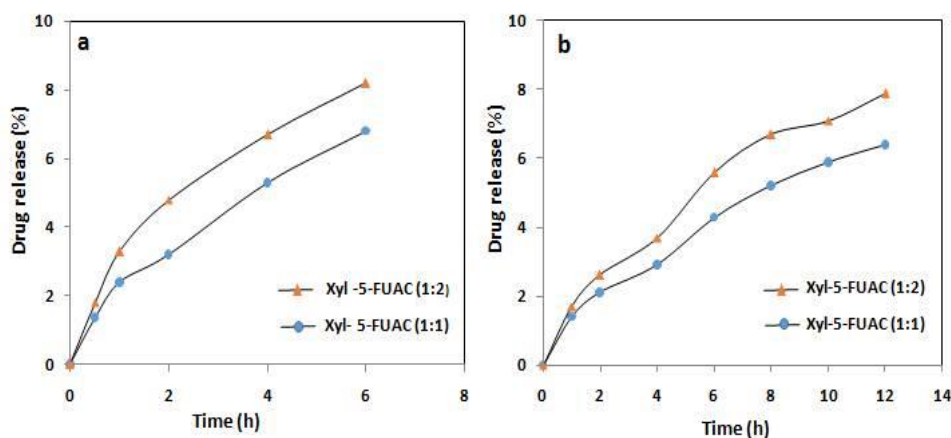


**Fig.5.** Stability studies of Xyl-5-FUAC conjugates at (a) acidic pH 1.2, and (b) pH 7.4 buffer. All values are expressed as mean ± SD (n=3).

3.5. In-vitro drug release study in gastrointestinal contents of rats

It is expected that the ester linkage between the polymer and drug could be hydrolyzed by the esterase enzyme of the upper gastrointestinal tract as well as polymer chain is degraded by the colonic microflora in the cecum and colonic region (Varshosaz et al., 2009). Thus the release of the drug from the conjugates was studied in the presence of rat's gastrointestinal content. The release study was performed in acidic buffers of pH 4.5, and phosphate buffer of pH 7.4 and 6.8 in the presence of gastrointestinal content (4wt %). In order to determine the effect of GI content on drug release profile, the release study was conducted for 6, 12 and 24h for the gastric, small intestine and colon respectively. The release patterns of the conjugates (Fig. 6a), shows 3-4% of the drug was released in the presence of gastric contents whereas 5-7% was released in presence of small intestine contents up to 2 and 4h respectively (Fig. 6b). These results suggest that the ester linkage of the conjugates is resistant to ester hydrolyzing enzymes; it may be due to steric hindrance of the polymeric chain of xylan. This showed that the conjugates might be stable against the proteolytic enzymes presented in the upper GI tract. (Fig. 6c), shows rapid drug release 53-61% within 24h in presence of cecum and colonic contents which indicates the significant role of the colonic microflora for hydrolysis of conjugates. The total amount of 5-

FUAC released in the presence of contents gastric, small intestine as well as cecum and colon was calculated (Fig. 6d). This observation indicates the different release behavior of the conjugats in presence of gastrointestinal contents and most of the drug was relesed in cecum and colonic contents.
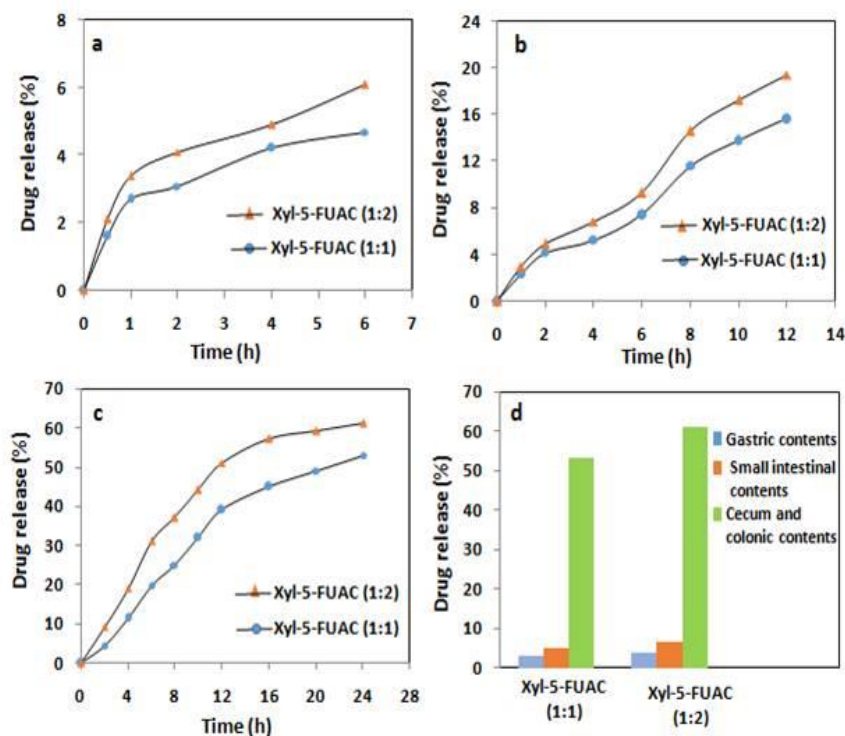


**Fig.6.** Release profiles of 5-FUAC from the conjugates in presence of (a) gastric contents at pH 4.5, (b) small intestine contents at pH 7.4, (c) cecum and colonic contents at pH 6.8, (d) total amount of 5-FUAC released in the presence of contents of each segment of the rat GI tarct. All values are expressed as mean ± SD (n=3).

3.6 In-vitro cytotoxicity

*In-vitro* cell cytotoxicity of the Xyl-5-FUAC conjugates and free drug (5-FU or 5-FUAC) was evaluated against HCT-15 (Fig. 7a), and HT-29 (Fig. 7b) at equivalent drug concentrations against HCT-15 and HT-29 for 12, 24 and 48h. Cell viability results clearly indicate that with increase in treatment time span and drug dosage levels there is subsequent increase in cytotoxicity of respective drug formulations. Apart from this, cytotoxicity study also confirmed that therapeutic efficacy of drug (5-FU or 5-FUAC) was drastically improved after conjugation

with xylan which is probably an outcome of drug transition from crystalline to amorphous state. For instance, $IC_{50}$ of 5-FU against HT-29 cells (i.e. 4.545 (µg/ml) declined drastically to a value of 1.818 (µg/ml) and 1.454 (µg/ml) in case of Xyl-5FUAC (1:1) and Xyl-5-FUAC (1:2) respectively. Similarly in case of HCT-15 also 5-FU, $IC_{50}$value declined from 2.54 µg/ml to 2.09 µg/ml and 1.727 µg/ml for Xyl-5-FUAC (1:1) and Xyl-5FUAC (1:2) respectively, (Table S2, Supplementary data). In summary, it can be stated that, Xyl-5-FUAC conjugates synthesised in this work exhibits better cytotoxicity against colon cancer cells (i.e. HCT-116 and HT-29) as compared to free drug as such. The profound increase in cytotoxicity was attributed to the enhanced aqueous solubility and lipophilicity of the drug upon conjugating it to xylan polymer ( Radwan & Alanazi, 2014; Sharma et al., 2014).
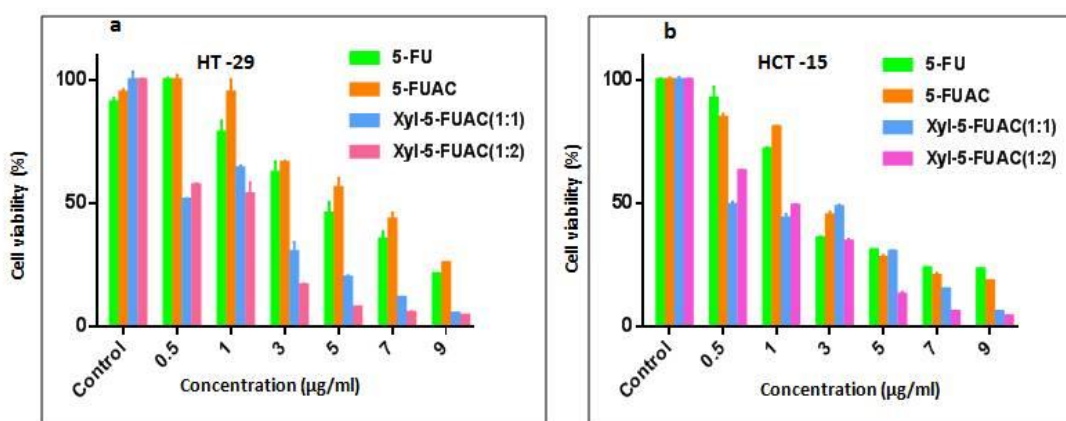


**Fig.7.** MTT viability assay of (a) HT-29 cells, and (b) HCT-15 cells, treated with 5-FU (0.5-9µg/ml), 5-FUAC (0.5-9µg/ml),  and Xyl-5-FUAC conjugates(0.5-9µg/ml), for 24 h. All values are expressed as mean ± SD (n=3).

In order to further ascertain the cell viability results, AO/EB staining was carried out to assess the cytotoxicity of drug and drug-polymer conjugates on a qualitative basis (S. U. Kumar & Gopinath, 2015; Raveendran, Bhuvaneshwar, & Sharma, 2016). Combination of two dyes AO and EB were used for the study, AO is a cell permeant dye which non-specifically labels the cell nucleus by intercalating with DNA and also stains cytoplasmic components to certain extent. On the other hand, although EB also intercalates with double stranded DNA, it can do so only in case of cells with compromised membranes (apoptotic cells). Thus, upon overlay of images of cells stained with AO and EB the cell population could be categorized into non-apoptotic, early apoptotic and late apoptotic. The non-apoptotic cells are labeled uniformly with AO alone, as

indicated in control, whereas early apoptotic cells appear bright green with spotted nucleus (Fig. 8). In contrast to this, late apoptotic cells with compromised membrane appear orange in color as their nucleus was stained by both AO and EB. The representative images (a-i) of (Fig. 8a and b) of treated HCT-15 and HT-29 cells clearly indicate that with increase in treatment time span there is consequent increase in apoptotic cells (orange to red cells). It is also evident from the images that Xyl-5-FUAC (1:2) could inflict higher apoptosis as compared to Xyl-5-FUAC (1:1) due to higher drug loading. Thus, in summary AO/EB staining results corroborated well with the cell viability results and illustrated the improved therapeutic efficacy of Xyl-5-FUAC conjugated as compared to 5-FU as such.
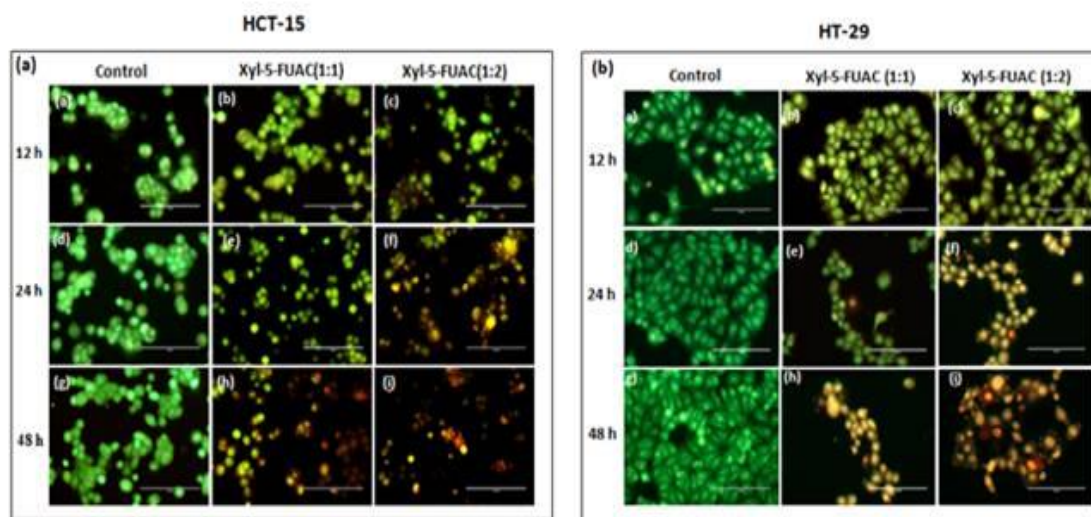


**Fig.8**. Fluorescence images (a-h) of live (green) and dead (red) cells (a) HT-29 cells, and (b) HCT-15 cells treated with  (5µg/ml) for 12, 24 and 48h.

## 4. Conclusion

In this study, Xyl-5-FUAC conjugates as colon specific prodrugs were synthesized using carbodiimide (CDI) coupling chemistry. The synthesized conjugates showed better stability in upper gastrointestinal tract. More importantly, synthesized conjugates released high amount of drug in colonic contents relative to the gastric or intestinal contents. *In-vitro* cytotoxicity study clearly indicates that these prodrugs conjugates are more active against human colorectal cancer cell lines (HCT-15, HT-29), as compared to the free drug. Based on these findings, the Xyl-5-FUAC conjugates as prodrugs are the potential candidates for colon specific drug delivery.

## References

Bosmans, T. J., Stépán, A. M., Toriz, G., Renneckar, S., Karabulut, E., Wågberg, L., & Gatenholm, P. (2014). Assembly of debranched xylan from solution and on nanocellulosic surfaces. *Biomacromolecules*, *15*(3), 924–930.

Chourasia, M. K., & Jain, S. K. (2003). Pharmaceutical approaches to colon targeted drug delivery systems. *Journal of Pharmacy and Pharmaceutical Sciences*, *6*(1), 33–66.

Daumar, P., Decombat, C., Chezal, J.-M., Debiton, E., Madesclaire, M., Coudert, P., & Galmier, M.-J. (2011). Design, synthesis and in vitro drug release investigation of new potential 5-FU prodrugs. *European Journal of Medicinal Chemistry*, *46*(7), 2867–2879.

Daus, S., & Heinze, T. (2010). Xylan-based nanoparticles: Prodrugs for ibuprofen release. *Macromolecular Bioscience*, *10*(2), 211–220.

Deutschmann, R., & Dekker, R. F. H. (2012). From plant biomass to bio-based chemicals: Latest developments in xylan research. *Biotechnology Advances*, *30*(6), 1627–1640.

Ebringerová, A., & Heinze, T. (2000). Xylan and xylan derivatives - Biopolymers with valuable properties, 1: Naturally occurring xylans structures, isolation procedures and properties. *Macromolecular Rapid Communications*, *21*(9), 542–556.

Fonseca Silva, T. C., Habibi, Y., Colodette, J. L., & Lucia, L. a. (2011). The influence of the chemical and structural features of xylan on the physical properties of its derived hydrogels. *Soft Matter*, *7*(3), 1090.

Fundador, N. G. V, Enomoto-Rogers, Y., Takemura, A., & Iwata, T. (2012). Acetylation and characterization of xylan from hardwood kraft pulp. *Carbohydrate Polymers*, *87*(1), 170–176.

Gao, C., Ren, J., Kong, W., & Chen, Q. (2015). RSC Advances xylan-based hydrogels : their properties and drug. *RSC Advances*, *5*, 90671–90681.

Grace, N., Fundador, V., Enomoto-rogers, Y., Takemura, A., & Iwata, T. (2012). Acetylation and characterization of xylan from hardwood kraft pulp. *Carbohydrate Polymers*, *87*(1), 170–176.

Hua, S., Marks, E., Schneider, J. J., & Keely, S. (2015). Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease : Selective targeting to diseased versus healthy tissue. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *11*(5), 1117–1132.

Huang, P., Wang, D., Su, Y., Huang, W., Zhou, Y., Cui, D., Yan, D. (2014). Combination of small molecule prodrug and nanodrug delivery: Amphiphilic drug-drug conjugate for cancer

therapy. *Journal of the American Chemical Society*, *136*(33), 11748–11756.

Kumar, S., & Negi, Y. S. (2012). Corn cob xylan-based nanoparticles: Ester prodrug of 5-aminosalicylic acid for possible targeted delivery of drug. *Journal of Pharmaceutical Sciences and Research*, *4*(12), 1995–2003.

Kumar, S. U., & Gopinath, P. (2015). Controlled delivery of bPEI-niclosamide complexes by PEO nanofibers and evaluation of its anti-neoplastic potentials. *Colloids and Surfaces B: Biointerfaces*, *131*, 170–181.

Li, H.-P., Wang, Z., & Yu, T. (2011). Microwave irradiation synthesis of novel ramose chitosan-based-5-fluorouracil and research on its in vitro release. *Medicinal Chemistry Research*, *20*(1), 67–73.

Liebert, T. F., & Heinze, T. (2005). Tailored cellulose esters: Synthesis and structure determination. *Biomacromolecules*, *6*(1), 333–340.

Peng, F., Peng, P., Xu, F., & Sun, R. C. (2012). Fractional purification and bioconversion of hemicelluloses. *Biotechnology Advances*, *30*(4), 879–903.

Petzold-Welcke, K., Schwikal, K., Daus, S., & Heinze, T. (2014). Xylan derivatives and their application potential - Mini-review of own results. *Carbohydrate Polymers*, *100*, 80–88.

Philip, A. K., & Philip, B. (2010). Colon targeted drug delivery systems: a review on primary and novel approaches. *Oman Med J*, *25*(2), 79–87.

Plyduang, T., Lomlim, L., Yuenyongsawad, S., & Wiwattanapatapee, R. (2014). Carboxymethylcellulose-tetrahydrocurcumin conjugates for colon-specific delivery of a novel anti-cancer agent, 4-amino tetrahydrocurcumin. *European Journal of Pharmaceutics and Biopharmaceutics*, *88*(2), 351–360.

Radwan, A., & Alanazi, F. (2014). Design and Synthesis of New Cholesterol-Conjugated 5-Fluorouracil: A Novel Potential Delivery System for Cancer Treatment. *Molecules*, *19*(9), 13177–13187.

Raveendran, R., Bhuvaneshwar, G. S., & Sharma, C. P. (2016). Hemocompatible curcumin-dextran micelles as pH sensitive pro-drugs for enhanced therapeutic efficacy in cancer cells. *Carbohydrate Polymers*, *137*, 497–507.

Sharma, P. R., & Varma, A. J. (2014). Functionalized celluloses and their nanoparticles: Morphology, thermal properties, and solubility studies. *Carbohydrate Polymers*, *104*(1), 135–142.

Sharma, R., Rawal, R. K., Gaba, T., Singla, N., Malhotra, M., Matharoo, S., & Bhardwaj, T. R. (2013). Design, synthesis and ex vivo evaluation of colon-specific azo based prodrugs of anticancer agents. *Bioorganic and Medicinal Chemistry Letters*, *23*(19), 5332–5338.

Sharma, R., Rawal, R. K., Malhotra, M., Sharma, A. K., & Bhardwaj, T. R. (2014). Design, synthesis and ex-vivo release studies of colon-specific polyphosphazene-anticancer drug conjugates. *Bioorganic and Medicinal Chemistry*, *22*(3), 1104–1114.

Sun, Z.-J., Sun, B., Sun, C.-W., Wang, L.-B., Xie, X., Ma, W.-C., Dong, D.-L. (2012). A poly(glycerol-sebacate-(5-fluorouracil-1-acetic acid)) polymer with potential use for cancer therapy. *Journal of Bioactive and Compatible Polymers*, *27*(1), 18–30.

Varshosaz, J., Emami, J., Tavakoli, N., Fassihi, A., Minaiyan, M., Ahmadi, F., & Dorkoosh, F. (2009). Synthesis and evaluation of dextran-budesonide conjugates as colon specific prodrugs for treatment of ulcerative colitis. *International Journal of Pharmaceutics*, *365*(1-2), 69–76.

Wang, Q. W., Liu, X. Y., Liu, L., Feng, J., Li, Y. H., Guo, Z. J., & Mei, Q. B. (2007). Synthesis and evaluation of the 5-fluorouracil-pectin conjugate targeted at the colon. *Medicinal Chemistry Research*, *16*(7-9), 370–379.

Yallapu, M. M., Gupta, B. K., Jaggi, M., & Chauhan, S. C. (2010). Fabrication of curcumin encapsulated PLGA nanoparticles for improved therapeutic effects in metastatic cancer cells. *Journal of Colloid and Interface Science*, *351*(1), 19–29.

Yang, H., Bremner, D. H., Tao, L., Li, H., Hu, J., & Zhu, L. (2016). Carboxymethyl chitosan-mediated synthesis of hyaluronic acid-targeted graphene oxide for cancer drug delivery. *Carbohydrate Polymers*, *135*, 72–78.