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SYNTHESIS AND *IN-VITRO* EVALUATION OF PEPTIDE LINKED PRODRUGS OF SELECTED CEPHALOSPORIN

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Keywords:

Trisaccharide drug carrier, Oral bioavailability, Typhoid, Ceftriaxone, Peptide linker, Enzyme specific prodrugs

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ABSTRACT: Cephalosporins are drug of choice for many infectious microorganisms even against many antibiotic resistant cases. Ceftriaxone, a third generation cephalosporin is effective in many infections including fluoro-quinolone resistant strains of *Salmonella typhi*, the causative pathogen for enteric fever (typhoid). The antibiotic is supposed to act in the intestinal cells but gastric instability limits the oral use of ceftriaxone. Present research aims to develop an orally bioavailable formulation of ceftriaxone which is otherwise available as parenteral owing to its gastric instability. Natural polysaccharides have been used as excipients and drug delivery carriers since long but are not good for linking to form prodrugs. Hydrolysis resistant fraction of Almond gum, whose structure has been elucidated as a trisaccharide is used to synthesize the prodrugs of ceftriaxone. Two peptide linkers *i.e*., glycine and phenylalanine have been used to link the trisaccharide with the cephalosporin in three steps. Both the prodrugs were characterized and evaluated *in-vitro*. IR and NMR data confirmed the synthesis of polymeric prodrugs and *in-vitro* evaluation shows sustained drug delivery without degradation of the drug. Results of assessment in simulated gastric fluid and intestinal fluid suggests that the drug was released by action of GI enzymes on the peptide linker. This research work resulted into development of enzyme specific prodrugs of ceftriaxone using natural polysaccharide as well as the concept can be further used to design new class of antibiotics.

INTRODUCTION: Use of cephalosporins in treatment of Salmonella infections is very common due to stability towards beta-lactamases and affinity for penicillin binding proteins. *Salmonella species* have been associated with a wide range of infections and characterized by resistance to a wide range of antibiotics including floroquinolones [1], macrolides, aminoglycosides, penicillins *etc*. [2]

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Present research work entails synthesis and *in-vitro* evaluation of synthesized polymeric prodrugs of selected third generation cephalosporin *i.e*., ceftriaxone. It has been proven that the drug is labile to acidic degradation and hence parenteral formulation is only available [3]. The dose is too large and leads to high amount of toxicity [4].

The research work is focused to develop orally bioavailable formulation of the drug. We used gum obtained from *Prunus Amygdalus-dulcis* (Sweet almond), a natural polysaccharide with established kinetic properties [5] and conjugated chemically with the drug *via* small peptide linkers. The pathogenesis of infection is characterized by prevalence of the causative organism, *Salmonella*

typhi in the intestinal mucosa [6]. Although *Salmonella typhi* colonizes in the liver, spleen and bone marrow in addition to the intestine and the mesenteric lymph nodes being a systemic infection [7]. The prepared polymeric prodrugs may deliver ceftriaxone in the intestine without getting affected by the acid, while the gastro-intestinal peptidases will break the peptide linked polysaccharide apart. Purified and lyophilized, hydrolysis resistant fraction of almond gum is used for chemical conjugation [8]. The hydrolysis resistant fraction is assumed to be a trisaccharide (AG) having molecular weight in range to the drug. We have used purified gum in our previous work but faced many challenges during synthesis of polymeric prodrugs [9]. Hydrolysis resistant fraction has better solubility and as mentioned the molecular weight is approximately 492 [8]. The resulting prodrugs resemble a glyco-peptide structure and may be compared with glycopeptide antibiotics.

The antimicrobial assay (published elsewhere after all activities) may show difference in the potency of the synthesized prodrugs as these are structurally similar to glycopeptide antibiotics also. Almond gum possesses high commercial benefits because it is easily available, economical and also the processing requirement of this polysaccharide requires little investment which eventually makes the formulation profitable [10]. Moreover, this polysaccharide also has a high rate of productivity and reproducibility making the process pertinent for industrialization [11, 12].

Accordingly, the present invention provides a drug for the oral sustained delivery of a natural extract based prodrug wherein the natural extract is an extract of *Prunus amygdalus dulcis* and wherein this prodrug is able to release about 75% of the drug in 24 h. The model drug used here is ceftriaxone which is a third generation cephalosporin antibiotic and whose anti typhoid action is well proven [13].

MATERIALS AND METHODS:

Preparation and Characterization of Polysaccharide:

Extraction and Purification of Almond Gum: The gum was purified by precipitating it from aqueous dispersion using ethanol following a method described earlier [8]. Crude gum was powdered and passed through 80 mesh sieve, dispersed in water and kept aside for 24 h. The swollen gum was precipitated using equal volume of ethanol. Precipitated gum was filtered using vacuum filtration on a Buchner funnel [8].

Hydrolysis of Gum: [8] The purified gum was subjected to acid hydrolysis, carried out in sealed ampoules at 100 ºC by using 2M HCl for 10 h. The hydrolyzed product was filtered and filtrate was purified by using activated charcoal. Lyophilized white powder was subjected to chromatographic and spectral analysis. Thin layer chromatography (TLC) of the hydrolyzed gum was done using silica gel G plates in n-butanol-pyridine-water (4:4:3) as mobile phase and Anisaldehyde - sulphuric as spraying reagent. The FTIR spectra of purified gum and hydrolyzed gum were recorded by KBr pellet technique. The 1H NMR/Mass spectra of purified and hydrolyzed gum were recorded using NMR spectrometer (Bruker, Germany) and Mass spectrometer (JMS - T100LC, Accu TOF) respectively.

Synthesis and Characterization of Prodrug:

Preparation of Chloro Derivative (AG-Cln): [9, 14, 15] Obtained hydrolysis product (2 g) was slowly poured with stirring into a beaker containing Chloral hydrate (20 g) pre-dissolved in 200 mL dimethylformamide (DMF) and kept for one week with occasional shaking. 20 mL methane sulphonyl chloride was added drop wise for 1.5 h with constant stirring at 75 °C. The temperature was maintained at 75 °C for another 1.5 h with stirring. The mixture was then brought to room temperature and poured into 250 mL chilled water with stirring. Finally neutralized using 2N Na_2CO_3 solution and kept overnight. Then the solvent was evaporated under reduced pressure and the residue was washed several times with ethanol.

Covalent binding of peptides to AG-Cln: [9, 15, 16] 20 mmol peptides were added in small portions for 1 h to solution of CP (4 g) and pyridine (0.4 mL) in DMF (50 mL), under stirring at room temperature. Reaction mixture was refluxed for 8 h with continuous stirring at 120 °C. Obtained residue after filtration was washed several times with ethanol and dried in a hot air oven. Both the compounds were characterized by spectral analysis.

Synthesis of Drug Conjugates: [16] A solution containing 3 mmol N, N'-dicyclohexylcarbodiimide and 1.2 mmol 4-dimethylaminopyridine in 15 mL of DMF was added drop wise to a solution of 2.5 g glycine / 3.0 g phenylalanine intermediates dissolved in 20 mL DMF. 3 mmol ceftriaxone was added in small portions to this mixture, maintained at 0 °C for 10 min with constant stirring. The coupling reaction was carried out with constant stirring for 96 h at room temperature. The obtained residue was filtered, washed several times with ethanol and dried in a hot air oven. The drug conjugates were characterized by spectral analysis.

Estimation of Drug Content: [17] The amount of drug in conjugates was determined by UV spectrophotometer. Drug conjugates were dispersed in 0.1N NaOH solution, sonicated for 10 min and kept for 24 h at room temperature. Filtered solutions were diluted using pH 7.4 phosphate buffer and absorbance taken at 241 nm.

***In-vitro* Drug Release Studies:** [16]

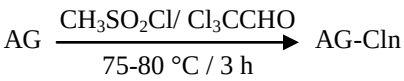
Studies in Different Buffers: The *in-vitro* drug release from the prodrugs was evaluated using USP XXIII dissolution apparatus. The absorbance was taken at the λ_{max} of drug *i.e.* 265 and 241 nm. Drug release pattern was recorded.

Enzymatic Hydrolysis: Studies were conducted in Simulated Gastric Fluid (SGF) of pH 1.2 containing pepsin and Simulated Intestinal Fluid (SIF) of pH 7.4 containing trypsin-chymotrypsin enzymes.

RESULTS AND DISCUSSION:

Synthesis of Prodrugs:

Synthesis of AG-Cln: Product was obtained in good yield (MP 250 °C). IR spectra of AG and AG-Cln are given in **Fig. 1** and **2**, respectively. 1H NMR and 13C NMR for AG-Cln is given in **Fig. 3** and **4** respectively. AG exhibits characteristic bands at 3500 cm^{-1} (OH) and 1250 cm^{-1} (C-O stretching). AG-Cln exhibits bands at 550-800 cm^{-1} and nil absorption for OH at 3300 - 3500 cm^{-1}, indicating the replacement of –OH by –Cl. ^{1}H NMR (D_2O) signals at δ 2.826 and no signal at δ 2.0 indicates replacement of hydroxyl by chloride. ^{13}C NMR (D_2O) signals at δ 38.72 indicates the presence of C-Cl bond.



SCHEME 1: SYNTHESIS OF AG-Cln

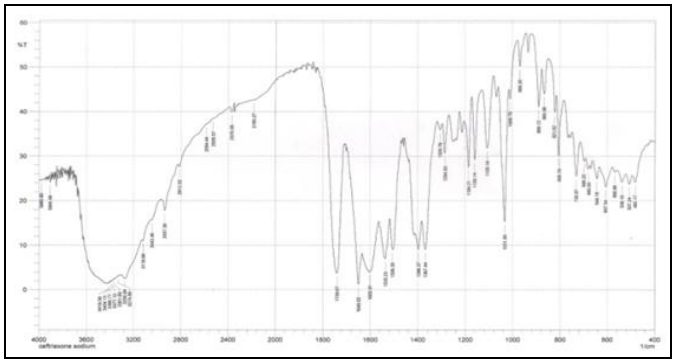


FIG. 1: IR SPECTRUM OF AG

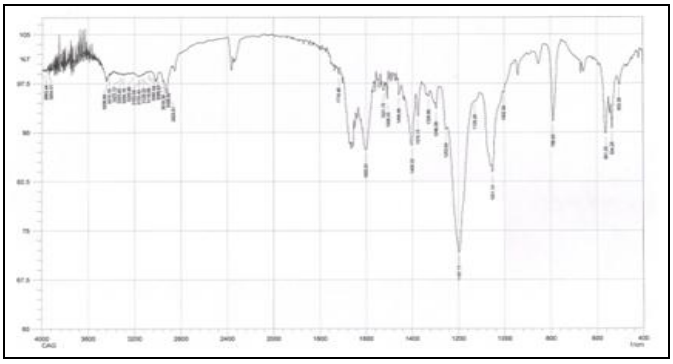


FIG. 2: IR SPECTRUM OF AG-Cln

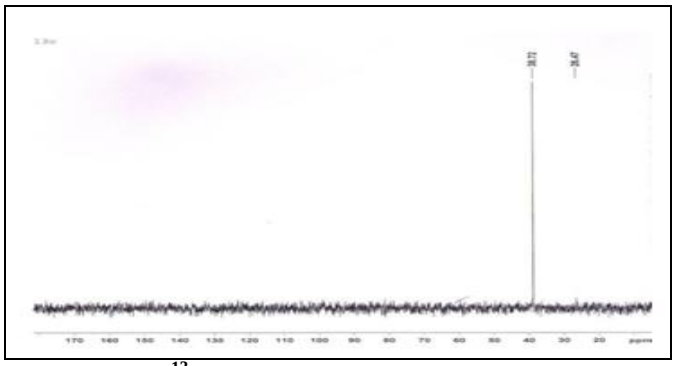


FIG. 3: ^{13}C NMR SPECTRUM FOR AG-Cln

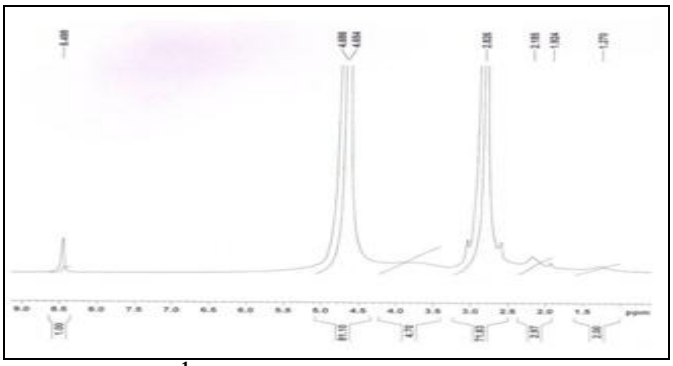
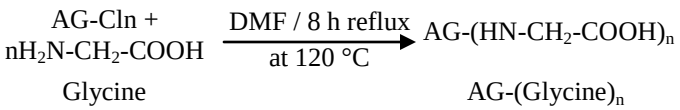


FIG. 4: ^{1}H NMR SPECTRUM OF AG-Cln

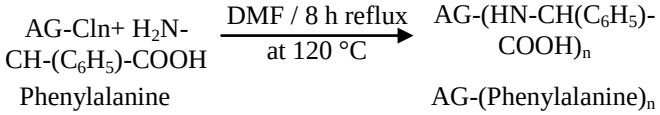
Covalent Binding of Glycine to AG-Cln: The coupling of AG-Cln with glycine is given in **Scheme 2**.



SCHEME 2: SYNTHESIS OF AG-(HN-CH_2-COOH)$_n$

AG-(Glycine)$_n$ was obtained in good yield. The IR and ^{1}H NMR are given in **Fig. 5** and **6** respectively. IR (KBr) spectrum shows single peak between 3200 - 3500 cm^{-1} (-NH stretch), 1660 cm^{-1} (C=O stretch), 1020-1220 cm^{-1} and 1402 cm^{-1} (C-N vibrations). ^{1}H NMR (D_2O) signals at δ 2.70 (-CH_2COOH).

Covalent Binding of Phenylalanine to AG-Cln: AG-(Phenylalanine)$_n$ was obtained in good yield (MP 235 °C). The IR and 1H NMR are given in **Fig. 7** and **8** respectively. IR (KBr) spectrum shows single peak between 3200-3500 cm^{-1} (-NH stretch), 1660 cm^{-1} (C=O stretch), 1020 - 1220 cm^{-1} and 1402 cm^{-1} (C-N vibrations). ^{1}H NMR (D_2O) exhibit AG signals at δ 3.9 for the methine (-CH-NH_2), multiplet at δ 7.3 for aromatic and signals δ 2.722 and 2.779 for benzyl protons of phenylalanine.



SCHEME 3: SYNTHESIS OFAG-(HN-CH(C_6H_5)-COOH)$_n$

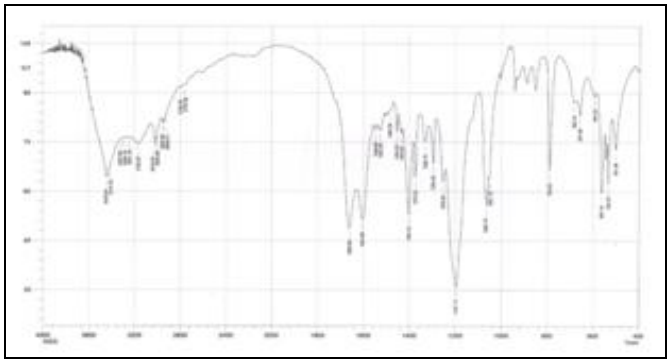


FIG. 5: IR SPECTRA OF AG-(GLY)$_n$

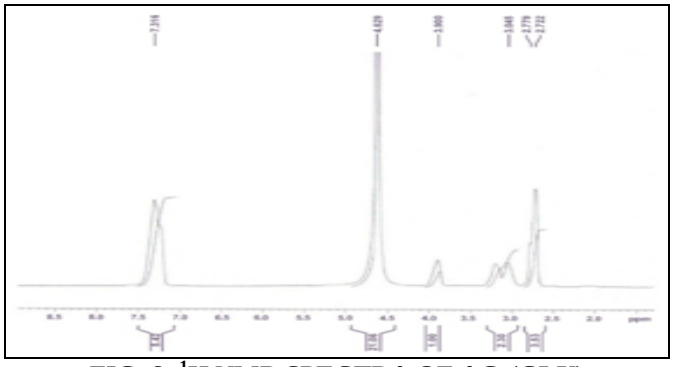


FIG. 6: ^{1}H NMR SPECTRA OF AG-(GLY)$_n$

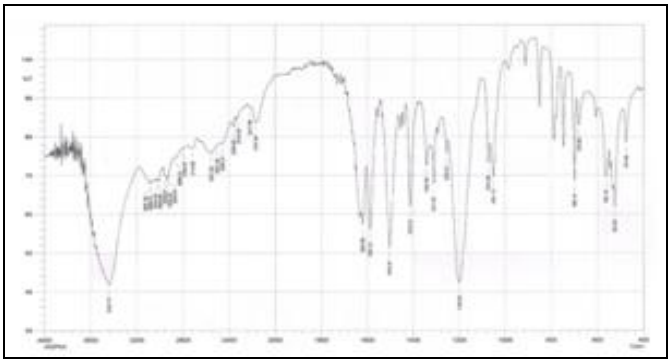


FIG. 7: IR SPECTRA OF AG-(PHE)$_n$

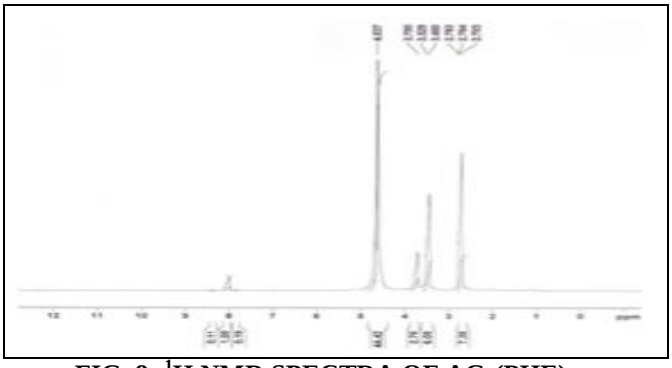


FIG. 8: ^{1}H NMR SPECTRA OF AG-(PHE)$_n$

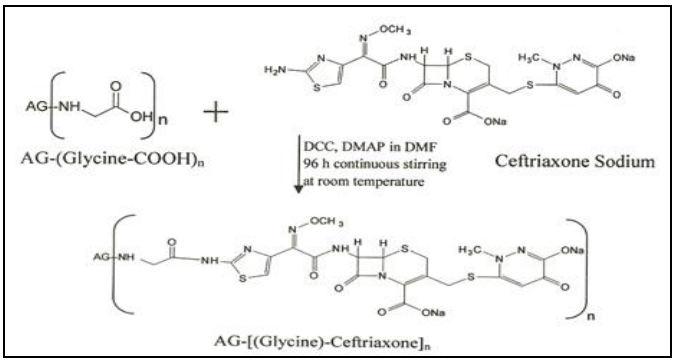
Synthesis of AG-[(Glycine)-Ceftriaxone]$_n$: AG-[(Glycine)-Ceftriaxone]$_n$ was obtained in good yield (MP 44 °C). The IR and ^{1}H NMR spectra are given in **Fig. 9** and **10**.

IR (KBr) spectrum exhibits broad band at 3200 cm^{-1} (-CONH, OH), 1737 cm^{-1} (-CONH), 1600 cm^{-1} (C=N) and 1050 - 1150 cm^{-1} (-C-O-C-). ^{1}H NMR exhibit signals at δ 5.0 and 5.6 for propiolactam protons, δ 3.64 and 3.49 for terahydrofuran, δ 3.57, 3.2 and 3.0 for methylene protons and δ 2.43 for methyl protons **Scheme 4**.

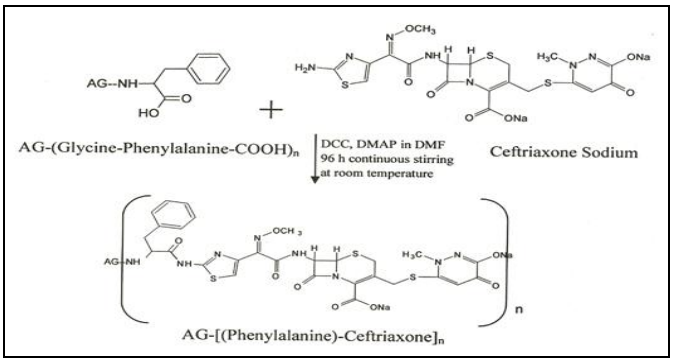
Synthesis of AG-[(Phenylalanine)-Ceftriaxone]$_n$: AG-[(Phenylalanine)-Ceftriaxone]$_n$ was obtained in good yield (MP 82 °C). The IR and ^{1}H NMR spectra are given in **Fig. 11** and **12**.

IR (KBr) spectrum exhibit AG broad band at 3200 - 3000 cm^{-1} (-CONH), 1750 - 1620 cm^{-1} (C=O), 1587 cm^{-1} (C=N) and 1153 cm^{-1} (-C-O-C-). ^{1}H NMR exhibit signals at δ 5.0 and 5.6 for propiolactam protons, δ 3.64 and 3.49 for terahydrofuran, δ 3.57, 3.2 and 3.0 for methylene protons and δ 2.43 for methyl protons.

Other than these signals to glycine prodrug it also showed multiple AG at δ 7.2-7.3 indicating benzyl protons of phenylalanine. Spectral data confirmed the synthesis of prodrugs hence validates the chemical method developed for the synthesis



SCHEME 4: SYNTHESIS OF AG-[(GLYCINE)-CEFTRIAXONE]$_n$



SCHEME 5: SYNTHESIS OF AG-[(PHENYLALANINE)-CEFTRIAXONE]$_n$

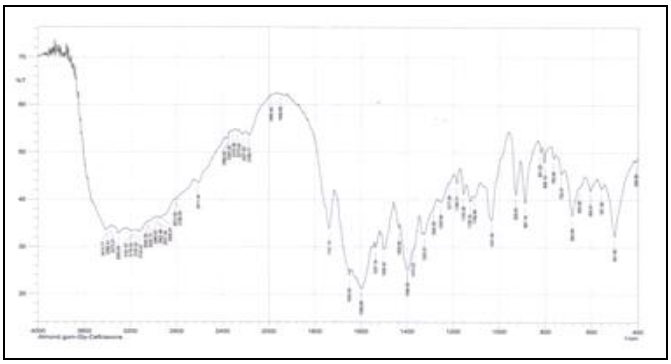


FIG. 9: IR SPECTRA OF AG-[(GLY)-CEFTRIAXONE]$_n$

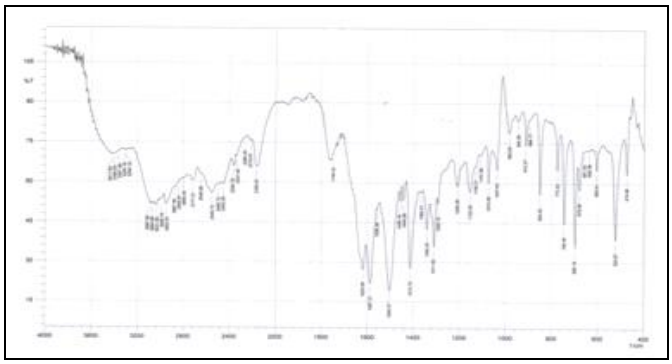


FIG. 10: IR SPECTRA OF AG-[(PHE)-CEFTRIAXONE]$_n$

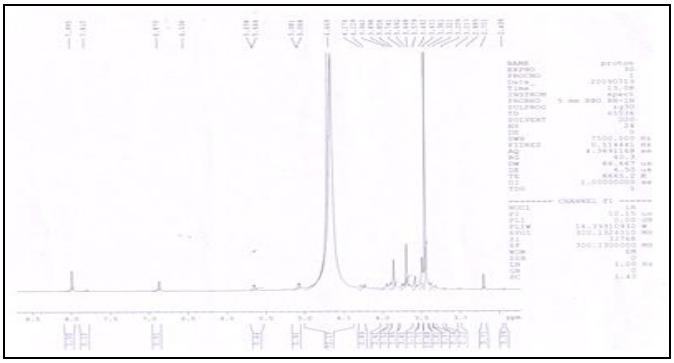


FIG. 11: 1H NMR SPECTRA OF AG-[(GLY)-CEFTRIAXONE]$_n$

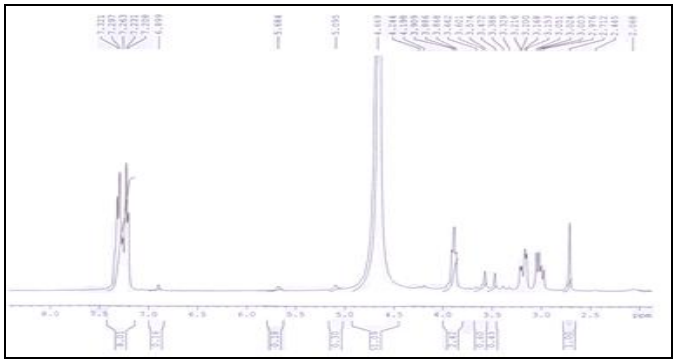


FIG. 12: 1H NMR SPECTRA OF AG-[(PHE)--CEFTRIAXONE]$_n$

Estimation of Drug Content: The amount of drug present in each of the prodrugs was estimated spectrophotometrically and is given in **Table 1**. The drug content of glycine linked prodrug is more, based on the obvious fact that the linker having smaller molecular weight also links similar amount of antibiotic per molecule. Additionally the attachment of ceftriaxone with the intermediate, containing more bulky aromatic amino acid may be less than glycine.

***In-vitro* Drug Release Studies:** [13, 14] Selected amino acids can be cleared by GI enzymes, releasing the drug molecule without getting degraded into inactive metabolites. In general the most favorable condition for action on peptide bonds is present in the intestine. Result AG of *in vitro* studies in pH 1.2, 7.4, SGF and SIF are shown in **Table 2** and **Fig. 13**.

TABLE 1: ESTIMATED DRUG CONTENT OF ALMOND GUM PRODRUGS

S. no.	Almond gum prodrugs	Drug content / 100mg of prodrug
1	AG-[(Glycine)-Ceftriaxone]$_n$	45.033 mg
2	AG-[(Phenylalanine) Ceftriaxone]$_n$	25.713 mg

TABLE 2: CUMULATIVE DRUG RELEASE (%) FROM AG-(GLY-CEFTRIAXONE)$_n$ AT DIFFERENT PHYSIOLOGICAL CONDITIONS

Time (min)	pH 1.2	SGF	pH 7.4	SIF
0	0	0	0	0
15	12.96	13.55	28.67	30.3
30	25.03	26.06	30.47	31.45
45	32.32	34.01	38.17	40.62
60	42.49	41.7	45.54	48.81
90	49.27	50.57	49.8	51.93
120	60.09	61.12	58.81	61.75
180	66.34	67.77	65.69	67.16
240	69.21	68.42	71.92	70.93
360	71.56	71.81	76.17	73.71
480	73.51	72.99	77.65	74.53
720	74.61	73.51	78.47	75.52
1440	78.2	78.46	80.93	80.27

TABLE 3: CUMULATIVE DRUG RELEASE (%) FROM AG-(PHE-CEFTRIAXONE)$_n$ AT DIFFERENT PHYSIOLOGICAL CONDITIONS

Time (min)	pH 1.2	SGF	pH 7.4	SIF
0	0	0	0	0
15	10.196	1.44	52.5	55.37
30	15.218	13.65	57.37	57.09
45	21	18.18	58.24	60.53
60	25.87	20.73	59.24	62.11
90	31.8	25.56	61.25	63.26
120	38.65	28.83	62.83	64.26
180	48.85	34.35	63.4	66.27
240	58.85	38.61	64.55	68.28
360	61.17	45.69	66.56	69.42
480	64.06	57.18	67.99	71.29
720	68.17	62.35	69.86	73.15
1440	68.78	62.72	70.29	75.88

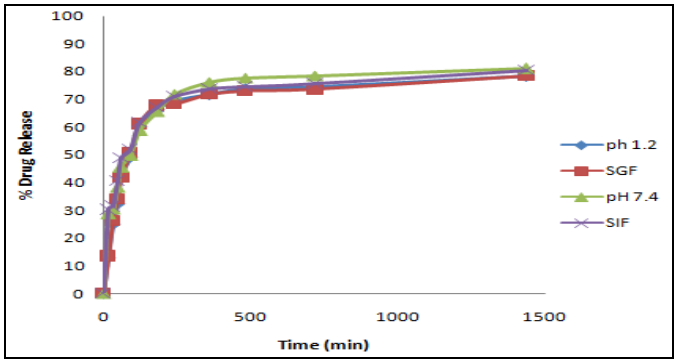


FIG. 13: *IN-VITRO* DRUG RELEASE FROM AG-(GLY-CEFTRIAXONE)$_n$

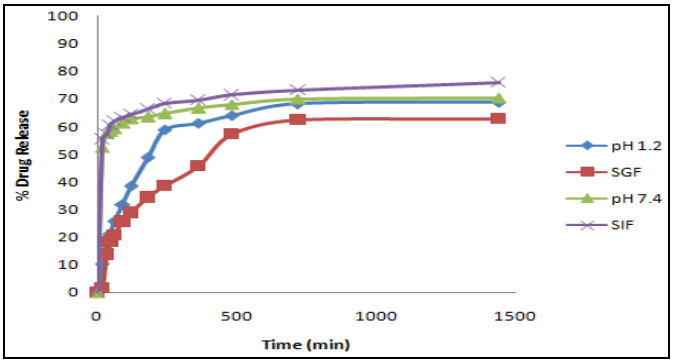


FIG. 14: *IN-VITRO* DRUG RELEASE FROM AG-(PHE-CEFTRIAXONE)$_n$

This is evident that phenylalanine containing prodrug (PP) can release only about 15% antibiotic in SGF and more than 55% in SIF within 30 min while the glycine derivative (GP) can release about 25% in SGF and 30% in SIF. Another set for major comparison is at 2 h interval where PP can release about 65% drug in SIF and less than 30% in SGF while GP can release about 60% drug in all physiological conditions. Considering the mean residence time (MRT) in stomach being 2 h, release profile discussed above confirms enzyme specificity of trypsin - chymotrypsin (Intestinal Enzymes) for PP.

Additionally the release profile beyond 2 h is also interesting for PP as it can release about 70% drug in 6 - 8 h while GP can release more than 75% the same. It can be concluded by above data that the action of GP is sustained in SIF but affected by pepsin. While PP is not effected by pepsin / acid and can release more than 65% within 2 h in SIF.

CONCLUSION: The research work states that not only the gum obtained from *Prunus amygdalus* can be used as a drug carrier while its hydrolysis product (trisaccharide) can also be used, even better, owing to its kinetic properties. Developed method was found suitable for preparing prodrugs of sustainable release profile (more than 75% in 24 h). Present data also supports development of formulation of ceftriaxone which can withstand the effect of gastric environment and release the drug at desired site (Intestine). The drug itself is able to penetrate well but owing to its acid instability, used only as injection in a high dose while delivery to intestine (particularly in typhoid) may help in reducing the dose substantially. The phenylalanine containing prodrug is even selective for intestinal enzymes and is able to release major amount of drug in the intestine.

Additionally, the developed prodrugs have sugar (trisaccharide), β-lactam skeleton with multiple peptide bonds and degradable peptide linker which itself comparable to glycopeptide antibiotics. Antimicrobial assay to evaluate its potential are further proposed. It can be finally concluded that the hydrolysis resistant fraction which is reported to be a trisaccharide can be exploited for the synthesis of orally active prodrugs.

This research work resulted into development of enzyme specific prodrugs of ceftriaxone using natural polysaccharide as well as the concept can be further used to design new class of antibiotics.

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CONFLICTS OF INTEREST: There is no conflict of interest.

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