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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and pharmacological evaluation of mutual prodrugs of aceclofenac with quercetin, vanillin and L-tryptophan as gastrosparing NSAIDS

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Abstract Synthesis, physicochemical characterization and pharmacological evaluation of mutual prodrugs of aceclofenac with quercetin, vanillin and L-tryptophan have been attempted to develop novel gastrosparing NSAIDs, devoid of ulcerogenic side effects. The structures of synthesized prodrugs were confirmed by IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy. The hydrolysis kinetics studies were performed in simulated gastric fluid, simulated intestinal fluid and rat fecal matter. Its anti-inflammatory and ulcer index were analyzed along with estimation of biochemical parameters (GWM and Hexosamine), oxidative parameters (LPO, GSH, CAT, and SOD) and protein estimation. The results indicated that the synthesized prodrugs are chemically stable, biolabile and possesses optimum lipophilicity. They also exhibited retention of anti-inflammatory activity with reduced ulcerogenicity. The study showed that the mutual prodrugs are better in action compared to the parent drug and have fewer gastrointestinal side effects.

Keywords Quercetin · Vanillin · Tryptophan · Hydrolysis kinetics · Anti-inflammatory activity · Ulcerogenicity · Biochemical parameters

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Introduction

Aceclofenac (ACF), 2-[2-[2-[(2,6-dichlorophenyl) amino] phenyl] acetyl] oxy acetic acid, a newer derivative of diclofenac, is one of the emerging NSAIDs for the treatment of various inflammatory diseases. The successful treatment of the disease depends on the maintenance of effective drug concentration level in the body for which a constant and uniform supply of drug is desired. However, the problems of side effects after long-term administration of these drugs, such as irritation and ulceration of the GI mucosa, have arisen in clinical trials (Martindale, 1999; Kay and Alldred 2003). These gastroenteropathies are generally believed to be resulted from the direct contact effect, which can be attributed to the combination of local irritation produced by the free carboxylic group in the molecular structure and by local blockage of prostaglandin biosynthesis in the GI tract. Therefore, the development of new NSAIDs without these side effects has long been awaited. The use of prodrugs to provisionally hide the acidic group of NSAIDs was reported with better lipophilicity, reduced gastric irritancy than parent drug, improved therapeutic index through the prevention of GI irritation and bleeding, improved anti-inflammatory activity, reduced GI erosive properties and synergistic analgesic effect (Stella *et al.*, 1985; Bundgaard, 1989).

During recent years, it has been well established that generation of reactive oxygen species (ROS) plays a significant role in the formation of gastric mucosal lesions associated with NSAIDs therapy (Yoshikawa *et al.*, 1993; Ivey, 1988). These studies establish that co-administration of antioxidants to form mutual prodrugs may possibly decrease the risk of NSAIDs-induced GI ulcerogenicity (Madhukar *et al.*, 2010). The mutual prodrugs are designed with improved physicochemical and pharmacological properties and release the parent drugs at the site of action (Shruti

et al., 2012). The present study aimed at conjugating quercetin, a naturally occurring polyphenolic flavonoid with ACF to obtain ACF-quercetin mutual prodrug. Quercetin is well known for anti-ulcer activity due to its antioxidant properties (Martin *et al.*, 1998; Cotelle, 2001). Although linking of quercetin with ACF in 1:1 ratio is difficult due to the presence of a number of hydroxyl groups, an attempt has been made to conjugate this in the form of its derivative, quercetin tetramethyl ether (QTME) with ACF.

Literature survey reveals that many efforts had been made to synthesize amino acid ester, glycolamide ester and amide prodrugs using various amines. But few attempts were made to develop ester prodrugs using antioxidants and amide prodrugs using amino acids. With this background, an attempt has been made to synthesize aceclofenac–vanillin and aceclofenac-L-tryptophan mutual prodrugs. With this approach, it is expected to get non-toxic prodrugs with minimal GIT disturbance but maintaining the useful physicochemical and pharmacological properties.

Materials

Quercetin, vanillin and tryptophan were obtained from M/s Sigma-Aldrich, Mumbai, India, and aceclofenac was obtained as gift sample from Alkem Laboratories, Mumbai, India. The other reagents and solvents used were of analytical grade. The melting points were recorded using melting point determination apparatus by Sigma Instrument, Chennai, and are uncorrected. The elemental analysis was performed using Carlo-Erba Model 1108 Analyzer, Italy. The infrared and mass spectra were recorded on IR spectrophotometer (Shimadzu 8201 PC) and mass spectrophotometer (Jeol SX-102 (FAB), Japan), respectively. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO on a Bruker DRX 400 Fourier transform spectrometer with TMS as internal standard. Chemical shifts are expressed as δ (ppm) values. The hydrolysis data and drug content determination were performed by UV spectrophotometer (Shimadzu, Japan) at a range of 200–400 nm. The pharmacological evaluation was carried out in Department of Pharmacology, Alshifa College of Pharmacy, Kerala. The histopathological studies were carried out using Primostar Carl Zeiss trinocular microscope in Department of Pathology, Al Shifa hospital, Kerala.

Experimental procedures

Synthesis of prodrugs

The study involves synthesis of aceclofenac with quercetin tetramethyl ether, vanillin and L-tryptophan and shown in Schemes 1, 2 and 3, respectively.

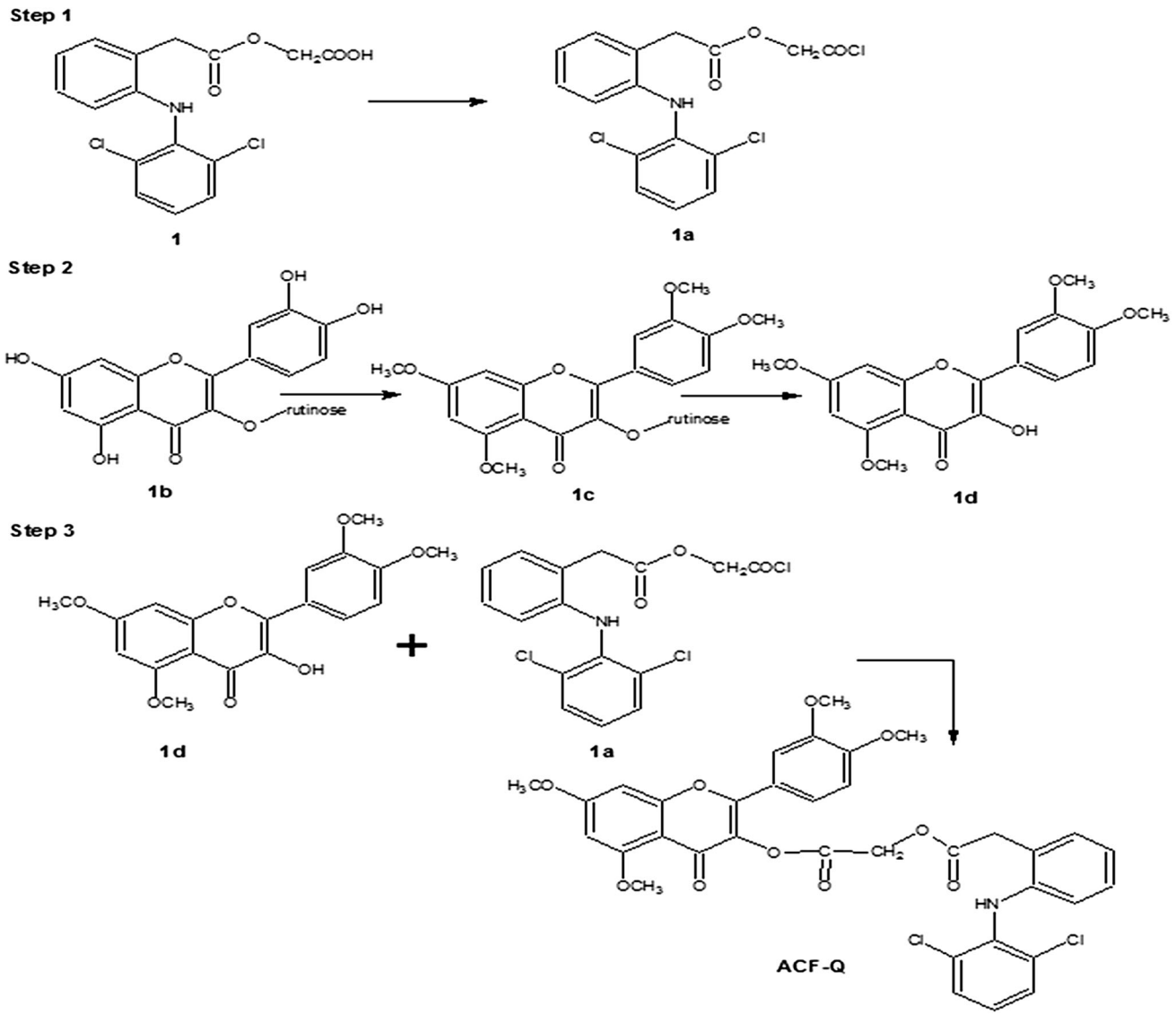
Synthesis of aceclofenac-quercetin mutual prodrug (Martin et al. 1998)

Step 1: Synthesis of aceclofenac acid chloride: Aceclofenac **(1)** (2.12 g, 0.01 mM) was added to thionyl chloride (1.44 g, 0.96 ml, 0.012 M) and stirred at room temperature for 18 h. The excess of thionyl chloride was removed under reduced pressure to give aceclofenac acid chloride **(1a)** as yellow amorphous solid.

Step 2: Synthesis of quercetin 5, 7, 3′, 4′ tetramethyl ether : To a fine solution of rutin hydrate **(1b)** (2 g, 0.003 M) in dry acetone (20 ml), anhydrous potassium carbonate (8 g, 0.056 M) and dimethyl sulfate (8 ml, 0.059 M) were added and the reaction mixture was refluxed for 60 h. The solution was filtered, and insoluble potassium salts were washed with acetone. The washings were combined with the filtrate, and the solvent was removed under reduced pressure to obtain methylated glycoside as semisolid residue. The product was refluxed with ethanolic sulfuric acid (2 %, 50 ml) for 2 h. The solvent was removed under reduced pressure, and the residue obtained was recrystallized from ethanol to give quercetin 5, 7, 3′, 4′ tetramethyl ether (QTME) **(1d)**.

Step 3: Aceclofenac-QTME mutual prodrug (ACF-Q): QTME (1.74 g, 0.005 M) was dissolved in dichloromethane (20 ml) containing triethyl amine (5 drops) and 4-dimethyl amino pyridine (1 pinch). The reaction mixture was cooled to −10 °C, and aceclofenac acid chloride (2.057 g, 0.01 M) dissolved in dichloromethane (30 ml) was added dropwise over a period of 1 h. The reaction mixture was stirred overnight, and the solvent was removed under reduced pressure and recrystallized from ethanol to obtain ACF-Q.

Characterization of 2-(5-(5,7-dimethoxy-4-oxochroman-2-yl)-2,3-dimethoxy-phenoxy)-2-oxoethyl-2-(2-(2,6-dichlorophenyl) amino) phenyl acetate (ACF-Q): brownish needles (from EtOH) (it was obtained as a brown solid); mp 198 °C; UV (MeOH) λ_{max} 300 (SGF), 324 (SIF), 287 (PBS) nm; IR (KBr) ν_{max}: 3424 (NH Stretch), 1580, 1498 (aromatic ring stretch), 1309, 1231 (primary or secondary, OH in plane bend), 1188, 1146 (secondary amine, NH bend), 1084 (primary amine), 766 (1,2 disubstitution (ortho, meta)) cm^{-1}; ^{1}H NMR (DMSO): $\delta = 4$ (1H, aromatic C–NH), 7.28 (2H, Ar–H), 6.48 (1H, Ar–H), 3.72 (2H, OCH_3), 2.50 (1H, CH in ring); ^{13}C NMR (DMSO): $\delta = 169.4$ (ester C=O), 171 (C-4′), 164 (C-7), 159 (C-9), 149 (C-2), 141.3 (C-4′), 140.7 (C-4′), 140.2 (C-3), 135 (C-3), 132.4, 130, 128.7, 127.2, 126.9 (12-C Ar–C biphenyl compound) 56.1 (OCH_3); Mass (*m/z*) 695.13 [M] + (100); $C_{35}H_{31}Cl_2NO_{10}$; (calcd. 696.43); Anal. Calcd. for $C_{35}H_{31}Cl_2NO_{10}$: C, 60.35; H, 4.49; N, 2.01; O, 22.97. Found: C, 60.11; H, 4.45; N, 1.98; O, 22.86.

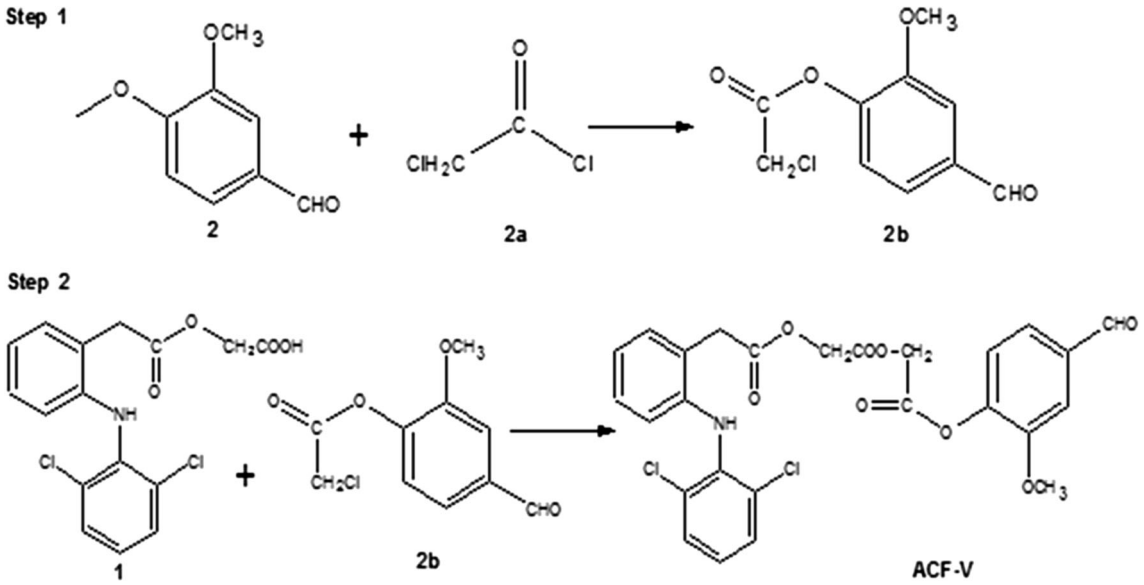


Scheme 1 Synthesis of aceclofenac–QTME mutual prodrug

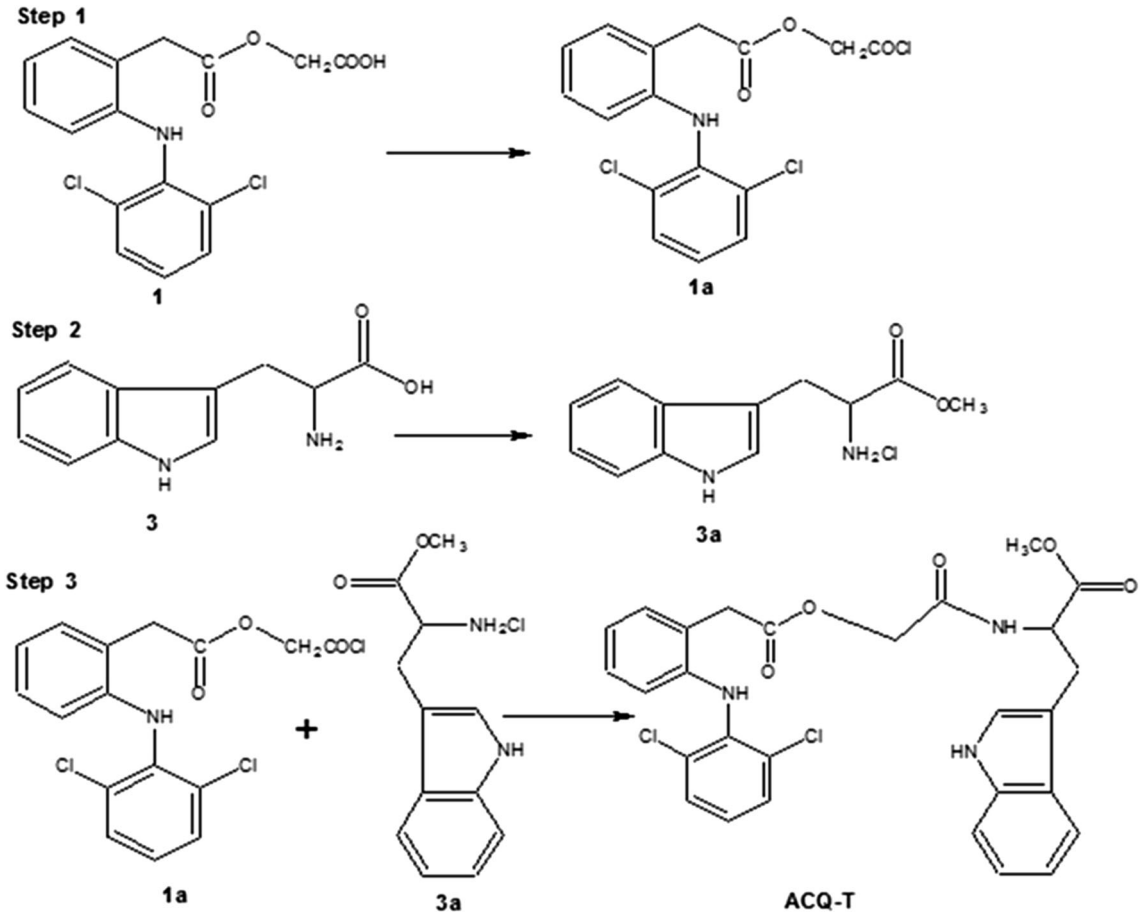
Synthesis of aceclofenac–vanillin mutual prodrug (Redasani and Bari, 2012)

Step 1: Synthesis of vanillin chloroacetyl derivatives: A mixture of appropriate antioxidant vanillin **(2)** (1.52 g, 0.01 M), tri ethyl amine (0.01 M) in dichloromethane (25 ml) was cooled in an ice salt mixture to −10 °C. To this, chloroacetyl chloride **(2a)** (0.01 M) in chloroform (25 ml) was added dropwise with constant stirring over a period of 1 h, maintaining the temperature constant. The reaction mixture was stirred further for 5 h, washed with HCl (5 %), sodium hydroxide (5 %) and finally with brine solution. The organic layer was dried over anhydrous sodium sulfate and filtered, and the solvent was removed under reduced pressure to obtain the corresponding antioxidant vanillin chloroacetyl derivative **(2b)**. It is then recrystallized from petroleum ether and ethyl acetate.

Step 2: Synthesis of aceclofenac–vanillin mutual prodrug (ACF-V): A mixture of appropriate antioxidant vanillin chloroacetyl chloride (0.01 M), aceclofenac (2.96 g, 0.01), TEA (0.01 M) and sodium iodide (0.01 M) in DMF (25 ml) was stirred over night at room temperature. The reaction mixture was poured into freshly crushed ice with stirring and extracted with chloroform. The combined organic layer was washed with sodium thio sulfate (2 %), HCl (5 %), sodium hydroxide (5 %) and finally with brine solution. The organic layer was dried over anhydrous sodium sulfate and filtered, and the solvent was removed under reduced pressure to obtain semisolid residue. The final product (ACF-V) was obtained as solids and recrystallized from petroleum ether and ethyl acetate.



Scheme 2 Synthesis of aceclofenac–vanillin mutual prodrug



Scheme 3 Synthesis of aceclofenac-L-tryptophan mutual prodrug

Characterization of 2-(4-formyl-2-methoxy-phenoxy)-2-oxoethyl 2-(2-(2,6-dichlorophenyl amino)phenyl)acetate (ACF-V): yellow needles (Pet Ether); (it was obtained as yellow solids); mp 209 °C; UV (MeOH) λ_{max} 287 (SGF), 333 (SIF), 299 (PBS); IR (KBr) ν_{max}: 3436 (NH Stretch), 1725 (ester), 1693, 1604 (aromatic stretch), 1308 (primary or secondary OH in plane bend), 1269 (CO ester stretch), 1194 (phenol, CO stretch), 922, 858, 822, 777, 739 (aromatic CH out of plane bend) cm^{-1}; ^{1}H NMR (DMSO): $\delta = 4$ (1H aromatic C–NH), 7.28 (3H, Ar–H), 7.53 (2H, C=O), 5.02 (methylene); ^{13}C NMR (DMSO): $\delta = 179.5$ (Ar–CH_2COO), 190.99 (CHO), 171 (OCH_2COO) 151.71 (ArC–OCH_3), 118–135 (Ar–CH_2COO), 60.85 (OCH_2 COO), 56.15 (OCH_3); mass (*m/z*) 487.06 [M] + (100); $C_{24}H_{19}Cl_2NO_6$; (calcd. 488.32); Anal. Calcd. for $C_{24}H_{19}Cl_2NO_6$: C,59.03; H, 3.92; N, 2.87; O,19.66. Found: C, 59.00; H, 3.82; N, 2.80; O, 19.15.

Synthesis of aceclofenac-L-tryptophan mutual prodrug (ACF-T) (Mishra et al., 2008)

Step 1: Synthesis of aceclofenac acid chloride: Aceclofenac **(1)** (17.70 g, 0.05 M) was dissolved in minimum amount of chloroform, and freshly distilled thionyl chloride (0.05 M, 6 ml) was added slowly to it. The mixture was refluxed for 15 h at 60–70 °C with continuous stirring on magnetic stirrer. The viscous liquid was immediately poured on petri dish and was vacuum-dried to give yellow-colored crude aceclofenac acid chloride **(1a)**.

Step 2: Synthesis of methyl ester hydrochlorides of amino acids: Freshly distilled (0.05 M, 6 ml) of thionyl chloride was slowly added to methanol (100 ml) with cooling, and L-tryptophan **(3)** (10.05 g, 0.05 M) was added to it. The mixture was refluxed for 6–8 h at 60–70 °C with continuous stirring on magnetic stirrer. Excess thionyl chloride and solvent were removed under reduced pressure, giving crude amino acid methyl ester hydrochloride. It was treated with 20 ml portion of cold ether at 0 °C until the excess of dimethyl sulfate was removed. The resulting solid product was collected and dried under vacuum. It was recrystallized from hot methanol by slow addition of 15–20 ml ether followed by cooling at 0 °C. The crystals were collected on next day and washed twice with ether–methanol mixture (5:1) followed by pure ether and dried under vacuum to give pure amino acid methyl ester hydrochloride **(3a)**.

Step 3: Synthesis of aceclofenac-L-tryptophan mutual prodrug (ACF-T): The step 3 involved the acylation of L-tryptophan ester with aceclofenac acid chloride and is carried out using Schotten–Baumann technique. Ice cold, aqueous sodium hydroxide solution (5 %) was taken in 250-ml beaker, and methyl ester of amino acid (0.05 M) was added to it. The reaction mixture was mechanically stirred for 30 min at room temperature, after which the beaker was transferred to an ice bath kept on mechanical stirrer, maintaining the temperature at 10 °C. Aceclofenac acid chloride (0.01 M, 3.72 g) was added in small portions with continuous stirring for 7–8 h. The solid that separated out was filtered using vacuum pump and dried. The crude prodrug was recrystallized from methanol to obtain amide prodrugs of aceclofenac (ACF-T).

Characterization of 2-(1-(isoindolin-1-yl)-2-methoxyethyl amino)-2-oxoethyl2-(2-(2,6-dichlorophenyl amino) phenyl)acetate (ACF-T): brown needles (from MeOH); (it was obtained as brown solids); mp 205 °C; UV (MeOH) λ_{max} 275 (SGF), 280 (SIF), 311 (PBS); IR (KBr) ν_{max}: 3421 (NH stretch), 2841 (methoxy methyl ether O-CH_3, CH stretch), 1726 (amide), 1607 (aromatic ring stretch), 1356, 1305, 1235 (primary or secondary, OH in plane bend), 1194, 1164, 1094, 1018 (aromatic C-H out of plane bend), 779, 743 (1, 2 disubstitution meta), 635, 601(alkyne C-H bend) cm^{-1}; ^{1}H NMR (DMSO): $\delta = 2$ (1 NH-amine), 4 (aromatic C-NH), 8.3 (secondary amine) 4.52 (1-CH), 3.86 (2H,OCH_3); ^{13}C NMR (DMSO): $\delta = 172.2$ (ester C=O), 168.2 (amide C=O), 139.6 (C-4 aromatic), 135.3 (C-1 aromatic), 128.9 (C-2 aromatic), 127 (C-3 aromatic), 59.3 (C-OCH_3); mass (*m/z*) 527.14 [M] + (100); $C_{27}H_{27}Cl_2N_3O_4$; (calcd. 528.43); Anal. Calcd. for $C_{27}H_{27}Cl_2N_3O_4$: C,61.37; H, 5.15; N, 7.95; O,12.11. Found: C, 61.15; H, 5.10; N, 7.91; O, 12.10.

Characterization of drugs and prodrugs

The various physicochemical properties such as purity, melting point, molecular weight, solubility, elemental and spectral analyses, partition coefficient and protein binding of ACF and their prodrugs ACFG, ACFV and ACFT were determined (Budavari and O'Neil, 1996; Indian Pharmacopoeia 1996; Sherma and Fried, 1996).

Thin-layer chromatography (TLC)

TLC was performed on pre-coated silica G plates using iodine vapor as detecting agent. The solvent system used for the study was mixture of acetone/chloroform/acetic acid/water (3:2:1:4).

Melting point determination

Melting point of drugs and prodrugs was determined by capillary fusion method using melting point determination apparatus.

Molecular weight determination

Mass spectrum of drugs and prodrugs was recorded to prove the exact mass or *M/Z* ratio, with field desorption

technique in magnetic sector of mass spectrometer, with an applied voltage of 70 eV for the fragmentation of molecular ions. Field desorption technique in mass spectroscopy was specially used for evaluation of peptide-type drugs.

Solubility studies

Solubility can be defined quantitatively as the concentration of the solute in a saturated solution at a certain temperature. Approximately 5 mg of drug was dissolved in 5 ml of each solvent at 37 ± 1 °C in glass test tubes. The solvents used were 0.1 N NaOH, 0.1 N HCl, 0.1 N KOH, methanol, ether, chloroform, acetone and water. Test tubes were gently shaken, and solubility was observed. In case of any observed insoluble fractions, the known amount of solvent was further added to ascertain the solubility of the compound. The same procedure was repeated for ACF as well as their prodrugs.

Elemental analysis

The elemental analysis was carried out to find the percentage of C, H and N in the prodrugs.

Spectral analysis

The characterization of drugs and anticipated structures of their prodrugs were confirmed by various spectral analyses using IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy. For UV absorption studies, the solution of ACF and their prodrugs were prepared in a concentration of 10–20 μg/ml and the absorbance was measured at a range of 200–400 nm.

Partition coefficient determination (Leo et al., 1971)

The partition coefficient of drugs and prodrugs was determined by shake flask method between *n*-octanol saturated with phosphate buffer (pH 7.4). A 20 mg of drug was weighed and dissolved in 20 ml octanol, and l0 ml phosphate buffer (pH 7.4) is added. The contents were thoroughly shaken for 24 h at room temperature followed by its transfer to a separating funnel. The octanol layer was dried under high vacuum, and the residue obtained was again dissolved in methanol (l0 ml). Two milliliters of this solution was further diluted to 100 ml with methanol. From this solution, an aliquot of 25 ml was withdrawn and was mixed with 4.5 phosphate buffer (pH 7.4). Volume was finally made to l00 ml by addition of methanol. The resulting solution of drug and prodrugs was analyzed, and the absorbance was measured spectrophotometrically at 230 nm.

Protein binding studies

A solution of the synthesized prodrug (10 mg/ml) was prepared in phosphate-buffered saline (PBS, pH 7.4). A total of 100 ml of this solution was placed in a beaker. The cellophane membrane (molecular mass cutoff in the range of 10,000–12,000 Da obtained from Hi-Media, India) was first washed with distilled with water and then with buffer solution (pH 7.4). It was tied at the opening end of a dialysis tube; the dialysis tube containing (6 %) egg albumin was dipped into the drug solution and covered. The whole assembly was placed on a magnetic stirrer and set at low revolutions per minute. The temperature was maintained at 37 ± 0.5 °C. After each 1 h, 1 ml of the PBS containing drug solution was replaced with fresh 1 ml of PBS. The withdrawn sample was further diluted with 1 ml phosphate buffer, and the concentration of the conjugate was estimated using a spectrophotometer at 230 nm.

Hydrolysis rate determination of prodrugs

In vitro hydrolysis studies of prodrugs were carried out in simulated gastric fluid (SGF) at pH 1.2, simulated intestinal fluid (SIF) at pH 7.4 and rat fecal matter at pH 7.4.

In SGF or SIF (Nielsenw and Bundgaard, 1988)

A solution of 10 mg of prodrug was prepared in 90 ml of SGF (pH 1.2) or SIF (pH 7.4). An aliquot of 15 ml of this solution was withdrawn repeatedly and kept in test tubes maintained at 37 ± 0.5 °C. At a definite time interval (0.5 h, 1–8 h), an aliquot was withdrawn from different test tubes and was transferred to microcentrifuge tubes, followed by addition of methanol to make up the volume. The tubes were placed in a freezing mixture in order to arrest further hydrolysis, followed by vortexing at high speed for 5 min. After vortexing, the tubes were centrifuged at high speed (3000 rpm) for 5 min. Five milliliters of clear supernatant obtained from each tube was measured by a spectrophotometer for the amount of free aceclofenac released after the hydrolysis of prodrugs in SGF or SIF at 230 nm.

In Rat Fecal Matter (Dhaneshwar and Gautam, 2012; Arun et al., 2011a, b)

Male albino rats weighing 105–115 g and maintained on a normal diet were used for the study. Six rats were asphyxiated using carbon dioxide, and fresh rat fecal matter was collected. Drug/prodrug was dissolved in phosphate buffer (pH 7.4), and volume was made up to 10 ml. Five milliliters of this solution was added to 10-ml volumetric flask, and volume was made up to 10 ml with isotonic phosphate buffer (pH 7.4). This was considered as the stock

solution. To each Eppendorf tube, 0.9 ml of the stock solution of prodrug and 0.1 ml of fecal matter were added and kept in incubator at 37 ± 1 °C in anaerobic conditions (5 % CO2). The first Eppendorf tube (0 min) was taken out and centrifuged at 10,000 rpm at 4 °C for different interval of time (0.5, 1, 2 up to 8 h). The drug content of the sample (20 µl) was analyzed spectrophotometrically at 230 nm. The release was observed over a period of 8 h.

The kinetics of hydrolysis was monitored by the increase in free drug concentration with time and the order of the reaction, and half-life ($t_{1/2}$) was also calculated. The rate of hydrolysis was calculated using the equation:

$$k_{\mathrm{H}} = \frac{2.303}{t} \log \frac{a}{a-x} \quad (1)$$

where k_{H} represents the hydrolysis constant, t is the time in min, a is the initial prodrug concentration, x is the amount of prodrug hydrolyzed and (a–x) is the amount of the remaining prodrug.

Pharmacological screening of drugs and prodrugs

Experimental animals

Wistar albino rats were randomly divided into six groups each of 6 rats, including a control and a standard group. The selected animals were housed in acrylic cages at standard environmental conditions at 25 ± 2 °C, relative humidity of 45–55 %, in a well-ventilated room maintained at 12: 12 h light/dark cycle, and fed with standard rodent diet and water ad libitum. All the animals were acclimatized for a week before experiment. All animal experiments were carried out according to the guidelines of the Committee for the Purpose of Control of Experiments on Animals and approval of the Institutional Animal Ethical Committee (Reg. No. 1195/ac/08/CPCSEA), Alshifa College of Pharmacy, Perinthalmanna, Kerala, India, was obtained.

Drugs as well as the synthesized prodrugs were evaluated for pharmacological screening (Swingle *et al.*, 1985; Arun *et al.*, 2011a, b) such as anti-inflammatory activity, ulcerogenicity, histopathological studies (Brodie *et al.*, 1970; Hersey and Sachs, 1995) and biochemical analysis (Shah *et al.*, 2013) such as determination of gastric wall mucous (Corne *et al.*, 1974), hexosamine assay (Blumenkrantz and Asboe-Hansen, 1976), antioxidant activity, lipid peroxidation measurement (Utley *et al.*, 1967), reduced glutathione assay (Ellman, 1959), catalase assay (Sairam *et al.*, 2001) and SOD assay (Piacham *et al.*, 2006).

Anti-inflammatory activity

The anti-inflammatory activity was determined by hind paw edema method using carrageenan (0.1 ml, 1 % w/v) as phlogistic agent. The mean increase in the volume of the right hind paw of rats was compared with control and standard. The percent inhibition of paw edema was calculated as

$$\text{Percentage inhibition} = (1 - V_s/V_c) \times 100 \quad (2)$$

where V_t—mean relative change in paw edema volume in test group, V_c—mean relative change in paw edema volume in control group.

Ulcerogenicity

Gastrointestinal toxicity of the prodrugs was measured and compared with the parent drug by measuring mean ulcer index. The mean ulcer index (UI) was calculated by severity of gastric mucosal lesions which are graded as grade 1: <1 mm erosions, grade 2: 1–2 mm erosions and grade 3: more than 2 mm erosions. The UI was calculated as

$$\begin{aligned} \mathrm{UI} = [1 \times &(\text{number of lesions of grade 1}) + 2 \\ &\times (\text{number of lesions of grade 2}) \\ &+ 3 \times (\text{number of lesions of grade 3})]/10 \end{aligned} \quad (3)$$

Histopathological studies

The histopathological studies of stomach of rats (100–200 g) were carried out using hematoxylin and eosin stain at Pathology Department, Al Shifa hospital, Perinthalmanna.

Biochemical Parameters

Determination of gastric wall mucus (GWM)

GWM was determined by modified procedure. The glandular segment of excised stomach was taken, weighed and transferred immediately to 10 ml of 0.1 % alcian blue solution. Tissue will be stained for 2 h in alcian blue and excess dye removed by two successive rinses with 10 ml of 0.25 mM/l sucrose, after 15 min then after 45 min. Dye complexed with the gastric wall mucus was extracted with 10 ml of 0.5 mM/l magnesium chloride which was intermittently shaken for 1 min at intervals of 0.5 h for 2 h. Four milliliters of blue extract was shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 rpm for 10 min and the absorbance of aqueous layer recorded at 580 nm. The quantity of alcian blue extracted per gram of wet glandular tissue was calculated.

Hexosamine assay

The gastric tissues were hydrolyzed in acidic medium, and the hydrolysate was neutralized with 3 N sodium

hydroxide and diluted to 10 ml with distilled water. Acetyl acetone solution was added to an aliquot (1 ml), mixed well and heated on a boiling water bath for 15 min avoiding evaporation. After cooling, ethanol (5 ml) was added to the mixture followed by Ehrlich's reagent. The mixture was diluted to 10 ml with 95 % ethanol and allowed standing for 30 min, and its absorbance was read at 530 nm.

Antioxidant activity

Preparation of tissue homogenate: Stomach was dissected out from the killed animal and rinsed with distilled water. The glandular portion of gastric mucosa was then homogenized into ice cold 0.15 M Tris–HCl (pH 7.4) to give 10 % w/v homogenate. The homogenate was then centrifuged at 15,000 rpm for 15 min at 4 °C in cooling centrifuge. Supernatant was used for determination of antioxidant activity.

Lipid peroxidation measurement (LPO)

The extent of lipid peroxidation was estimated by measuring the secondary lipid peroxidation product—thiobarbituric acid-reactive substance formed in the lipid peroxidation processes. In this, 0.2 ml of tissue homogenate, 0.2 ml of 8.1 % sodium dodecyl sulfate (SDS), 1.5 ml of 20 % acetic acid and 1.5 ml of 8 % TBA were added. The volume of the mixture was made up to 4 mL with distilled water and then heated at 95 °C in a water bath for 60 min. After incubation, the tubes were cooled to room temperature and final volume was made to 5 ml in each tube. The mixture of 5 ml of *n*-butanol/pyridine (15:1) was added, and the contents were vortexes thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min, the upper organic layer was taken and absorbance taken at 532 nm against the blank without the sample.

Reduced glutathione (GSH) assay

In this method, 5,5′-dithiobis-(2-nitrobenzoic acid) is reduced by SH groups of glutathione to form 1 M of 2-nitro-5-mercaptobenzoic acid per M of SH. The mercapto benzoic acid anion has an intense yellow color and used to measure SH groups. The supernatant (40 µl) was mixed with 400 µl Tris HCl and 3360 µl water. Then 0.2 ml (200 µl) DTNB solution was added, and absorbance was measured at 412 nm. Standard curve for GSH was prepared using glutathione.

Catalase (CAT) assay

CAT is a ubiquitous heme protein that reduces hydrogen peroxide to water. Hence catalase activity was determined by measuring decreasing absorbance of hydrogen peroxide. A total of 100 µl of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1 ml of freshly prepared 30 mM hydrogen peroxide. The decrease in absorbance was read at 240 nm for 3 min at interval of 30 s. The activity was calculated using extinction coefficient of H_2O_2 (0.041 µM/cm^2). Results were expressed as micromole of H_2O_2 utilized/min/gm tissue. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm.

Superoxide dismutase (SOD) assay

In this assay, 1.2 ml sodium pyrophosphate buffer (pH 8.3, 052 M), 0.1 ml phenazine methosulfate (186 µl), 0.3 ml of 300 µM nitro blue tetrazolium, 0.2 ml NADPH (750 µM) added to the 0.1 ml supernatant and incubated at 30 °C for 90 s, and then 0.1 ml glacial acetic acid was added and stirred with 4 ml *n*-butanol. It was allowed to stand for 10 min. The content was centrifuged, and butanol layer was separated. Then the absorbance of content was measured at 560 nm for 2 min at 30 s intervals

$$\text{SOD activity } (\%\text{inhibition}) = \frac{\Delta A_{\text{control}} - \Delta A_{\text{sample}}}{\Delta A_{\text{sample}}} \times 100 \quad (4)$$

Statistical analysis

Statistical analysis of the pharmacological activity of the synthesized prodrugs on animals was evaluated using a one-way analysis of variance (ANOVA). Student's *t* test was applied for expressing the significance, and the experimental data are expressed as mean ± SD (standard deviation).

Result and discussion

Characterization of aceclofenac prodrugs

The synthesized prodrugs ACF-Q, ACF-V and ACF-T were subjected to physicochemical characterization and are presented in Table 1. The thin-layer chromatographic results revealed a single spot, confirming purity of the drug and prodrugs. Also, the high partition coefficient of prodrugs in *n*-octanol/phosphate buffer suggests an increase in lipophilicity of prodrugs and may therefore lead to the

Table 1 Physicochemical characterization of prodrugs

Prodrug/mol. formula	Mol. Wt	Color	Melting point (°C)	Yield (%)	R_f value	Protein binding	Elemental analysis		
							Calculated (%)		Found (%)
ACF	354.2	White	149	–	0.79	92.0	C	54.20	54.18
$C_{16}H_{13}Cl_2NO_4$							H	3.67	3.62
							N	3.95	3.85
							O	18.06	18.02
ACF-Q	696.43	Brown	198	38.77	0.62	71.65	C	60.35	60.11
$C_{35}H_{31}Cl_2NO_{10}$							H	4.49	4.45
							N	2.01	1.98
							O	22.97	22.86
ACF-V	488.32	Yellow	209	81.81	0.58	64.0	C	59.03	59.00
$C_{24}H_{19}Cl_2NO_6$							H	3.92	3.82
							N	2.87	2.80
							O	19.66	19.15
ACF-T	528.43	Brown	205	73.92	0.77	68.05	C	61.37	61.15
$C_{27}H_{27}Cl_2N_3O_4$							H	5.15	5.10
							N	7.95	7.91
							O	12.11	12.10

higher absorption of drug through lipoidal cell membrane. All the prodrugs were synthesized with a good yield. Increased R_f value of prodrugs was observed in comparison with the parent drug, indicating higher lipophilicity of synthesized prodrugs. The increase in melting point of prodrugs can be attributed to the effect of high melting amino acid esters and thereby confirms the product formation. ACF was acidic with poor aqueous solubility and remained unionized in the gastrointestinal tract. As the pH of the gastric environment increases, the drug undergoes ionization in the alkaline medium and the solubility of drug may increase. The results of the solubility studies revealed overall superiority of aqueous and organic solubility of prodrugs than parent drug, indicating the former's lipophilic behavior.

The results of the elemental analysis of synthesized prodrugs were in all cases within ±0.4 % of the theoretical value and confirmed the desired structure. Characterization of prodrugs establishes that they had been formed in pure form and conforms to the expected properties. The lower protein binding of prodrugs than the corresponding parent drug might facilitate its availability for hydrolysis in various biological fluids. The results of partition coefficient showed that the major fraction of the prodrugs was partitioned toward the organic phase. High partition coefficient of synthesized prodrug as compared to the parent drug was

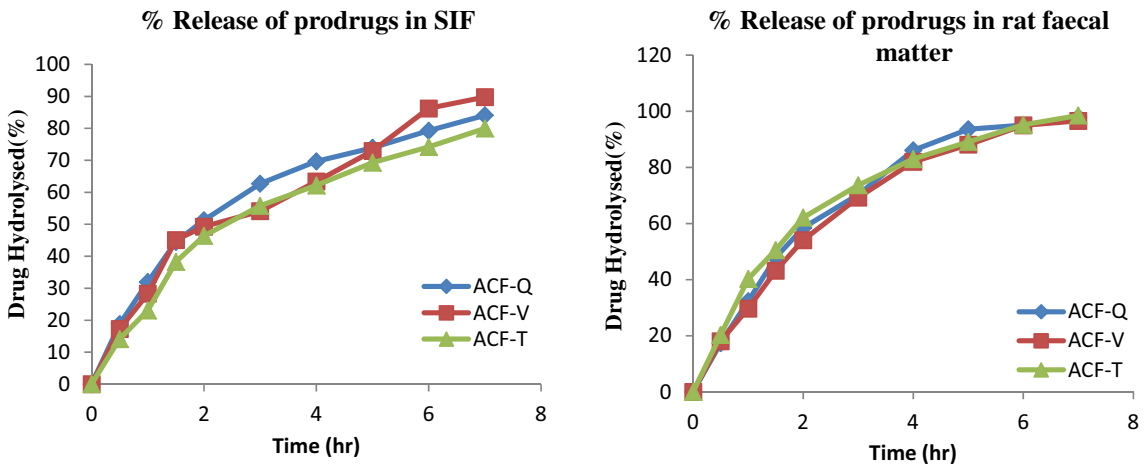


Fig. 1 Comparative percentage hydrolysis of aceclofenac prodrugs in SIF and rat fecal matter

observed, indicating the increased lipophilicity of the prodrugs. This may lead to the higher absorption of the compound through lipoidal cell membrane.

The IR spectra of prodrugs showed absorption bands at 3325–3350 cm^{-1} (–NH stretch), 1720–1737 cm^{-1} (C=O stretching of ester), 1700–1750 cm^{-1} (–NH deformation or amide I band) and 1650–1700 cm^{-1}(C–N stretching or amide band) which confirms the presence of ester group and formation of amide bond in ACF prodrugs, ester groups present in both ACF-Q and ACF-V prodrugs and amide groups present in ACF-T prodrug. In the IR spectrum of ACF, the above peaks were absent. Instead, peaks were observed at 1716.67 cm^{-1} (OH), 1255.35 cm^{-1} (CO stretching of COOH), 1417.83 cm^{-1} (OH bending of COOH), 2900 cm^{-1} (OH stretching of COOH) and 926.36 cm^{-1} (OH bending out of plane of COOH), which showed the acidic nature of ACF due to the presence of free carboxylic acid group.

^{1}H NMR spectra of prodrugs of ACF showed chemical shift (ppm) at 1.12–2.5 (CH_3 terminal peaks), 8.18–9.77 (NH) and 7.28–7.82 (multiplicity of relative number of different proton of benzene ring), confirming the formation of amide bond in the compounds. The ^{13}C NMR spectra of ACF prodrugs range from 150 to 175 and 160 to 165, thereby establishing the formation of amide and ester linkages, respectively. The absorption maxima of all prodrugs were observed to be higher than those of parent drug. It was confirmed by the shifting of wavelength from lower to higher due to the additional auxochrome of conjugated amino acid and antioxidant with ACF. Thus, the characterization establishes that the prodrugs had been formed in pure form and conforms to expected properties.

Hydrolysis studies of aceclofenac prodrugs

The in vitro hydrolysis studies were designed to mimic the entire gastrointestinal tract pH, and hence as a primary

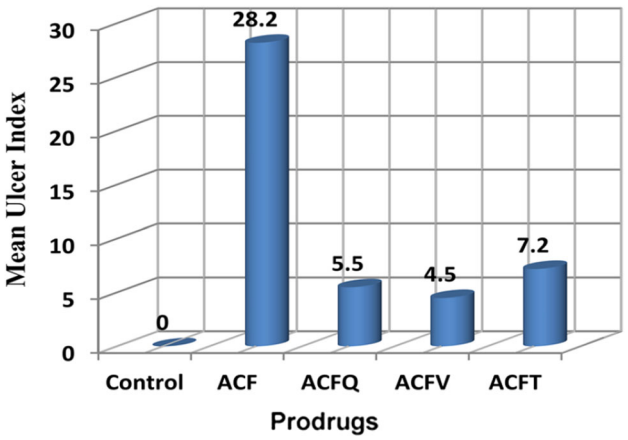


Fig. 2 Comparative ulcerogenicity of aceclofenac and its prodrugs

requirement, calibration curve in the experimental pH value related to the study was made. The minimum reversion was observed at gastric pH (SGF, pH 1.2), suggesting the stability of synthesized prodrugs in gastric pH, both in fasted and in fed state. The comparative analysis of the prodrugs of ACF performed in the SIF and rat fecal matter is shown in Fig. 1. The results showed that the amount of ACF regenerated on hydrolysis of ACF-Q, ACF-V and ACF-T in SIF was 84.11, 89.78 and 79.99, respectively. Similarly, respective amounts 97.85, 96.58 and 98.50 of AC were regenerated when the hydrolysis of ACF-Q, ACF-V and ACF-T was performed in rat fecal matter. This reveals that at higher pH values, i.e., in SIF representing intestine, the percentage reversion was significantly higher, thereby making the free drug available for absorption in the intestine. A much higher value was observed in rat fecal matter due to the enzyme-dependant hydrolysis taking place in colon. Also the process of reversion increases almost linearly with time at intestinal pH and physiological pH of blood. The kinetic studies revealed that all the prodrugs followed first-order kinetics

Table 2 Anti-inflammatory activity of aceclofenac and its prodrugs

Drug	Dose (mg/Kg)	Anti-inflammatory activity (%)[b]					
		1 h	2 h	3 h	4 h	5 h	6 h
ACF[a]	2.5	70.13 ± 0.21*[c]	69.00 ± 0.35*[c]	67.45 ± 0.93*[c]	64.15 ± 0.78*[c]	61.14 ± 0.16*[c]	59.55 ± 0.21*[c]
ACF-Q	3.5	61.15 ± 0.33*[c]	63.99 ± 0.87*[c]	65.11 ±-0.78*[c]	66.13 ± 0.71*[c]	67.01 ± 0.064*[c]	73.99 ± 0.023*[c]
ACF-V	3.8	63.77 ± 0.45*[c]	65.77 ± 0.55*[c]	68.11 ± 0.52*[c]	71.77 ± 0.65*[c]	72.77 ± 0.21*[c]	80.19 ± 0.71*[c]
ACF-T	3.6	62.65 ± 0.71*[c]	63.76 ± 0.87*[c]	65.87 ± 0.091*[c]	67.85 ± 0.97*[c]	69.88 ± 0.16*[c]	75.77 ± 0.33*[c]

[a] Normal control animals were administered orally 1 % CMC. [b] Values were expressed as mean ± SD of six observations. [c] Comparison between ACF Vs ACF-Q, ACF-V and ACF-T

* $p < 0.05$, statistical significant test comparison was done by one-way ANOVA followed by Dunnett's '*t*' test

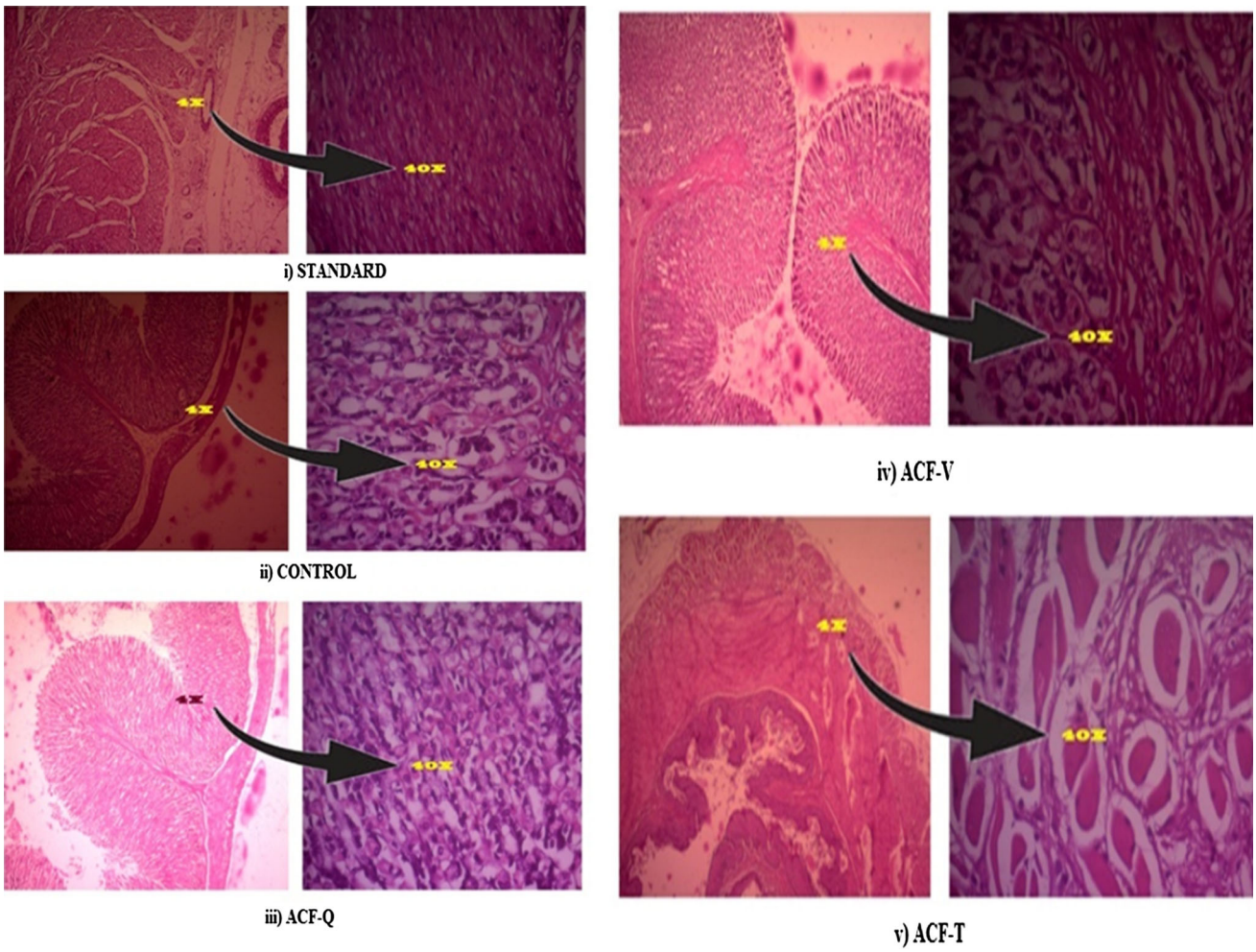


Fig. 3 Histopathological studies using aceclofenac and its prodrugs. **i** Standard aceclofenac, **ii** Control, **iii** ACF-Q, **iv** ACF-V, **v** ACF-T

and the $t_{1/2}$ of the prodrugs was observed to be at a higher value than that of the parent drug.

Pharmacological screening of drugs and prodrugs

The anti-inflammatory activity obtained after 1 and 6 h of administration of standard drug ACF was found to decrease from 70.13 to 59.55 %, while all prodrugs showed an increase in percentage anti-inflammatory activity as presented in Table 2. The maximum anti-inflammatory activity was observed at 6 h and remained practically constant up to 8 h. The results revealed that the increase in the anti-inflammatory activity of prodrugs with time is due to their higher bioavailability compared to the parent drug. The statistical significance was tested by one-way ANOVA followed by Dunnett's '*t*' test and showed that the anti-inflammatory activity of prodrugs was effective in comparison with ACF.

The mean ulcer index of standard drug ACF was found to be 28.2, while all the prodrugs showed relatively very less values as shown in Fig. 2. The minimized side effect obtained in the prodrugs might be due to the inhibition of direct contact of carboxylic acid group of the drug to the gastric mucosa which is mainly responsible for the damage (Fig. 3).

In histopathological studies, the gastric tissues were investigated microscopically and the tissue samples of control group rats showed normal histological findings. Microscopic investigation of ACF group revealed a focal erosive area in gastric mucosa and a zone (clear zone) in the basal regions of the gastric glands stained pale. This zone was parallel to the surface of the stomach lumen. In this zone, the structures of the gland were destroyed. They had disintegrated from the basal lamina and fallen into the lumen. The nuclei of these cells had become smaller and dense, and their cytoplasms were stained as dark eosinophilic bodies. Small hemorrhagic areas and patches of inflammatory cell infiltrations were present in the lumen of the glands and lamina propria. Normal histological findings were displayed for standard, control, ACF-Q, ACF-V and

Table 3 Biochemical properties of aceclofenac and its prodrugs

Groups	GWM (barrier mucus) μg/mg protein[b]	Hexosamine (μg/mL)[b]	LPO (nM MDA/mg protein)[b]	GSH (μM/100 g protein)[b]	Catalase (U/mg protein)[b]	SOD (% inhibition)[b]
Control[a]	285.68 ± 3.35	122.32 ± 4.63	4.38 ± 0.40	350.88 ± 45.65	26.05 ± 4.55	34.43 ± 7.43
ACF[c]	190.02 ± 7.14	136.44 ± 12.19	25.25 ± 0.95	412.67 ± 56.18	4.65 ± 1.78	36.09 ± 8.12
ACF-Q[c]	213.11 ± 2.45	165.59 ± 18.47	12.76 ± 0.96	331.14 ± 35.67	21.94 ± 4.86	28.98 ± 8.11
ACF-V[c]	209.87 ± 1.98	150.87 ± 19.09	10.98 ± 0.87	327.78 ± 48.98	20.87 ± 3.90	25.12 ± 7.90
ACF-T[c]	215.23 ± 1.87	169.17 ± 15.78	19.78 ± 0.76	313.16 ± 42.12	19.85 ± 3.12	27.76 ± 7.99

[a] Normal control animals were observed. [b] Values were expressed as mean ± SD of six observations. [c] Comparison between ACF, ACF-Q, ACF-V and ACF-T

* $p < 0.05$, statistical significant test comparison was done by one-way ANOVA followed by Dunnett's '*t*' test

ACF-T, revealing that the prodrugs did not produce any ulceration in the gastric region. The percentage protein binding of prodrugs ACF-Q, ACF-V and ACF-T was found to be 71.50, 64 and 68 %, respectively, which was lower when compared to the standard drug, whose value is 92 %. The low protein binding of the prodrugs increases its availability for hydrolysis in plasma, and the required dose will be lower.

The effect of ACF and its prodrugs was studied on various peripheral markers such as GWM, hexosamine, proteins and oxidative stress parameters including LPO, GSH, CAT and SOD activity as presented in Table 3. GWM content showed that there is significant deduction in GWM when ACF was administered while the prodrugs maintained its content. Hexosamine and protein concentration got enhanced when taking ACF-Q, ACF-V and ACF-T when compared with that of the one administrated with ACF. The studies revealed that GWM, hexosamine and protein concentration provide cytoprotection. LPO levels were found to be significantly increased in ACF-treated group than control, indicating gastric damage. However, lower LPO levels of prodrugs were observed, exhibiting protective effect against gastric damage that may be due to the inhibition of lipid peroxidation and cell damage. GSH level was observed more with ACF than control, while prodrugs significantly reduced GSH. Decreased level of SOD indicates increased generation of oxygen radical and increased gastric mucosal injury. Catalase activity was determined by measuring decreased absorbance of hydrogen peroxide. Decreased level of catalase indicates increased gastric damage. The studies suggested that ACF-Q, ACF-V and ACF-T exhibited significant activity as compared to ACF.

Conclusion

The study attempted to develop synthetic routes for the prodrug structures of ACF reacted with flavonoid, antioxidants and amino acids and evaluated their capabilities to improve site specificity and reduce gastrointestinal toxicity by maintaining the desired pharmacological properties. The prodrugs were synthesized and characterized by modern analytical techniques. The study also determined the important properties of synthesized prodrugs such as protein binding, lipophilicity and hydrolytic behavior in various simulated fluids for the development of safe and effective drugs. The prodrugs were observed to be chemically stable and biolabile. These prodrugs exhibited retention of anti-inflammatory activity along with reduced ulcerogenicity as compared to ACF. Histological studies showed that the prodrugs do not produce any ulceration in the gastric region. They also possess less protein binding and better absorption than parent drug and show good result in all the biochemical parameters. The study proved that prodrug approach could successfully attain the goal of minimized gastrointestinal toxicity by improving the desired physicochemical properties and pharmacological activities of the drug.

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