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Original Article

Synthesis, Characterization and Release Studies of Ethylene Diamine Tetraacetic Acid (EDTA)-Antimicrobial Drug Conjugates for Colon Release

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ABSTRACT

Background: The colon targeted drug delivery system is mainly useful for the topical treatment of colon diseases such as ulcerative colitis, Crohn's disease OR colorectal cancers where we attain high local concentration and minimum side effects. In the present study we have synthesized some colon targeted Ethylene diamine tetra acetic acid (EDTA)-antimicrobial drug conjugates. **Objectives:** The goal of synthesizing EDTA-antimicrobial drug conjugates are that they reach intact in the colon and not be absorbed in the upper GIT, this is because of high molecular weight (>500) of the conjugates (Lipinski's rule of 5). **Materials and Methods:** A series of EDTA-antimicrobial drug conjugates (E1-E5) were synthesized by stirring tetra sodium EDTA with antimicrobial drugs viz. Metronidazole (MTZ), Ornidazole (OZ), Ciprofloxacin (CF), Norfloxacin (NF) and Sulfamethoxazole (SM) in presence of EDAC ((1-ethyl-3(3-dimethylaminopropyl) carbodiimide). All the synthesized conjugates were characterized by FTIR, NMR (^{1}H and ^{13}C), Mass spectrometry and elemental analysis to identify structural components. The synthesized conjugates were screened for antimicrobial (antibacterial) and colon release studies. **Results and Discussion:** On the basis of chemical and spectral analysis EDTA-antimicrobial drug conjugates were synthesized and synthesized conjugates showed good antimicrobial (antibacterial) activity against tested stains. *In-vitro* release studies of the synthesized conjugates of the series have shown good release results which indicates that synthesized conjugates release the parent drug (antimicrobial drugs) to the colon. **Conclusion:** Synthesized conjugates improve drug delivery to the intestinal region. Release studies result suggest that the drugs begin to release from the conjugates in the distal intestinal region and appreciable release in the colon has been observed.

Keywords: Anti-microbial drugs, Metronidazole, IBDs, Colitis, Colon targeted drug delivery.

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INTRODUCTION

Colon targeted drugs are becoming increasingly important for the topical treatment of colonic IBDs (inflammatory bowel diseases) such as ulcerative colitis (UC) and Crohn's disease (CD). In ulcerative colitis, inflammation and sores (ulcers) on the superficial lining of the large intestine i.e. the bolus (colon) and rectum whereas in Crohn's disease it is characterized by inflammation of the lining of the digestive tract, which can usually, affect the deeper layers of the digestive tract. Symptoms are characterized by diarrhea, rectal bleeding, abdominal pain, weight loss and fatigue.[1] Crohn's disease and ulcerative colitis have a high prevalence rate worldwide. Inflammatory bowel diseases (IBDs) are historically called Western disease. However, over the past two decades, the prevalence of lesion colitis and Crohn' disease have been higher in Asian countries.[2]

In India, the prevalence rate for colitis was reported to be 443/100,000 individuals, whereas the age standardized prevalence rate of colon cancer in India are reported to be 4.2 and 3.2 per 100,000 for males and females respectively.[3] In other countries, particularly in North America, the incidence of people with Crohn's disease is higher and statistics data shows that approximately 129,000 people are living with the same disease in Canada. The medical history of IBDs reveals that around 01 in 700 persons have IBDs within the UK that sometimes happens between the ages of 15 and 40, though any age are often affected. Although disease onset usually occurs in adulthood, IBDs are increasingly being diagnosed in children. Treatment of IBDs typically assumes the utilization of medicines which will reduce the symptoms and inflammation within the lining of the colon. Types of drugs together with 5-aminosalicylic acid are often used for treatment of IBDs. Smoking is perhaps the foremost vital environmental issue for the event of CD.[4-5]

EDTA and its derivatives are usually referred to as chelating agents approved by the United State Food and Drug Administration for the therapy of serious metal poisoning[6-7] since early clinical trials shows that treatment with chelation therapy mediated by EDTA has systematically ability to cleanse the body from the metals and other items deposits which are responsible for atherosclerosis, cardiovascular disease and tumors.[8-13] EDTA is additionally used as distinction enhancing agents for magnetic resonance imaging.[14] If acceptable substitutions are made, the chelating properties of EDTA are basically preserved and fully new applications are transferred to the new compounds. For example, throughout the 1970s, long hydrophobic alkyl group chains were connected to EDTA via amide OR ester bonds and a brand-new variety of chemical agent surfactant (chelator) were born.[15] The synthesis of alkyl amide derivatives of EDTA incorporates a washing ability which ends in a very commercially obtainable detergent powder, which has less encrustation of the fabric material and this property is additionally the chelating property of EDTA.[16] Bile acid bind to EDTA for various

purposes i.e. derivatives of bile acid with EDTA might be used to deliver significant atoms into the hepatobiliary system in animals and metal complexes are used as radiopharmaceuticals and as distinction agents for magnetic resonance imaging.[17] The adamantyl derivatives of EDTA are compounds of interest as a result of high biological activity, specially an antiviral activity.[18-19] Cyclodextrin-EDTA conjugates was synthesized and their release results on the gut have been studied.[20] It seems that the antibacterial drug activity of EDTA is because of the chelation of cations from the outer membrane of the bacteria[21] Russell *et al*. disclosed that EDTA made a creosote-like bacterial growth inhibition zone. However, lower concentrations of EDTA produced a reduced zone of non-inhibition.[22] Kotula *et al*. reveals that the antimicrobial effect of metallic element EDTA was maintained till the chelators forms bonds with the metal ions.[23]

The present work was divided into three parts, first was to synthesized Ethylene diamine tetraacetic acid (EDTA)- antimicrobial drug conjugates by forming ester and amide bonds between the carboxylic acid groups of EDTA and hydroxyl OR amino group of the antimicrobial drugs. Second characterization, third was stability and release studies. In these EDTA-antimicrobial drug conjugates the pharmacological effects are because of antimicrobial drugs used i.e. Nitroimidazole derivatives (MTZ-Metronidazole, OZ-Ornidazole), Fluoroquinolones (CF-Ciprofloxacin, NF-Norfloxacin) and Sulphonamide derivatives (SM-Sulfamethoxazole).

Metronidazole is the main elegance of the nitroimidazoles and was included by the WHO in the sixteenth edition of the major drug lists[24] and observed in the mid-1950s by Rhône Poulenc in the search for a treatment for trichomoniasis, a sexually transmitted disease,[25] caused by *Trichomonas vaginalis*. Nitroimidazole derivatives are pronounced with various activities such as antiprotozoal activity, antivaginal, giardiasis, Chagas disease (trypanosomiasis),[26-27] amoebiosis caused by *Entamoeba histolytica*,[28] infections with anaerobic microorganisms, together with gram-negative *Bacteroides fragilis*, that produces peritoneal infections, gram positive *Clostridium difficile*, which causes pseudomembranous inflammation in colon,[29] and *Helicobacter pylori*, which causes stomach ulcers,[30] MTZ is also used as antituberculous,[31] and in immunological disorder.[32]

Metronidazole was first declared potent in the treatment of CD by Ursing and Kamme in 1975[33] and has become more potent than sulfasalazine in the treatment of CD[34] and the recovery of perianal fistulas. Nitroimidazoles such as MTZ, OZ are used as part of the remedy and prophylaxis against anaerobes, and excessive concentrations of these microorganisms are present in the ileocolic region.[35-37] Sulfonamides are one of the cheapest drugs, and this factor largely explains their increased use in developing countries. These drugs are useful for infections of the gastrointestinal (GI) tract, for this gastrointestinal infection, sulfonamides, viz. Sulfamethoxazole and sulfadiazine can be used.[38-39]

This study aimed to develop a colon-targeted drug delivery system for nitroimidazole, fluoroquinolone, and sulphonamide derivatives by conjugates synthesis. For this purpose, two ester conjugates of EDTA with MTZ, OZ and three amide conjugates of EDTA with CF, NF and SM were synthesized in presence of EDAC ((1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride). The synthesized conjugates were characterised by chemical analysis and analytical ways FTIR, NMR (^{1}H and ^{13}C), mass spectroscopy and elemental analysis. The synthesized conjugates showed potent antimicrobial (antibacterial) activity against human pathogenic microorganisms and release studies were performed by *in-vitro* method.

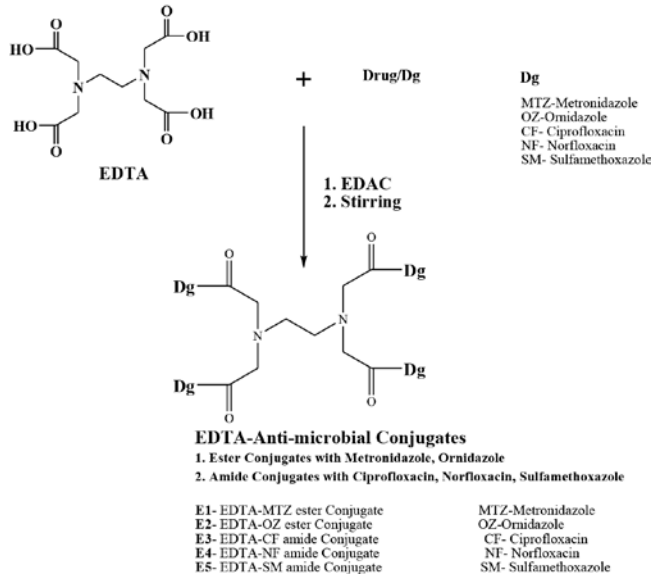
MATERIALS AND METHODS

Considering the significance of colon targeted prodrugs, we report the synthesis of EDTA-antimicrobial drug conjugates. Synthesis involves the reaction between the tetra sodium EDTA and antimicrobial drugs viz Metronidazole (MTZ), Ornidazole (OZ), Ciprofloxacin (CF), Norfloxacin (NF) and sulfamethoxazole (SM) in presence of EDAC ((1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride), this mixture was stirred for 5 days. Then part of the solvent was removed under vacuum and the residue was dialyzed against distilled water for 48 hr to purify the final products (SCHEME).

All the reactions were performed under specific laboratory conditions. All the synthesis was performed by procuring laboratory grade reagents and analytical grade solvents purchased from Hi Media, CDH and Sigma Aldrich. The drug viz. Metronidazole IP, Ornidazole IP, Ciprofloxacin IP, Norfloxacin IP and Sulfamethoxazole IP as a gift sample were obtained from Aishwarya Healtcare Baddi Himachal Pradesh. The Melting points were determined by a Thiele tube and digital melting point apparatus.

The structures of the synthesized conjugates were determined by FTIR, NMR (^{1}H and ^{13}C), mass spectroscopy and elemental analysis. FTIR spectra were recorded on KBr discs (cm^{-1}) using a SHIMADZU IR spectrometer from GLA Mathura, UP, INDIA. NMR spectra were measured on a Bruker Avance-II 300 MHz NMR spectrometer using $CDCl_3$ as solvent and TMS (tetra methyl silane) as an internal standard from IIT ROORKEE, Uttarakhand, INDIA. The Chemical shift values are reported as ppm relative to TMS (δ=0). The mass spectrum was recorded on Waters Micromass Q-TOF Micro, mass spectrometer and Elemental analysis of compounds was carried out by company Thermo Finnigan from SAIF Punjab University, Chandigarh PUNJAB, INDIA. The purification of the conjugates was carried out by column chromatography and progress of the reaction was confirmed by thin layer chromatography (TLC) where chloroform: methanol (4: 1) and ethyl acetate: chloroform: ammonia (25: 25: 1) was the mobile phase and the spots were visualized in the UV lamp and iodine chamber. All the synthesized conjugates (E1-E5) were screened for antimicrobial (antibacterial) activity and release studies.

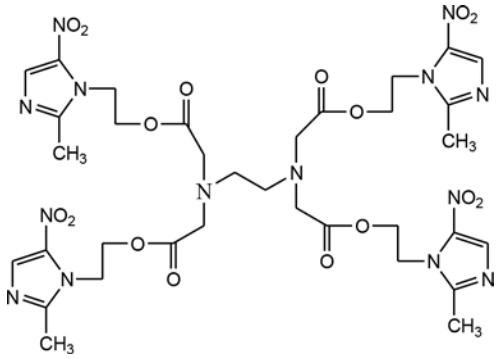
SCHEME: EDTA-Anti-microbial drug Cojugates



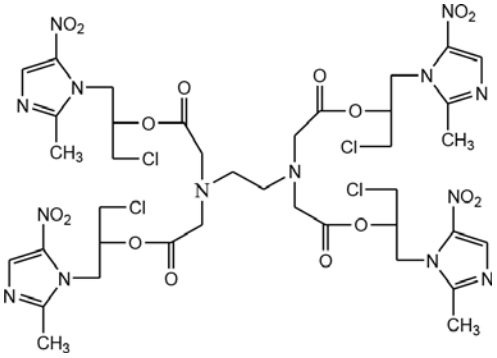
Structure of synthesized conjugates

A. EDTA-Antimicrobial ester Conjugates

i. EDTA-Metronidazole (E1) Conjugate

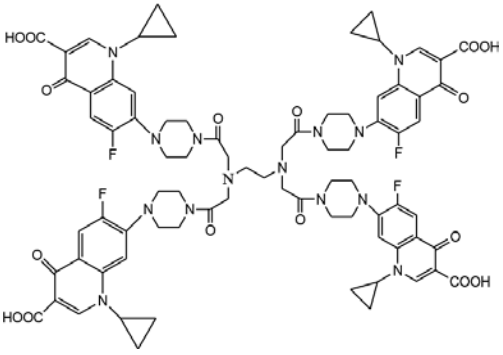


ii. EDTA-Ornidazole (E2) Conjugate

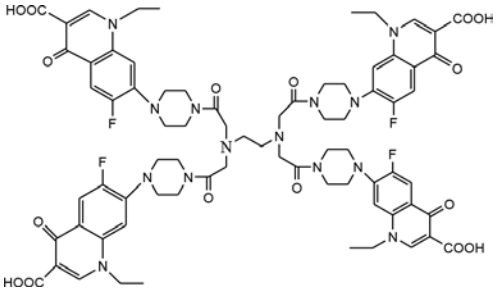


B. EDTA-Antimicrobial amide Conjugates

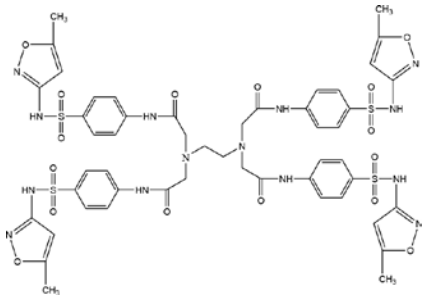
i. EDTA-Ciprofloxacin (E3) Conjugate



ii. EDTA-Norfloxacin (E4) Conjugate



iii. EDTA-Sulfamethoxazole (E5) Conjugate



General procedure for the synthesis of Ethylene diamine tetraacetic acid (EDTA)-Antimicrobial drug conjugates (E1-E5)

Synthesis of Ethylene diamine tetraacetic acid–Metronidazole (EDTA-MTZ) conjugates (E1)

The EDTA-MTZ, EDTA-OZ, EDTA-CF, EDTA-NF and EDTA-SM (E1-E5) conjugates were synthesized by coupling antimicrobial drugs (MTZ, OZ, CF, NF, SM) with carboxyl groups of the EDTA.

Tetra sodium EDTA (0.38 g, 1 mmol) was dissolved in water and EDAC ((1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride) (0.191 × 4 = 0.76 g, 1 × 4 mmol) was also in water, then the EDAC solution was added to the EDTA solution with stirring at room temperature. The mixture was stirred at room temperature for 2 hr to activate the carboxyl group of EDTA, after the activation of the carboxyl group a solution of metronidazole (0.171 × 1.25 = 0.21 g, 1.25 × 4 mmol) in methanol was added. The reaction mixture was stirred at room temperature for 5 days, then part of the solvent was removed under vacuum and the residue was dialyzed against distilled water for 48 hr to purify the final product. Finally, the EDTA-MTZ ester conjugates (E1) was obtained as white cotton like powder with a yield of 76%. The purity of the EDTA-MTZ ester conjugate was carried out by TLC on silica gel G using chloroform: methanol (4:1) and ethyl acetate:chloroform:ammonia (25:25:1) as the mobile phase. The single spots obtained with Rf values equal to 0.72 confirm the purity of the conjugates.[40-43]

The remaining compounds (E2-E5) were synthesized similarly.

Characterization data of synthesized Ethylene diamine tetraacetic acid–Metronidazole (EDTA-MTZ) conjugates (E1)

IR (KBr cm^{-1}): 2910 (CH*str*, Aliph.), 1740 (C=O*str* ester), 1597 (C-C*str* Aro.), 1550 (N-O *str*), 1275 (C-O*str* ester); ^{1}HNMR (300 MHz, $CDCl_3$) δ ppm: 2.21 (t, 2H, -CH_2-, -N (C) C-), 2.48 (s, 3H, -CH_3, -N(C) C-), 3.32 (s, 2H, -CH_2-CO-O-), 4.02 (t, 2H, -CH_2O), 4.60 (t, 2H, -CH_2N), 7.91(s, H, -CH=C- imidazol); ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 14.0 (-C-CH_3), 45.0 (-N-CH_2-CH_2-O-), 52.5 (30-65 CH_2-CH_2-N=), 56.5 (N-C-CO-), 63.0 (50-90 C-C-O), 128.8 (125-150 -C=C- imidazol), 134.0 (125-150 -C=C- imidazol), 150.5 (125-150 -C=C-imidazol), 166.6 (160-185 C=O ester); MS (70 eV) *m/z*: 905 [M^+]; Anal. Calcd. for $C_{34}H_{44}N_{14}O_{16}$: C, 45.13; H, 4.90; N, 21.67. Found: C, 45.03; H, 4.87; N, 21.03%

Characterization data of synthesized Ethylene diamine tetraacetic acid–Ornidazole (EDTA-OZ) conjugates (E2)

IR (KBr cm^{-1}): 2981 (CH*str*, Aliph.), 1735 (C=O*str* ester), 1585 (C-C*str* Aro.), 1540 (N-O *str*), 1261 (C-O*str* ester),711 (C-Cl*str*); ^{1}HNMR (300 MHz, $CDCl_3$) δ ppm: 2.32 (t, 2H, -CH_2-, -N (C) C-), 2.49 (s, 3H, -CH_3C), 3.38 (s, 2H, -CH_2CO-O), 3.74 (s, 2H, -CHO-CH_2N), 4.30 (m, 1H,-4CH_2-CH_2Cl), 7.86 (s, H, -CH=C- imidazol); ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 13.8 (-CH_3-), 34.0 (N-C-C-O-), 47.1 (C-O-C-Cl), 51.0 (-N-C-C-), 70.0 (50-90 -C-C-O), 132.5 (125-150 –C=C-imidazol), 138.5 (125-150 –C=C-imidazol), 151.5 (125-150 -C=C-imidazol), 172.5 (160-185 C=O ester); MS (70 eV) *m/z*: 1098 [M^{++}]; Anal. Calcd. for $C_{38}H_{48}Cl_4N_{14}O_{16}$: C, 41.54; H, 4.40; N, 17.85. Found: C, 41.48; H, 4.32; N, 17.78%

Characterization data of synthesized Ethylene diamine tetraacetic acid–Ciprofloxacin (EDTA-CF) conjugates (E3)

IR (KBr cm^{-1}): 3344 (NH*str*, amide), 3060 (C-H*str*, Ali.), 2765 (OH*str*, Carb. acid), 1660 (C=O*str* amide), 1598 (C-C*str* Aro.), 1321 (C-F*str*); ^{1}HNMR (300 MHz, $CDCl_3$) δ ppm: 1.28 (p, 1H, $-CH_2-CH_2-$ cyclopropane), 2.45 (t, 2H, $-CH_2-$, -N (C) C-), 3.26 (s, 2H, -CO-N-CH_2), 3.44 (t, 2H,-CH_2N), 3.65 (t, 2H, -CH_2N), 6.21 (d, 1H, -C=C-), 7.26 (d, 1H, -C=C-), 7.89 (s, H, -C=C-), 11.2 (1H, OH carboxylic acid); ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 8.1 (-C-C cyclopropane), 42.2 (-C-C-N), 53.0 (N-C-C-N=), 59.0 (-N-C-CO), 113.5 (-C=C- Ar-C), 128.0(-C=C- Ar-C), 129.1 (-C=C- Ar-C), 170 (165-175 C=O amide) 179 (175-185 C=O acid); MS (70 eV)*m/z*: 1545 [M^+]; Anal. Calcd. for $C_{78}H_{80}F_4N_{14}O_{16}$: C, 60.62; H, 5.22; N, 12.69. Found: C, 60.58; H, 5.16; N, 12.58%

Characterization data of synthesized Ethylene diamine tetraacetic acid–Norfloxacin (EDTA-NF) conjugates (E4)

IR (KBr cm^{-1}): 3344 (NH*str*, amide), 3070 (C-H*str*, Ali.), 2785 (OH*str*, Carb. acid), 1654 (C=O*str* amide), 1598 (C-C*str* Aro.), 1323 (C-F*str*); ^{1}HNMR (300 MHz, $CDCl_3$) δ ppm: 1.28 (t, 3H, -CH_2N), 2.48 (t, 2H, $-CH_2-$, -N (C) C-), 3.12 (q, 2H, CH_3), 3.28 (s, 2H, -CO-N-CH_2), 3.47 (t, 2H,-CH_2N), 3.68 (t, 2H, -CH_2N), 6.08 (d, 1H, -C=C-), 7.16 (d, 1H, -C=C-), 7.92 (s, 1H, -C=C-); ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 16.0 (-CH_3-), 41.8 (C-C-N), 49.0 (-N-C-C-N-), 52.5.0 (30-65 N-C-CO), 106.2 (-C=C-Ar-C), 114.0(-C=C- Ar-C), 118 (-C=C- Ar-C), 128.0 (-C=C- Ar-C), 131.0 (-C=C- Ar-C), 147 (Ar-C-F), 167 (165-175 C=O amide) 170 (175-185 C=O acid); MS (70 eV)*m/z*: 1497 [M^+]; Anal. Calcd. for $C_{74}H_{80}F_4N_{14}O_{16}$: C, 59.35; H, 5.38; N, 13.09. Found: C, 59.28; H, 5.34; N, 13.08%

Characterization data of synthesized Ethylenediamine tetraacetic acid–Sulfamethoxazole (EDTA-SM) conjugates (E5)

IR (KBr cm^{-1}): 3300 (NH*str*, amide), 3050 (C-H*str*, Aro.), 2895 (CH*str*, Aliph.), 1654 (C=O*str* amide), 1595 (C-C*str* Aro.), 1363 (S=O*str*, Sulfo.); ^{1}HNMR (300 MHz, $CDCl_3$) δ ppm: 2.36 (s, 3H, -C=C-), 2.48 (t, 2H, -CH_2N), 3.32 (d, 2H, -CO-N), 7.94(s, 1H, -C=C); ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 12.1 (-CH_3-), 48.1 (-N-C-C-N-), 58.5 (N-C-CO), 95.33 (-C=C-O- Isoxazole), 128.5 (-C=C- Ar-C), 129.1 (-C=C- Ar-C), 133.0 (-C-SO), 167.0 (-CO-NH, amide); MS (70 eV)*m/z*: 1233 [M^+]; Anal. Calcd. for $C_{50}H_{52}N_{14}O_{16}S_4$: C, 48.69; H, 4.25; N, 15.90; S, 10.10; Found: C, 48.62; H, 4.22; N, 15.84; S, 10.04 %

Biological evaluation of synthesized conjugates

Anti-Bacterial activity

The antibacterial activity of synthesized conjugates (E1-E5) screened against strains of Gram positive, *Staphylococcus aureus* (MCC 2408), *Bacillus subtilis* (MCC 2010) and Gram-negative *Escherichia coli* (MCC 2552) and *Pseudomonas aeruginosa* (MCC 2907) using the cylinder plate method.[44] Culture media used were prepared using aseptic and sterilization techniques.[45] The incubation time was 24 hr at 37 °C to activate the bacterial strain. All the test compound solutions were prepared by dissolving 1 mg of the test sample in 1 ml of DMF (N, N-dimethylformamide), this gives the sample concentration 1000 µg/ml or 1000 ppm. Different dilutions such as 200 µg/ml and 100 µg/ml were prepared from the sample solution. The DMF solution (10%) was used as a control. Pure cultures of *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* were obtained from the Microbial Culture Collection, (Now called as National Centre for Microbial Resource, NCMR) Pune, India and grown in an Erlenmeyer flask also known as conical flask (100 ml) containing Potato Dextrose Agar (PDA). The evaluation of antibacterial activity was carried out by measuring the zone of inhibition in different Petri dishes and calculating the average of each strain. The results of antibacterial activity are depicted in Table 1. Tested compounds (conjugates) showed slight to moderate antibacterial activity.

Table 1: Antimicrobial (Antibacterial) screening data of synthesized EDTA-antimicrobial drug conjugates (E1-E5).

Conjugates Code	Average diameter of zone of inhibition in mm			
	Gram Negative		Gram Positive	
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
E-1	21	22	20	19
E-2	19	20	21	18
E-3	27	26	24	23
E-4	24	25	26	27
E-5	21	23	22	21
Standard (Ciprofloxacin)	25	23	22	23
Control (DMF)	0	0	0	0

Release Studies

In-vitro Release studies

Colon-specific prodrugs must survive intact while passing through the stomach and small intestine to reach the colon, where they must be broken by enzymes of the colonic microflora. Therefore, to obtain information about the behaviour of the conjugates in acidic and alkaline environments, the stability OR release study of the conjugates *in-vitro* in acidic and basic buffers was investigated.[46] This is because the ester and amide bonds in the conjugates are prone to hydrolysis due to their chemical structure. The amount of free drug released from E1-E5 conjugates such as MTZ, OZ, CF, NF and SM was measured in 6 hr studies to assess the stability of the conjugates. The *In vitro* stability OR release studies was performed at pH = 1.2 and pH = 7.4.[47]

Release Studies in Simulated Gastric Fluid (SGF, pH 1.2)

Each of the synthesized conjugates (E1-E5, 10 mg) were gently spread on the surface of 900 ml of HCl buffer (Simulated Gastric Fluid, SGF) present in separate baskets rotated at 100 rpm and temperature was maintained at 37 ± 1°C.[48] Perfect sink conditions were maintained during the dissolution of the drug. The solutions were shaken and 5 ml aliquots were removed from the dissolution vessel at 15 min time intervals. The study was conducted over a 6 hr period. The withdrawn samples were assayed using UV spectrophotometer at λ_{max} 292 nm, 332 nm, 282 nm, 298 nm and 278 nm, respectively. Free released drugs from the synthesized conjugates did not interfere with the absorption of E1, E2, E3, E4 and E5 because their λ_{max} was significantly different from the conjugates.

Release Studies in Simulated Intestinal Fluid (SIF, pH 7.4)

The experiments for drug release in alkaline (Simulated Intestinal Fluid, SIF, pH 7.4) were conducted using an approach similar to that is given above, except that HCl buffer (Simulated Gastric Fluid, SGF, pH 1.2) was replaced with Simulated Intestinal Fluid (SIF).[49]

Release Studies in Simulated Colonic Fluid (SCF, pH 7.0)

Each of the conjugates (E1-E5) was dissolved in SCF (pH 7.0) so that the final concentration of the solution was 250 µg/ml. Fresh rat (wistar rat) feces (approximately 1 g) were weighed and placed in several sets of test tubes. 1 ml of the conjugate's solution was added to the tube and diluted to 5 ml with SCF (50µg/ml). The tubes were then incubated at 37°C for different time intervals. For the analysis, the concentrations of the drug release from the conjugates (E1-E5) were estimated directly using UV spectrophotometer (Shimadzu UV 1700) at λ_{max} 292 nm, 332 nm, 282 nm, 298 nm and 278 nm respectively. The percentage of free drug release from its conjugates is shown in the table and the Figure.[50]

RESULTS

Chemistry

The synthesis of all the synthesized conjugates were carried as per the scheme out lined. In the first step activation of the carboxyl group of EDTA was achieved by coupling it with the water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). After the activation of the carboxyl group, a solution of antimicrobial drugs viz. Metronidazole (MTZ), Ornidazole (OZ), Ciprofloxacin (CF), Norfloxacin (NF) and Sulfamethoxazole (SM) were added and the mixture was stirred for 5 days to obtain the corresponding EDTA-antimicrobial drug conjugates (E1-E5). The physical constants are shown in Table 2 and the yields of the synthesized conjugates were found to be in the range of 74 to 78%.

Anti-Bacterial activity

The screening outcomes of antibacterial activity are shown in Table 1. Among all of the synthesized conjugates, E-3 and E-4 displayed good activity against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* as compared to the standard; this might be because of synergistic effect of EDTA and antimicrobial drugs.

In-vitro Release studies

In-vitro release studies results of the synthesized conjugates are shown in Table 3 and Figure 1-8, it showed that all the synthesized conjugates were stable in SGF (pH 1.2) and negligible release observe in SIF (pH 7.4). A substantial release was observed in SCF (pH 7.0), 90.48 of E1, 87.40 of E2, 88.55 of E3, 88.55 of E4 and 89.05 % of E5 (Table 3, Figure 8).

Table 2: Physical constants of synthesized EDTA-antimicrobial drug conjugates.

Conjugates Code	Molecular Formula	Mol. Weight	Melting Point (0°C)	Yield (%)	R_f Value*
E-1	$C_{34}H_{44}N_{14}O_{16}$	905	278-282	76	0.72
E-2	$C_{38}H_{48}Cl_4N_{14}O_{16}$	1098	282-286	74	0.82
E-3	$C_{78}H_{80}F_4N_{14}O_{16}$	1545	328-332	78	0.66
E-4	$C_{74}H_{80}F_4N_{14}O_{16}$	1497	264-268	75	0.68
E-5	$C_{50}H_{52}N_{14}O_{16}S_4$	1233	284-288	77	0.64

*Mobile Phase: Chloroform: methanol (4:1) and ethyl acetate: chloroform: ammonia (25:25:1)

DISCUSSION

In this research carboxylic acid group of EDTA converted to ester conjugates with Metronidazole (MTZ), Ornidazole (OZ) and amide conjugates with Ciprofloxacin (CF), Norfloxacin (NF) and Sulfamethoxazole (SM). The structures of synthesized conjugates were examined on the basis of physical constant, Infrared spectroscopy (FTIR), Nuclear Magnetic Resonance (^{1}H and ^{13}C), Mass spectroscopy and Elemental analysis. We studied physical constant (Table 2) viz. molecular formula, molecular weight, melting point and percentage yield. Infrared spectrum of the synthesized conjugates (E1 and E2) showed the characteristics absorption bands at 1750-1735 cm^{-1} due to carbonyl (-C=O) ester group, 3000-2850 cm^{-1} for aliphatic CH stretching, 1550-1475 cm^{-1} for nitro group stretching and synthesized conjugates (E3, E4 and E5) showed the absorption bands at 1690-1630 cm^{-1} due to carbonyl amide group, 3100-3000 cm^{-1} for aromatic CH stretching, 1400-1000 cm^{-1} for C-F stretching and 1363 cm^{-1} for S=O (E-5) stretching.[51] In ^{1}H NMR, the characteristics chemical shifts observed for conjugates (E1-E5) were the protons of aliphatic region δ 2.21- 2.32 [s, 2H] –CH_2-, δ 2.48-2.49 [s, 3H] -CH_3C, δ 3.32-3.38 [s, 2H] –CH_2-CO-O-, proton of imidazol δ 7.86-7.91 [s, 1H] -CH=C-, proton of aromatic region δ (6.08-7.94) Ar-H. In ^{13}CNMR, the characteristics chemical shifts observed for conjugates (E1-E5) showed δ ppm value at 0-35 for R-CH_3-, 50-90 -CO-, 125-150 Ar-C-, 160-185 C=O ester and 160-175 C=O for amide.[52] The mass spectra of E1, E2, E3, E4 and E5 showed parent ion/ molecular peak (m/z) at 905, 1098, 1545, 1497 and 1233 respectively,[53] which confirms the molecular weight of the synthesized conjugates. The elemental analysis OR CHN studies also confirm the synthesis of synthesized conjugates. The detail spectral data are shown in characterization data of synthesized conjugates.

All the synthesized conjugates were screened for antimicrobial (antibacterial) activity compared to the standard drug Ciprofloxacin (Table 1). Results reveal that the known standard antibiotic (Ciprofloxacin) produces a average diameter of zone of inhibition in the range of 22-25 mm. Synthesized conjugates E-3 and E-4 displayed good activity against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*. This is only occurs due to synergistic effect of EDTA and antimicrobial drugs. Other conjugates (E-1, E-2, and E-5) exhibited less pronounced antimicrobial (antibacterial) activity against the tested strains.

The *in-vitro* release studies of the synthesized conjugates E-1, E-2, E-3, E-4 and E-5 showed that they were stable in SGF (pH 1.2), implying that they did not undergo hydrolysis and could be stable in acidic gastric pH and in SIF (pH 7.4), negligible release was observed (Figure 2). This fulfilled

Table 3: % Release of drug from EDTA-antimicrobial drug conjugates (E1-E5) in rat fecal matter.

Time (min)	% Release of drug from EDTA-antimicrobial drug conjugates (E1-E5) in rat fecal matter														
	E-1	SD	SEM	E-2	SD	SEM	E-3	SD	SEM	E-4	SD	SEM	E-5	SD	SEM
0	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000
15	9.24	0.240	0.139	8.58	0.507	0.293	9.77	0.503	0.291	9.05	0.507	0.293	9.11	0.271	0.157
30	16.86	0.572	0.330	19.11	0.502	0.290	17.29	0.571	0.330	18.65	0.100	0.058	19.64	0.087	0.050
45	28.32	0.572	0.330	26.94	0.452	0.261	30.34	0.649	0.375	26.40	0.391	0.225	27.48	0.483	0.279
60	35.64	0.635	0.367	37.11	0.519	0.300	35.48	0.359	0.207	37.55	0.136	0.079	37.64	0.087	0.050
75	46.98	0.550	0.317	45.48	0.524	0.303	46.80	0.677	0.391	46.05	0.500	0.289	46.00	0.035	0.020
90	54.66	0.550	0.317	55.85	0.523	0.302	55.80	0.678	0.392	57.25	0.305	0.176	56.38	0.626	0.361
105	62.52	0.275	0.159	63.95	0.557	0.322	66.21	0.219	0.127	66.05	0.492	0.284	64.48	0.507	0.293
120	70.92	0.550	0.317	74.70	0.538	0.311	75.21	0.602	0.348	74.15	0.458	0.265	75.22	0.557	0.321
240	81.54	0.334	0.193	82.64	0.607	0.350	83.96	0.590	0.341	83.55	0.512	0.295	83.17	0.329	0.190
360	90.48	0.365	0.211	87.41	0.571	0.330	88.55	0.633	0.365	88.55	0.687	0.397	89.05	0.098	0.057

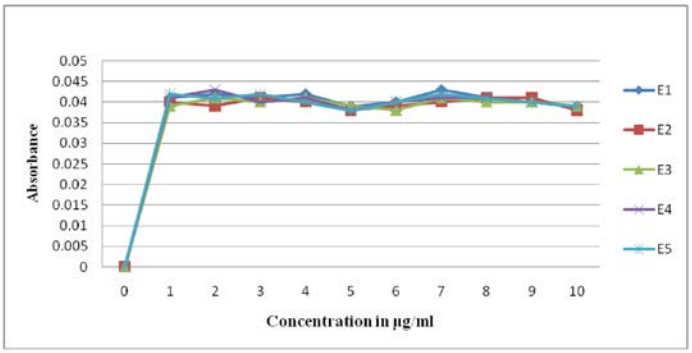


Figure 1: Release studies of synthesized EDTA-antimicrobial drug conjugates (E1-E5) in acid (pH 1.2/SGF) at 37°C.

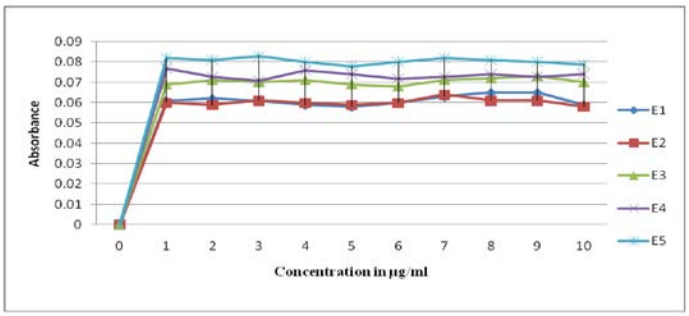


Figure 2: Release studies of synthesized EDTA-antimicrobial drug conjugates (E1-E5) in basic (pH 7.4/SIF) at 37°C.

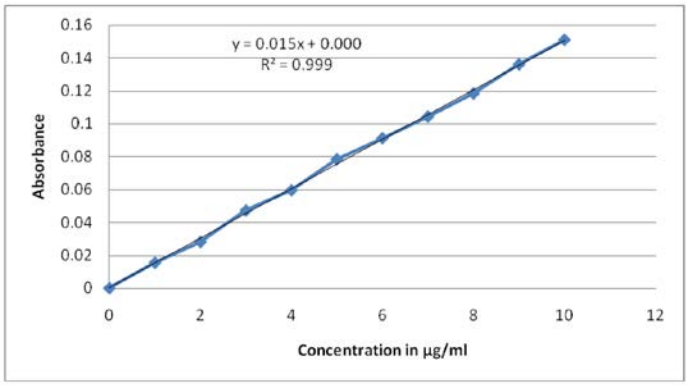


Figure 3: Standard Curve of E1 conjugates in SCF at λ_{max} 292 nm.

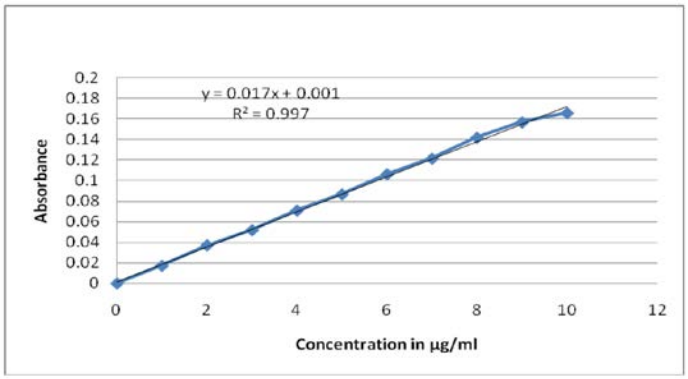


Figure 4: Standard Curve of E2 conjugates in SCF at λ_{max} 332 nm.

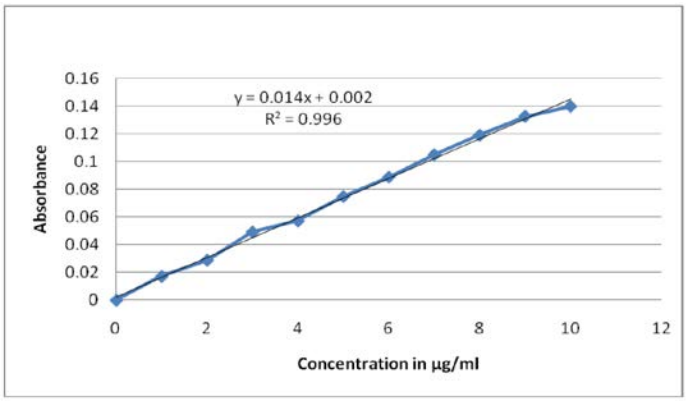


Figure 5: Standard Curve of E3 conjugates in SCF at λ_{max} 282 nm.

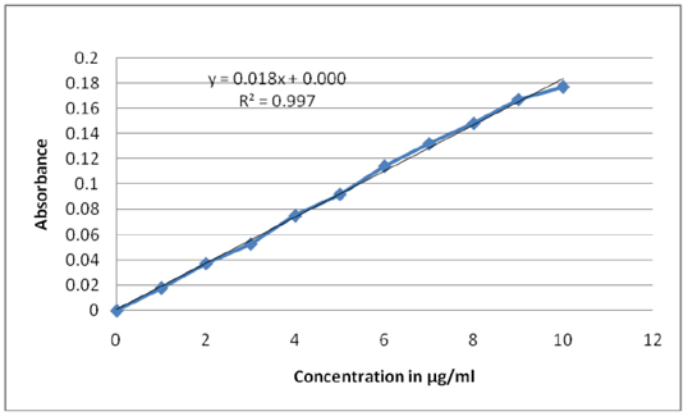


Figure 6: Standard Curve of E4 conjugates in SCF at λ_{max} 298 nm.

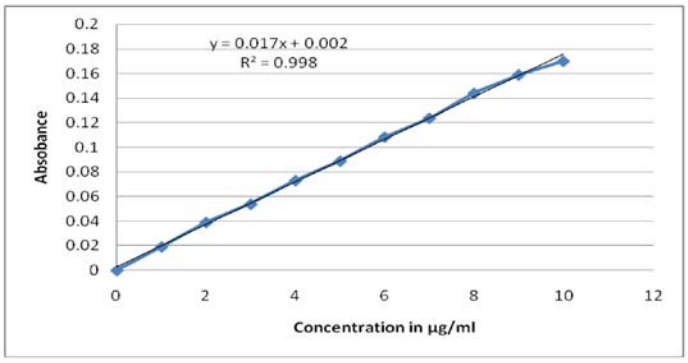


Figure 7: Standard Curve of E5 conjugates in SCF at λ_{max} 278 nm.

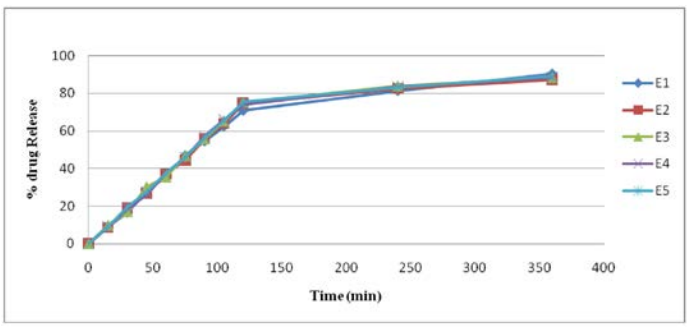


Figure 8: % Release of drug from synthesized EDTA-antimicrobial drug conjugates (E1-E5) in rat fecal matter.

the intention of bypassing upper GIT without releasing free drugs. In order to confirm the colonic release of the synthesized conjugates, the kinetics were studied in SCF (7.0 pH), indicating a substantial release of free drugs from the conjugates, with a release percentage of 90.48 of E1, 87.40 of E2, 88.55 of E3, 88.55 of E4 and 89.05 of E5. (Table 3, Figure 8).

CONCLUSION

In conclusion, we successfully synthesized some EDTA-antimicrobial drug conjugates, from these studies, it can be concluded that the formation of EDTA-antimicrobial drug conjugates of EDTA and antimicrobial drugs definitely improves drug delivery to the intestinal region this is because of high molecular weight of synthesized conjugates (> 500), and as per the Lipinski Rule of Five it suggested that these conjugates will not be absorbed by the upper GIT. Release studies suggest that the drugs begin to release from the conjugates in the distal intestinal region and appreciable release in the colon has been observed.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Human and Animal Rights

Animals were used for the release studies on which this investigation is based. Release studies of the synthesized conjugates were performed at the Department of Pharmaceutical Chemistry, Acharya Narendra Dev College of Pharmacy, Babhnan, Gonda UP-271313, India and their animal facility was approved by CPCSEA: 1585/PO/E/ 5/11/CPCSEA, Registration date: 23/12/2011 and Author permission IAEC approval Reference NO: IAEC/ANDCP/3/2021. The experimental protocols for these studies have been approved by the Institutional Animal Ethics Committee.

ABBREVIATIONS

IBD: Inflammatory bowel diseases; **^{13}C-NMR:** Carbon Nuclear Magnetic Resonance; **^{1}H-NMR:** Proton Nuclear Magnetic Resonance; **CD:** Crohn's disease; **CF:** Ciprofloxacin; **DMSO:** Dimethyl Sulfoxide; **E1:** EDTA-Metronidazole Conjugates; **E2:** EDTA-Ornidazole Conjugates; **E3:** EDTA-Ciprofloxacin Conjugates; **E4:** EDTA-Norfloxacin Conjugates; **E5:** EDTA-Sulfamethoxazole Conjugates; **EDAC:** 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; **EDTA:** Ethylene diamine tetraacetic acid; **FTIR:** Fourier Transform Infrared spectroscopy; **IP:** Indian Pharmacopoeia; **KBr:** Potassium Bromide; **m/z:** Mass/ Charge Ratio; **MCC:** Microbial Culture Collection; **MS:** Mass Spectroscopy; **MTZ:** Metronidazole; **NF:** Norfloxacin; **OZ:** Ornidazole; **SCF:** Simulated Colonic Fluid; **SD:** Standard Deviation; **SGF:** Simulated Gastric Fluid; **SIF:** Simulated Intestinal Fluid; **SM:** Sulfamethoxazole; **TLC:** Layer Chromatography; **TMS:** Tetra Methyl Silane; **UC:** Ulcerative colitis; **WHO:** World Health Organisation.

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