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Current Medicinal Chemistry, XXXX, *XX*, 1-00

RESEARCH ARTICLE

Mechanochemical Synthesis of Diclofenac Conjugates with Glucosamine and Chitosan Exhibiting COX-2 Selective Ulcer Safe Anti-inflammatory Activity

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Abstract: ***Introduction*:** Non-steroidal anti-inflammatory drugs are associated with severe gastrointestinal irritation upon prolonged use, largely due to their carboxylic (- COOH) functional group.

***Aim*:** To address this issue, we aimed to synthesize diclofenac conjugates with glucosamine and chitosan, converting the -COOH group into an amide (-CONH-) *via* a mechanochemical, environmentally friendly method.

***Methods*:** In this study, diclofenac acid was first converted to its acid chloride using thionyl chloride under mechanochemical conditions and subsequently reacted with glucosamine base and chitosan. The resulting conjugates were evaluated for anti-inflammatory activity through the rat-paw edema test, along with ulcerogenicity, COX inhibition assays, and cardiovascular assessment.

***Results*:** The mechanochemical approach provided high yields (>90%) and resulted in conjugates that significantly reduced paw edema (62.3 ± 2.3% for diclofenac-glucosamine and 58.5 ± 1.6% for diclofenac-chitosan) compared to diclofenac sodium (49.0 ± 1.3%) after 5 h. Notably, the conjugates were ulcer safe, as no gastric lesions were observed, unlike the multiple lesions detected in animals treated with diclofenac sodium. Both conjugates also demonstrated a high degree of COX-2 selectivity and cardiovascular safety.

***Conclusion*:** This study highlights the potential of mechanochemical synthesis for efficient amide formation, avoiding the need for hydroxyl group protection.

ARTICLE HISTORY

Received: September 10, 2024
Revised: November 09, 2024
Accepted: November 21, 2024

DOI:
10.2174/0109298673352652241217090558

Keywords: Diclofenac sodium, glucosamine, chitosan, conjugates, bio-conjugates, NSAIDs.

1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed to manage both acute and chronic pain associated with arthritis and other conditions [1]. Diclofenac (Fig. **1**) is one of the most frequently prescribed NSAIDs; in the United States alone, more than ten million prescriptions are issued each year [2]. Arthritis patients are major consumers of NSAIDs on a long-term basis [3].

Diclofenac, (2-[2-(2,6-dichloroanilino) phenyl] acetic acid), belongs to the phenylacetic acid class of drugs. It was first synthesized by Sallmann and Pfister and introduced by Novartis in 1973 [4]. Diclofenac is a potent non-selective inhibitor of cyclooxygenase (COX) enzymes [5], with a stronger inhibitory effect on COX-2 than on COX-1 [6], which probably contributes to its widespread use. In addition to its anti-inflammatory effects, diclofenac also influences platelet activation and aggregation [7].

Ideally, pain-relieving drugs should selectively inhibit the COX-2 enzyme to minimize adverse effects. Long-term NSAID use, however, is linked to

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0929-8673/XX $65.00+.00

an increased risk of gastrointestinal (GI) irritation that can lead to ulcers, as well as cardiovascular (CV) complications [8]. Similarly, diclofenac use is associated with GI discomfort, CV issues, and hepatotoxicity [9]. It has been linked to a heightened risk of atrial fibrillation, ischemic stroke, heart failure, myocardial infarction, and cardiac death across genders and age groups, even at low doses [10].

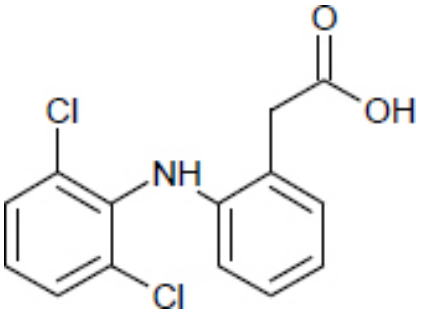


Fig. (1). Structure of diclofenac.

To address these challenges, advanced materials such as genetically engineered cellular nanovesicles [11], biomimetic self-propelled nanomotors [12], and functionalized bionanomaterials [13] are being developed for targeted therapies in cancer, inflammation, cardiovascular diseases, Parkinson's disease, and other degenerative conditions. These innovations hold great promise for overcoming the limitations associated with traditional NSAIDs.

GI erosion and bleeding are the most common toxic side effects, even at low prophylactic doses. It is estimated that around 50% of patients using NSAIDs long-term may develop mucosal damage in the small intestine [14]. GI toxicity is believed to result from the local irritation caused by the carboxylic acid functionality when it comes into direct contact with GI mucosal cells [15]. However, only one report [16] contests this hypothesis. To mitigate the effects of the carboxylic acid group, several approaches have been adopted [17]. One such approach involves minimizing GI toxicity without compromising anti-inflammatory efficacy by combining NSAIDs with agents that mask the effects of the carboxylic acid group [18]. In this context, several NSAID prodrugs, including ester and amide derivatives, have been prepared and evaluated [19, 20]. Such conjugations *via* amide linkages have been achieved in solution using diclofenac acid chloride. The chlorination of diclofenac has been attempted under three conditions [21] as: i) reflux with an excess of thionyl chloride for 2 h, resulting in degradation of diclofenac; ii) reaction with oxalyl chloride in methylene chloride at room temperature up to 72 h, which did not yield acid chloride; and iii) the use of DMF as catalyst in the oxalyl chloride reaction yielding 99% acid chloride. We report a green method (mechanochemical) for the synthesis of diclofenac acid chloride without the use of any solvent or catalyst. This method was also employed to form an amide linkage between the drug and glucosamine or chitosan in the present work. The choice of glucosamine and chitosan is significant due to their roles in the management of osteoarthritis.

Glucosamine (Fig. **2**), a natural amino-saccharide found in the body is a substrate for the biosynthesis of proteoglycans, which maintain the structural and functional integrity of cartilage having chondro-protective properties. Glucosamine lessens proteoglycan loss, delays cartilage deterioration and joint-space constriction, and ameliorates pain associated with arthritis. It has been reported that glucosamine exhibits anti-inflammatory effects by reducing the levels of pro-inflammatory mediators, such as tumor necrosis factor-alpha, interleukin-1, and interleukin-6, in addition to its chondro-protective properties [22]. It also minimizes cell death and improves the anabolic-catabolic balance of the extracellular cartilage matrix by producing anti-inflammatory mediators and proteases [23].

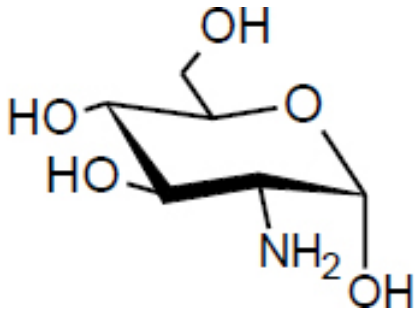


Fig. (2). Structure of glucosamine.

Glucosamine has an amine group, which can form an amide linkage with the carboxylic acid of NSAIDs. A couple of studies reported that diclofenac conjugated with per-*O*-acetylated glucosamine exhibits increased COX inhibition with enhanced selectivity towards COX-2 [1, 24]. To the best of our knowledge, there is no study reporting the conjugation of diclofenac with unacetylated glucosamine and its *in vivo* anti-inflammatory activity. The apparent reason for using per-*O*-acetylated glucosamine for conjugation with NSAIDs is to achieve amidation after protecting the hydroxyl groups therein. In the present work, we expected to achieve amide bond formation without protecting the hydroxyl groups by the mechanochemical method.

Chitosan is a linear polysaccharide of β-(1-4)-linked,2-amino-2-deoxy-glucopyranose (Fig. **3**) [25]. It

exhibits mild anti-inflammatory and antioxidant effects, which can help reduce the symptoms of osteoarthritis, a condition marked by inflammation and oxidative stress in the joints. Additionally, chitosan promotes cartilage repair and regeneration, which is crucial for managing osteoarthritis. It supports the growth of chondrocytes (cartilage cells) and the synthesis of the extracellular matrix, both of which contribute to joint health [26-28].

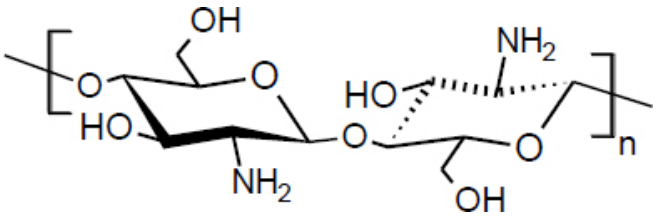


Fig. (3). Structure of deacetylated chitosan.

The primary amine group, in chitosan, is available for amide formation like glucosamine. Keeping in view the important chemical and pharmacological properties of glucosamine and chitosan, the objectives of the present work were set to: i) mechanochemically synthesize the conjugates of diclofenac with unacetylated glucosamine and chitosan and ii) investigate ulcer-safe anti-inflammatory activity and iii) enhance COX-2 selectivity. To the best of our knowledge, diclofenac has not been covalently attached to these materials, however, chitosan has been used as an excipient in an oral formulation for delivery of diclofenac [29]. There is only one report [30] claiming the synthesis of diclofenac-glucosamine prodrug using methanol as solvent. Under the conditions used by the authors, the formation of methyl ester of diclofenac by the reaction of diclofenac acid chloride with an abundant amount of methanol is unavoidable and the formation of the conjugate becomes questionable.

We hypothesize that the conjugates will provide the desired anti-inflammatory effect with enhanced COX-2 selectivity, without causing gastric irritation or cardiovascular complications, and will also offer synergistic benefits from glucosamine and chitosan.

2. MATERIALS AND METHODS

2.1. Materials

Diclofenac, chitosan (medium molecular weight ~6×10^4 g mol^{-1}, DDA ≥75%, Prod. #448877), glucosamine HCl, thionyl chloride (≥99%), methanol, diethyl ether, dimethyl sulfoxide (DMSO), DMSO-d_6 and sodium bicarbonate were procured from Sigma-Aldrich, USA. The spray solvents used for desorption electrospray ionization-mass spectrometry (DESI-MS) analysis were: methanol (Sigma-Aldrich), formic acid (Sigma-Aldrich), and dimethyl sulfoxide (DMSO, Fisher Scientific, Waltham, MA). All the materials were of analytical grade and used without further purification. Distilled water was used throughout this work.

2.2. Syntheses and Characterization

The glucosamine base was prepared by neutralizing an aqueous solution of glucosamine HCl with sodium bicarbonate. The conjugates with diclofenac were then synthesized using a mechanochemical technique. In this method, physical grinding promotes intimate mixing of the reactants, increasing the reaction rate by maximizing the contact surface area between them. The products were characterized using elemental analysis, Fourier-transform infrared (FT-IR) spectroscopy, electronic spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, and thermal analysis.

Elemental analysis (CHN) was performed using a CHNS-161 Analyzer (LECO Corporation, USA). The FT-IR spectra were recorded in the range of 4000-400 cm^{-1} with a resolution of ≤ 2 cm^{-1} in ATR mode, by placing a small amount of the sample on the diamond crystal of an FT-IR Spectrometer (Agilent Cary 630). Electronic spectra in the 200-800 nm range were obtained using a Varian Cary® 50 UV-Vis Spectrophotometer (Agilent Technologies, USA) in DMSO (1 mg mL^{-1}), with DMSO as the reference. DMSO was selected as the solvent because chitosan is soluble in it. DESI-MS Mass technique was used to determine the molar mass; this technique is suitable for mechanochemically synthesized products [31]. LCQ Fleet quadrupole ion trap (Thermo Scientific, San Jose, CA, USA) mass spectrometer was used for this.

The NMR spectra were recorded on Bruker 600 MHz NMR instrument (Avance Neo Bruker) using DMSO-d_6 as the solvent. Thermogravimetric analysis (TGA) of the conjugates was conducted on a thermal analyzer (SDT Q600, TA Instruments, USA) under a nitrogen flow at 15°C min^{-1} from ambient temperature to 1000°C using platinum cups.

2.2.1. Synthesis of Diclofenac Acid Chloride from Diclofenac Sodium

Diclofenac (1.00 g, 3.4 mmol) was mixed with thionyl chloride (1.0 mL, 13.7 mmol) and ground together by use of mortar and pestle. During the grinding

process, the reaction proceeded and the evolution of HCl and SO_2 gases was witnessed. Grinding was continued until gas evolution ceased, which was confirmed using an ammonia-dipped rod test. This ensured that all the diclofenac had reacted with the thionyl chloride and that the excess thionyl chloride was driven off. The nearly dry product was then washed with ether to remove any remaining traces of thionyl chloride, resulting in a fully dry product. Ether was chosen because it is non-reactive towards acyl chlorides and is a good solvent for removing organic impurities. UV-Vis absorptions (nm) in DMSO: 282 (π-π*). DESI-MS: $[C_{14}H_{10}Cl_3NO]^+$ (m/z found 314.5898 calculated 314.590); yield 92.3%. FT-IR (ATR) absorptions (cm^{-1}): 3076 (N-H str), 2884 (C-H str), 1692 (C=O str), 1603 (O=C-NH- str) 1157 (C-N str), 740 (C-Cl str), 662 (ring C=C bend). ^{1}HNMR in DMSO-d_6 δ (ppm) w.r.t. TMS: 3.7 (2H, s), 6.3 (1H, ddd, *J* = 8.1, 1.2, 0.6 Hz), 6.7 (1H, ddd, *J* = 7.9, 7.5, 1.2 Hz), 7.0-7.2 (3H, 7.08 (ddd, *J* = 8.1, 7.5, 1.3 Hz), 7.2 (ddd, *J* = 7.9, 1.3, 0.6 Hz), 7.2 (t, *J* = 7.9 Hz)), 7.5 (2H, dd, *J* = 7.9, 1.6 Hz), 9.3 (1H, s); ^{13}CNMR: 40.1 (1C, s), 120.2 (1C, s), 125.1 (2C, s), 128.6-128.5 (2C, 128.6 (s), 128.5 (s)), 129.0-129.6 (5C, 129.1 (s), 129.2 (s), 129.2 (s), 129.5 (s)), 139.6 (1C, s), 144.1 (1C, s), 172.7 (1C, s).

2.2.2. Preparation of Glucosamine Base from Glucosamine Hydrochloride

Glucosamine HCl (2.00 g, 9.23 mmol) was dissolved in water (10 mL). Sodium bicarbonate solution (0.78 g, 9.23 mmol in 2 mL H_2O) was added dropwise with continuous stirring into the glucosamine HCl solution until effervescence due to the evolution of carbon dioxide ceased. The resultant solution was dried at ~40°C in a rotary vacuum evaporator followed by freeze-drying. Elemental analysis for $C_6H_{13}NO_5$, % found (calculated): C 40.00 (40.22), H 7.14 (7.31), N 7.85 (7.82). DESI-MS: $[C_6H_{13}NO_5]^+$ (m/z found 179.1720, calculated 179.1720); melting point 149.9°C (lit. 150°C); yield 85.6%. UV-Vis absorptions (nm) in DMSO: 220, 270 (π-π*). FT-IR (ATR) absorptions (cm^{-1}): 3348 (O-H str), 3293 (N-H str), 1615 (NH_2 cissor), 1094 (sec O-H str). ^{1}HNMR in DMSO-d_6 δ (ppm) w.r.t. TMS: 2.7 (1H, dd, *J* = 2.7, 2.2 Hz), 3.0-3.2 (2H, 3.05 (dd, *J* = 2.7, 2.2 Hz), 3.1 (t, *J* = 2.2 Hz)), 3.5 (1H, td, *J* = 4.7, 2.7 Hz), 3.8 (2H, d, *J* = 4.7 Hz), 4.8 (1H, d, *J* = 2.7 Hz), 8.3 (2H, s); ^{13}CNMR: 55.2 (1C, s), 62.4 (1C, s), 71.2 (1C, s), 72.6 (1C, s), 78.1 (1C, s), 89.7 (1C, s).

2.2.3. Synthesis of Diclofenac-glucosamine Conjugate

Diclofenac acid chloride (0.629 g, 2.0 mmol) and glucosamine (0.358 g, 2.0 mmol) were transferred to a mortar; the mixture was ground until the HCl gas ceased to evolve. The product was washed with methanol and ether to get the dry product. Elemental analysis for $C_{20}H_{22}Cl_2N_2O_6$, % found (calculated): C 52.56 (52.53), H 4.78 (4.85), N 5.99 (6.13). DESI-MS: $[C_{20}H_{22}Cl_2N_2O_6]^+$ (m/z found 457.3041, calculated 457.3040); melting point: 190-192°C; yield: 98.8%. UV-Vis absorptions (nm) in DMSO: 220 (amide), 280 (π-π* in the benzene ring). FT-IR (ATR) absorptions (cm^{-1}): 3346 (O-H str), 3294 (N-H str), 1693 (C=O str), 1156 (C-N str), 1094 (sec O-H str), 742 (C-Cl str), 661 (ring C=C bend). ^{1}HNMR in DMSO-d_6 δ (ppm) w.r.t. TMS: 3.1 (1H, dd, *J* = 2.7, 2.2 Hz), 3.3 (1H, t, *J* = 2.2 Hz), 3.5 (1H, td, *J* = 4.6, 2.7 Hz), 3.6-3.8 (4H, 3.71 (s), 3.7 (s), 3.8 (d, *J* = 4.6 Hz)), 3.9 (1H, dd, *J* = 2.7, 2.2 Hz), 5.0 (1H, d, *J* = 2.7 Hz), 6.3 (1H, ddd, *J* = 8.1, 1.2, 0.6 Hz), 6.7 (1H, ddd, *J* = 7.9, 7.5, 1.2 Hz), 7.0-7.1 (2H, 7.06 (ddd, *J* = 7.9, 1.3, 0.6 Hz), 7.0 (ddd, *J* = 8.1, 7.5, 1.3 Hz)), 7.2 (1H, t, *J* = 7.9 Hz), 7.5 (2H, dd, *J* = 7.9, 1.6 Hz); ^{13}CNMR: 40.3 (1C, s), 53.3 (1C, s), 62.3 (1C, s), 71.0 (1C, s), 71.8 (1C, s), 78.0 (1C, s), 93.2 (1C, s), 120.0 (1C, s), 125.1 (2C, s), 128.3-128.5 (2C, 128.6 (s), 128.6 (s)), 129.0-129.3 (5C, 129.2 (s), 129.3 (s), 129.3 (s), 129.3 (s)), 139.8 (1C, s), 144.2 (1C, s), 169.0 (1C, s).

2.2.4. Synthesis of Diclofenac-chitosan Conjugate

Diclofenac acid chloride (0.5 g) and chitosan (0.1 g) were transferred to a mortar, the mixture was ground until the HCl gas ceased to evolve. The product was washed as above to get the dry product. Elemental analysis, % found (calculated on the basis of 100% substitution): C 51.40 (53.31), H 4.20 (4.62), N 7.50 (7.22). Melting point (decomp.) 220-221°C; yield 95.1%. UV-Vis absorptions (nm) in DMSO: 240 (amide), 280 (π-π* in the benzene ring). FT-IR (ATR) absorptions (cm^{-1}): 3442 (O-H str), 3294 (N-H str), 1693 (C=O str), 1650 (amide str). 1156 (C-N str), 1094 (sec O-H str), 742 (C-Cl str), 663 (ring C=C bend).

2.2.5. Stability Study of the Conjugates

The Diclofenac-glucosamine and Diclofenac-chitosan conjugates were subjected to stability study for six months. The samples were stored at 40 ± 1°C with equilibrated relative humidity (ERH) 75% in the dark for six months. A drug (diclofenac) assay was performed spectrophotometrically at 230 nm at different intervals of time for six months.

2.2.6. In Vitro Release of Diclofenac from the Conjugates

In vitro release of diclofenac from the synthesized conjugates was studied in simulated gastric fluid (SGF, Sigma-Aldrich, USA) and at pH 7.4 (phosphate buffered saline, PBS, Sigma-Aldrich, USA) using USP dissolution apparatus for 72 h. The assay was performed spectrophotometrically at 230 nm.

2.3. Anti-Inflammatory Activity

The synthesized conjugates were subjected to an acute anti-inflammatory test using a carrageenan induced rat paw edema model. The protocol used in this work was approved by the Institutional Review Board of Forman Christian College, Lahore (vide #IRB-412/03-2023 dated March 20, 2023) [32]. The carrageenan-induced rat paw edema test is considered a convenient method for assessing the anti-inflammatory activity of drug molecules [33], was employed to compare the anti-inflammatory activity of the synthesized diclofenac conjugates with glucosamine and chitosan.

The values are expressed as mean ± SD; the statistical significance was determined by comparing *p*-values against the saline group using one-way ANOVA followed by a post-hoc Tukey test.

2.3.1. Animals and Treatments

Healthy male Albino Wistar adult rats (300-350 g) were procured from Tollinton Market, Lahore. The animals were individually housed in polypropylene cages in the animal house of the Department of Pharmacy, Forman Christian College, Lahore. They were kept at 25 ± 2°C and 50 ± 3% relative humidity with a photo schedule of successive 12 h light and dark cycles. They were acclimated about a week prior to treatment and fed a regular pellet diet with water *ad libitum*.

The animals were randomly divided into four groups (of 5 each): positive control, negative control, and treatment groups A, B, C, and D. The positive control group received diclofenac sodium (10.0 mg kg^{-1} b. w. in terms of diclofenac) in 1 mL of carboxymethyl cellulose suspension (0.5% in water), the negative control received 1 mL of carboxymethyl cellulose suspension (0.5% in water), the treatment group A received the synthesized diclofenac-glucosamine conjugate (10.0 mg kg^{-1} b. w. in terms of diclofenac) in 1 mL of carboxymethyl cellulose suspension (0.5% in water) and the treatment group B received diclofenac-chitosan conjugate (10.0 mg kg^{-1} b. w. in terms of diclofenac) in 1 mL of carboxymethyl cellulose suspension (0.5% in water). The treatment groups C and D received equivalent doses of diclofenac sodium physically mixed (without grinding) with glucosamine and chitosan, respectively. The drugs were administered orally 1 h before the carrageenan injection. The edema was induced by injecting a freshly prepared carrageenan solution (0.1 mL, 1% w/v in sterile water) into the plantar side of the left hind paw of each rat [34]. The paw volume was measured (mL) by the plethysmometer (built in-house having an accuracy of 0.01 mL and a resolution of 0.01 mL) before and after 0, 1, 2, 3, and 5 h of the carrageenan injection. The paw volumes at 5 h were compared ($p<0.05$) among the groups and percent (%) inhibition was calculated by the formula $(V_c-V_t)/V_c\times100$, where V_t = Average volume of the paw at 5 h in the treatment group and V_c = Average volume of the paw of the negative control group.

2.3.2. Ulcerogenic Activity

The ulcerogenic effects of the conjugates were determined using the rat stress model [35]. For this, male Albino Wistar rats (330-400 g) were randomly divided into four groups (of 5 each) with the dosing regimen as described for the anti-inflammatory test. The animals were fasted for 24 hours prior to dosing and sacrificed 4 hours after administration of the dose. The rats were anesthetized with ketamine/xylazine mixture (75/2.5 mg kg^{-1} b.w.) *via* the intra-peritoneal route before sacrificing. After euthanasia, the stomach was removed along the greatest curvature, rinsed with normal saline and the gastric mucosa was examined for lesions (ulcers) using a magnifying glass. The ulcerogenic activity was determined by counting the gastric lesions as ulcer score (US) and calculating the ulcer index (UI). The severity was scored as: normal gastric mucosa - 0, vascular congestions - 1, one or two lesions - 2, more than 5 small lesions - 3, large (> 2 mm) 2-3 lesions - 4, and lesions with perforation of mucosa - 5. The results were reported as the ulcer index (UI) calculated by the formula UI = (ulcerated area/total stomach area) × 100.

2.3.3. COX Inhibitory Activity

The COX inhibitory activity of the conjugates was determined strictly following a reported method [1] using COX Fluorescent Inhibitor Screening Assay Kit 700,100 (Cayman Chemical, Ann Arbor, MI, USA). The solutions were prepared in DMF. Celecoxib was used as the COX-2 selective standard. The results were reported in terms of mean IC_{50} (μM) ± SD values (n = 3).

2.3.4. Pharmacokinetics

Six rats received a dose of 2 mg kg^{-1} of diclofenac sidium *via* oral gavage; the dosing volume was 2 ml kg^{-1}. Blood samples (1 ml each) were drawn from the orbital venous plexus at baseline (0 h) and at intervals of 2 min, 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 8 h following administration. The samples were collected in dipotassium-EDTA tubes and centrifuged at 2,500 × g for 10 min at 4°C. Plasma was subsequently separated and stored at -80°C before it was analyzed spectrophotometrically at 230 nm for drug assay.

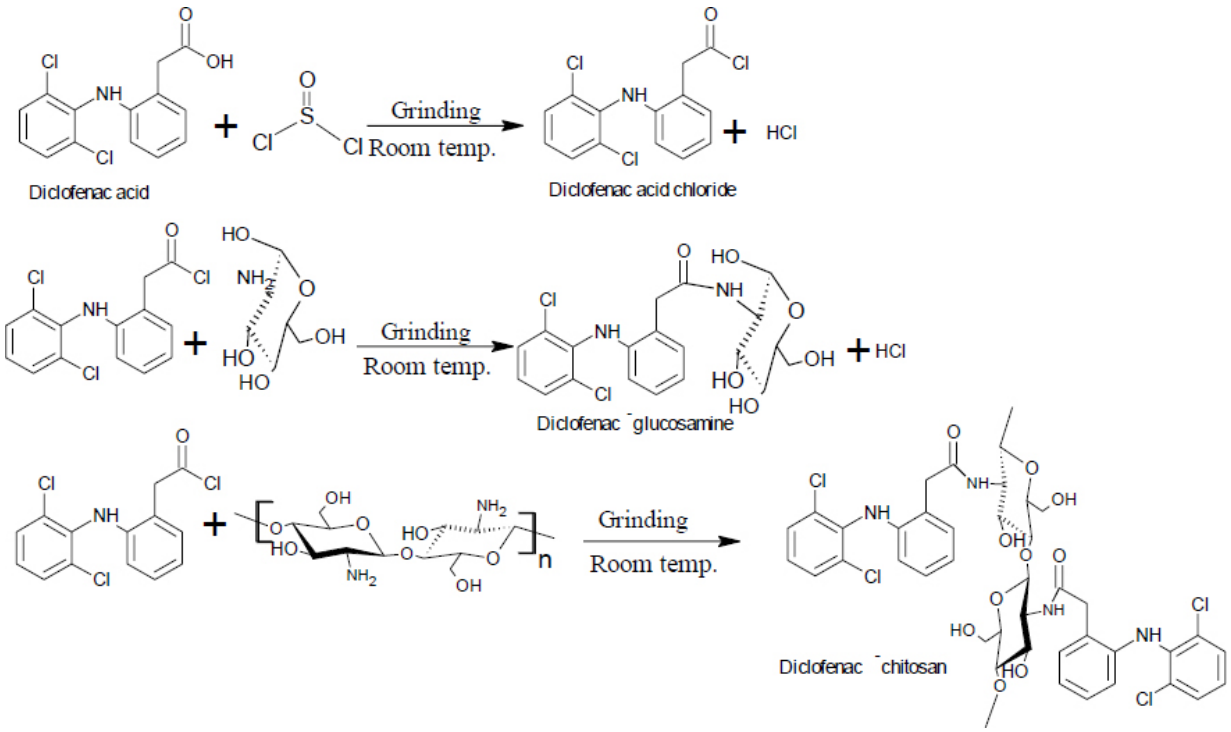
2.3.5. Electrocardiography

Cardiovascular complications in rats can be studied by various experimental models. Measures of blood markers like D-dimer, fibrinogen, and pro-inflammatory cytokines (*e.g.*, IL-6, TNF-alpha) are good indicators of thrombotic activity. Here we used electrocardiography (ECG) [36] as the basic tool to identify thrombotic events affecting cardiac or pulmonary function. PowerLab (ADInstruments Inc., USA) with appropriate electrode needles (Ag/AgCl electrodes) was used. Isoflurane was applied as anesthesia to keep the rat still. The animal was positioned on its back on an insulating surface to minimize electrical noise and interference. The electrodes were placed on the right forelimb, left forelimb, and left hindlimb. Recording was started with the animal at rest and baseline data was captured for comparison. Then recording was carried out for 10 min at a high sampling rate (1 kHz) to capture the rapid heart rate. P wave, QRS complex, T wave, and heart rate variability were analyzed. The MS, FT-IR and NMR spectra of diclofenac, glucosamine, chitosan and the conjugates have been provided as Supporting Supplementary Material (Fig. **S1-S16**).

3. RESULTS AND DISCUSSION

3.1. Syntheses and Characterization

Diclofenac was converted to diclofenac acid chloride by grinding it with thionyl chloride, as outlined in Scheme **1**. This reaction released HCl gas (confirmed by a positive ammonia-dipped rod test) and resulted in a loss of weight. The glucosamine base was isolated in pure form from its hydrochloride by neutralization with $NaHCO_3$. The acid chloride then reacted with glucosamine and chitosan *via* mechanochemical methods, forming amides without the use of any solvent. The conversion was indicated by the release of HCl gas and a decrease in weight, with high yields (>95%). The synthesized intermediates and final products were characterized using the following instrumental techniques.



Scheme 1. Mechanochemical synthesis of diclofenac-glucosamine and diclofenac-chitosan conjugates.

3.1.1. Elemental Analysis

The experimental CHN values for the intermediates, glucosamine base, diclofenac acid chloride, and the product, diclofenac-glucosamine conjugate closely matched the calculated values (within 0.4% variation) indicating their high purity. In the case of the diclofenac-chitosan conjugate approximately a 20% increase in these values compared to chitosan was observed. The compositions were confirmed by the mass spectral data.

3.1.2. Fourier Transform Infrared (FT-IR) Spectra

The FT-IR spectra of diclofenac acid chloride, glucosamine, diclofenac-glucosamine, chitosan, and diclofenac-chitosan conjugate are shown in Fig. (**4**). Most of absorption bands in the spectra of diclofenac, glucosamine and chitosan moieties were present in the spectra of the conjugates. The absorption at ~1800 cm^{-1} in the spectrum of diclofenac acid chloride, characteristic of ν(O=C-Cl) [37, 38], disappeared in the spectra of conjugates. The band at ~1603 cm^{-1} due to ν(O=C-N-H-) appeared on the formation of the amide bond in the spectra of conjugates. The ν(-NH) absorption at ~3286 cm^{-1} of primary amine in glucosamine shifted to ~3076 cm^{-1} in the diclofenac-glucosamine conjugate. These changes indicated the formation of the amide linkage.

3.1.3. Electronic Absorption Spectroscopy

The UV-Vis spectra of diclofenac sodium and the synthesized conjugates are shown in Fig. (**5**). The absorption at around 280 nm (π-π* in the benzene ring) characteristic of diclofenac and a new absorption in the 220-240 nm region due to amide formation were observed in both the conjugates.

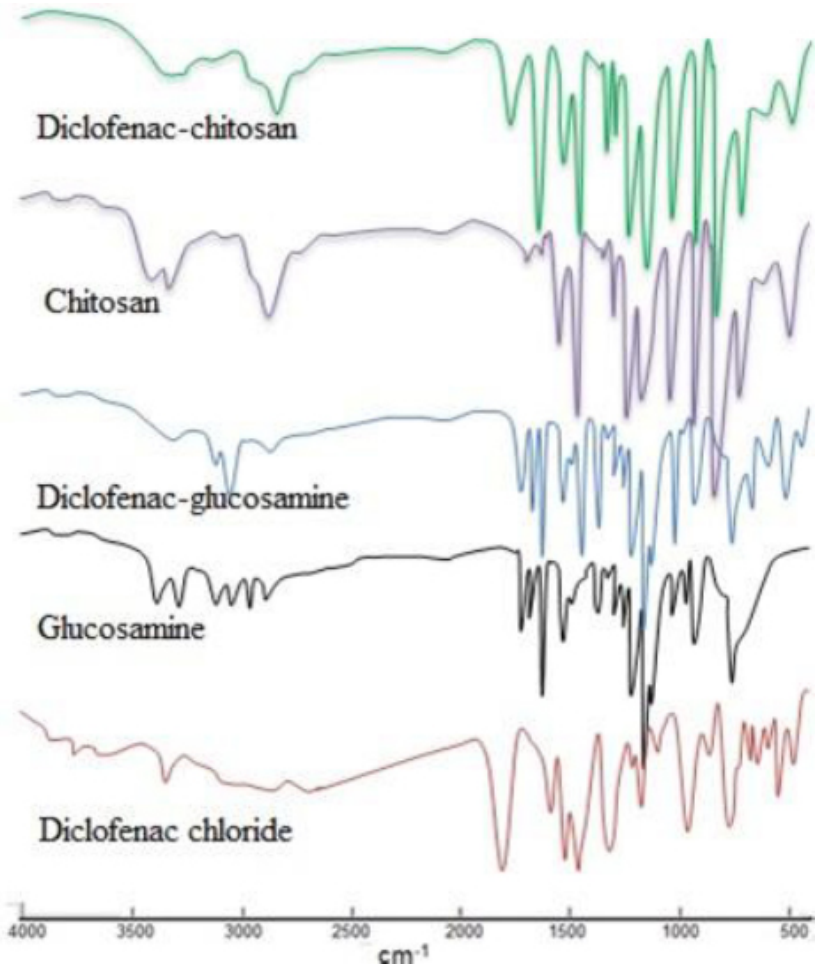


Fig. (4). FT-IR Spectra of diclofenac chloride, glucosamine, diclofenac-glucosamine conjugate, chitosan, and diclofenac-chitosan conjugate. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

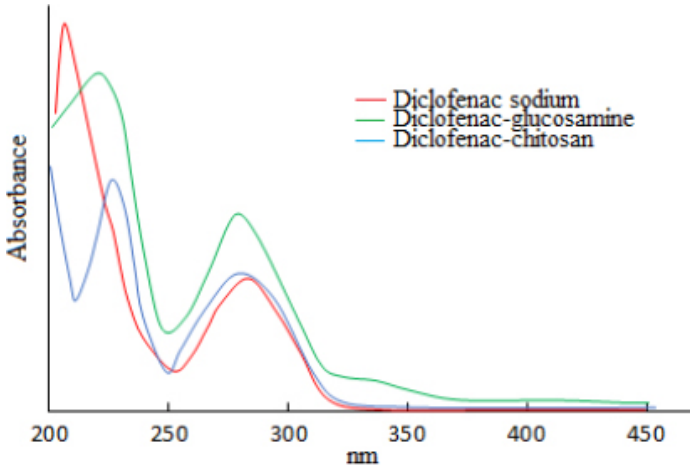


Fig. (5). UV-Vis spectra of diclofenac sodium, diclofenac-glucosamine and diclofenac-chitosan in DMSO. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.1.4. Nuclear Magnetic Resonance (^{1}HNMR) Spectra

The ^{1}HNMR spectra of diclofenac chloride, glucosamine, diclofenac-glucosamine conjugate, chitosan, and diclofenac-chitosan conjugate recorded in DMSO are shown in Fig. (**6**). Generally, the spectra of the conjugates included the chemical shifts related to glucosamine or chitosan present in them. The characteristic shifts around 9.3 ppm, due to Ar-NH-Ar, and the aromatic protons, in the region 7.5 - 8.4 ppm, in diclofenac chloride are noticeable in the conjugates. The chemical shift around 8.3 ppm due to -NH_2 functionality present in the spectra of glucosamine and chitosan was found to be replaced with/shifted to ~7.5 ppm due to the formation of -CO-NH- (amide linkage) in the conjugates [39, 40].

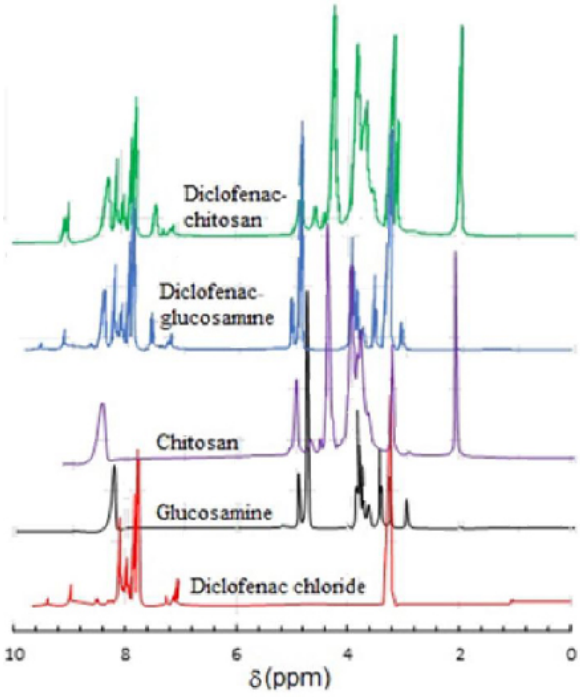


Fig. (6). ^{1}HNMR spectra of diclofenac chloride, glucosamine, chitosan, diclofenac-glucosamine, and diclofenac-chitosan in DMSO-d_6. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Thus, the elemental and spectroscopic analyses provided unambiguous evidence that the mechano-chemical technique is a convenient method to couple a carboxylic group with an amino group to form amides without the use of hazardous reagents and solvents. This method affords high yields compared to wet chemistry methods, where hazardous solvents and reagents like anhydrous tetrahydrofuran, hydroxybenzotriazole, N-(3-dimethylaminpropyl)-N-ethylcarbodiimide hydro-chloride, anhydrous dichloromethane and triethylamine are used and the product yield is very low (27%) [1]. Moreover, in wet reactions other reactive groups like -OH need to be protected.

3.1.5. Thermal Analysis

TGA was used to determine thermal stability and the fraction of volatile components in the products by monitoring the weight change on heating the sample at a constant rate. The thermograms (TGA) are shown in Fig. (**7**). The conjugates exhibited different degra-dation patterns from the starting materials indicating the formation of new compounds. The diclofenac-glucosamine conjugate was stable up to 120°C; the approx. 18% weight loss at this temperature was due to the absorbed moisture that was confirmed by the Karl-Fischer titration. A gradual stepwise weight loss was recorded after the moisture loss due to successive decomposition of the drug compounds. Similarly, approx. 10% weight loss was recorded around 110°C due to absorbed moisture in the diclofenac-chitosan conjugate followed by a gradual stepwise complete degradation of the drug and polymer components. The residues of glucose mine and chitosan and their conjugates contained traces of ash, whereas that of diclofenac sodium contained a high ash content due to the formation of Na_2O. The results of TGA exhibited distinct thermal decomposition profiles of the conjugates due to their different compositions and indicated that they are thermally stable up to 120°C (Fig. **7**).

3.2. Stability Study the Conjugates

The stability profiles of the conjugates stored at 40 ± 1°C (75% ERH) in solid form and at 37 ± 1°C in pH 7.4 PBS are shown in Fig. (**8**). In the solid state, the conjugates remained stable (assay >90%) under accelerated conditions, indicating that they would remain stable for approximately three years if stored at around 30°C in a dry place. In PBS, their assay remained >90% for approximately 90 days, suggesting good stability under physiological conditions.

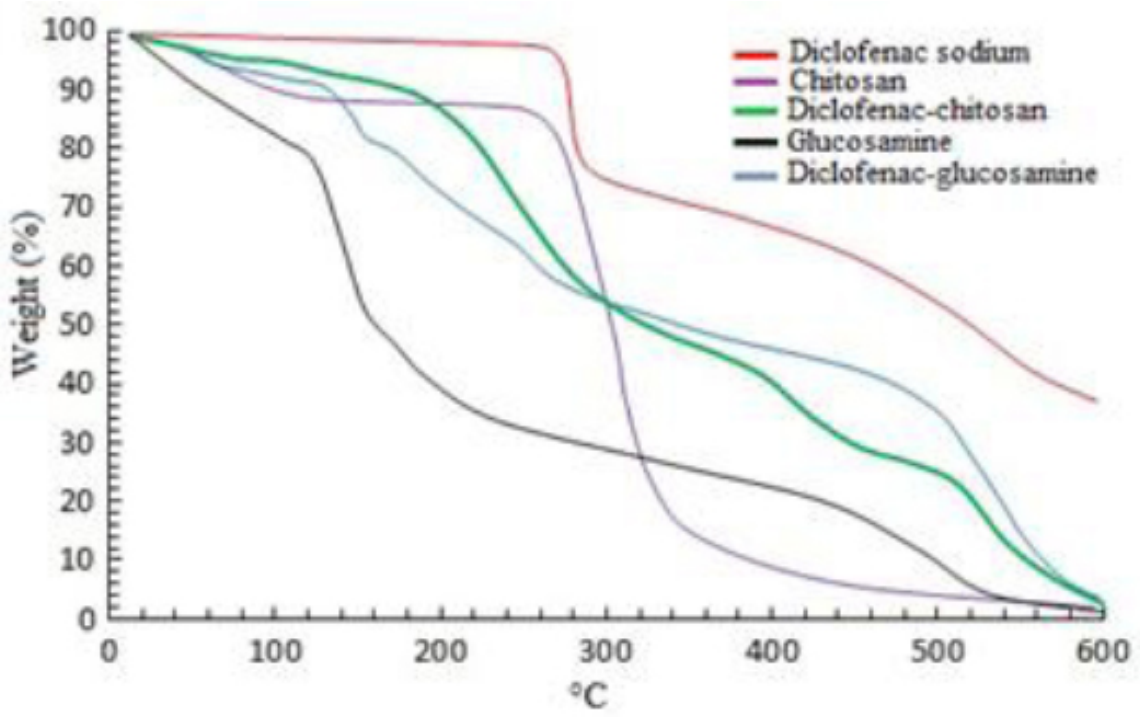


Fig. (7). Thermograms of diclofenac sodium, glucosamine, chitosan, diclofenac-glucosamine, and diclofenac-chitosan. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

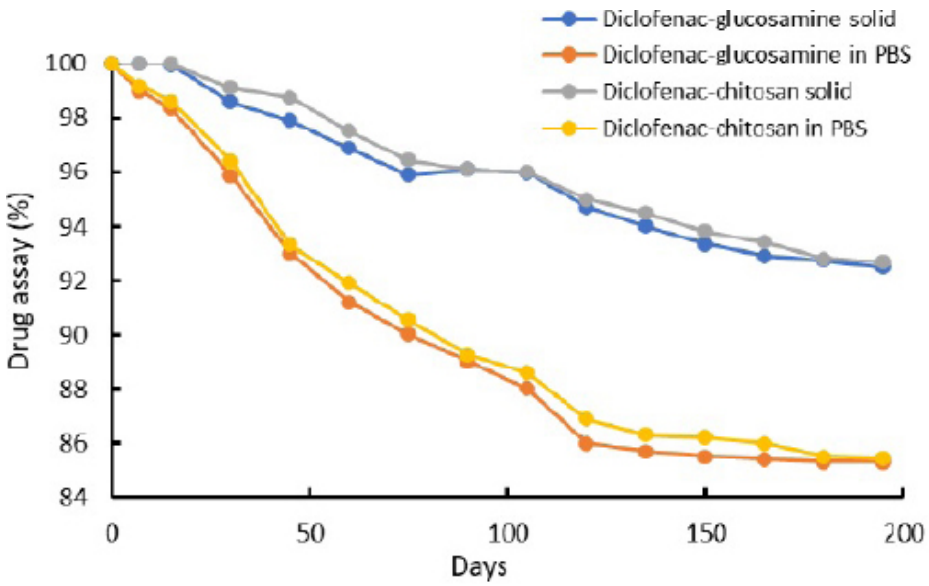


Fig. (8). Stability profiles of the diclofenac conjugates stored in solid state (at 40 ± 1°C, ERH 75%) and in pH 7.4 PBS (at 37 ± 1°C). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.3. *In Vitro* Release of Diclofenac from the Conjugates

The drug (diclofenac) is released by the conjugates on basic hydrolysis of the conjugates. The drug conjugates involving the amide linkage are considered better prodrugs compared to those involving ester linkage because of the greater stability of the amide bond due to the delocalization of lone pair on the nitrogen atom with the π-bond of C=O that results in a partial π-bond between nitrogen and the carbonyl carbon. The release profiles of diclofenac sodium and the synthesized conjugates are shown in Fig. (**9**). No significant release was observed in the acidic medium as diclofenac is insoluble at acidic pH. It is well understood that acid-catalyzed hydrolysis of amides is a reversible process. The forward reaction may be driven over to the product by using an excess of water as the solvent. On the other hand, base-catalyzed hydrolysis is essentially irreversible because the product is a non-electrophilic carboxylate salt. Approximately 75% of diclofenac was released from the diclofenac-glucosamine conjugate and 57% from the diclofenac-chitosan conjugate in 72 h at pH 7.4. The release from the latter was relatively more sustained because of its polymeric nature. Thus, the dissolution study demonstrated that the conjugates would function as the pro-drugs of diclofenac affording a sustained release over an extended period of time. The release data of the conjugates fitted well ($R^2 = 0.998$) into the Hixon-Crowell model [40], $(1\text{-}f_i)^{1/3} = 1 - K_\beta t$, where $f_i = 1 - (W_i/W_0)$ represents the fraction of drug dissolved (W_0, the initial amount of the drug in the system ÷ W_i, the amount remaining in the system) on time t and K_β is a release constant. This suggests that the drug release is controlled by dissolution rate and not by diffusion.

3.4. Anti-inflammatory Activity

The carrageenan-induced rat paw edema test is considered a reliable method for assessing the anti-inflammatory activity of drug molecules [33]. This test was employed to compare the anti-inflammatory activity of synthesized diclofenac conjugates with glucosamine, chitosan, and standard diclofenac sodium. The edema induced in the rats reached its maximum within 2 h. The results from the treated animals are presented in Fig. (**10**). It was observed that treatment with the synthesized conjugates significantly ($p<0.05$) enhanced the inhibition of edema, with 62.3 ± 2.3% inhibition observed with Diclofenac-glucosamine and 58.5 ± 1.6% with Diclofenac-chitosan after 5 h, compared to 49.0 ± 1.3% with standard diclofenac sodium. Activity of the conjugates was >19% more than diclofenac sodium. To verify that this enhanced activity was due to the chemically bonded conjugates and not the result of concomitant administration of physically mixed components, a separate experiment was performed. The results showed no significant difference in activity compared to the controls, providing straightforward evidence of a chemical reaction between the components

during the grinding process (Fig. **10**). The enhanced activity of the conjugates may be attributed to the synergistic effects of glucosamine and chitosan, which inhibit NF-κB signaling activation, thereby reducing the expression of pro-inflammatory cytokines such as TNF-α and IFN-γ [41], or through the inhibition of prostaglandins [42].

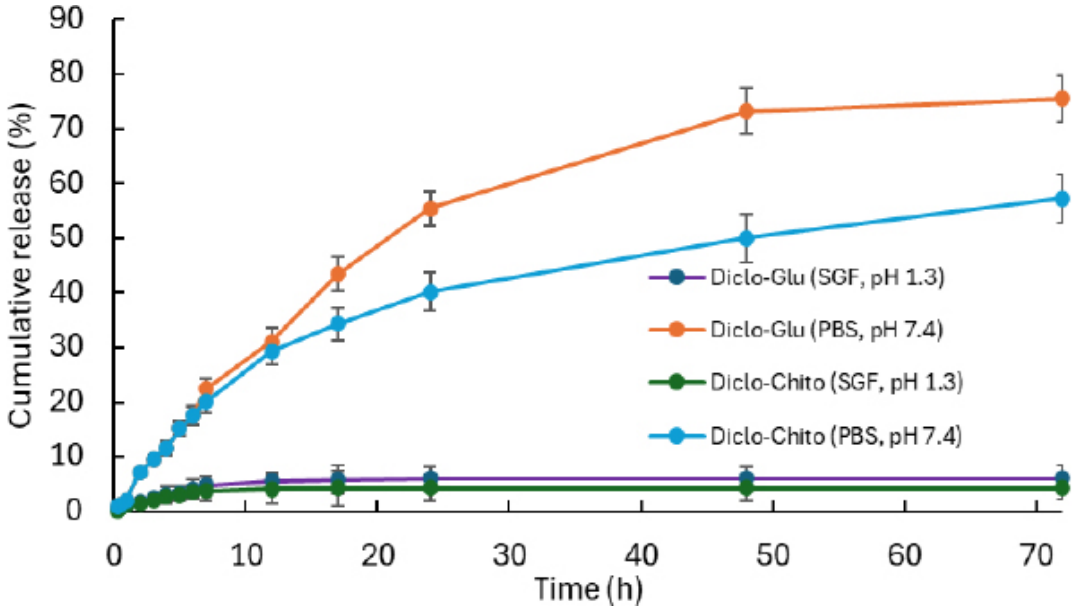


Fig. (9). *In vitro* cumulative release of diclofenac from Diclofenac-glucosamine (Diclo-Glu) and Diclofenac-chitosan (Diclo-Chito) in SGF (pH 1.3) and PBS (pH 7.4). The data points are mean (n=3) and error bars are ± SD. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

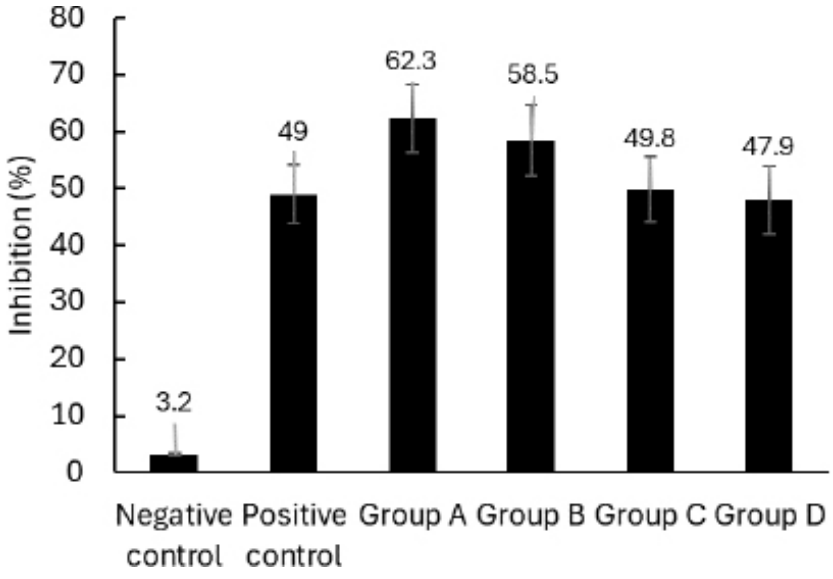


Fig. (10). Inhibition (%) of edema after treatment with diclofenac sodium (positive control), diclofenac-glucosamine conjugate (Group A), and diclofenac-chitosan conjugate (Group B), compared with negative control, after 5 h at doses of equivalent to 10.0 mg kg^{-1} b.w. of diclofenac. Groups C and D received equivalent doses of diclofenac sodium physically mixed (without grinding) with glucosamine and chitosan, respectively. The values are a mean of five and the error bars represent ± SD. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.5. Ulcerogenic Activity

The conjugates under investigation were subjected to *in vivo* evaluation using a rat-stress model to assess their expected ulcer-safe activity. The US and UI values after treatment with diclofenac sodium (US, 4; UI, 63.3±1.2) and its physical mixtures (without grinding) with glucosamine (US, 4; UI, 59.4±1.3) and chitosan (US, 4; UI, 60.1±1.0) were significantly higher (p<0.05) than those with diclofenac-glucosamine (US, 2; UI, 30±1.3) and diclofenac-chitosan (US, 1; UI, 16.6±1.2). The US and UI values for the control were 0 and 3.3, respectively. Representative micrographs of normal and treated gastric mucosa are shown in Fig. (**11**). These values are significantly lower for the synthesized conjugates due to masking of the carboxylic moiety in diclofenac [34, 43]. These results demonstrated that the synthesized conjugates are ulcer safe and possess better anti-inflammatory activity, which is the validation of our hypothesis.

3.6. COX Inhibitory Activity

As one of the objectives of this study was to synthesize selective COX-1/COX-2 derivatives of diclofenac, therefore, the synthesized diclofenac conjugates with glucosamine and chitosan were subjected to COX assays [1]; The results showed that the conjugates under investigation act as selective inhibitors of COX-2, as evidenced by significantly lower IC_{50} values for COX-2 compared to those for COX-1 (Table **1**). The possible reason for selective inhibition is understandably the structural differences in COX-1 and COX-2 active sites. COX-2 has a larger and more flexible active site compared to that of COX-1, primarily due to a smaller side chain in its binding site (valine in COX-2 instead of isoleucine in COX-1). This structural difference allows selective COX-2 inhibitors (such as celecoxib and rofecoxib) to fit into the COX-2 more effectively. The same explanation may apply to the conjugates under investigation as they are also larger in size than diclofenac. The bulky side chains in the conjugates exploit the spatial differences in the COX-2 active site. They bind in a way that selectively blocks the catalytic activity of COX-2 without interfering with COX-1. The conjugates are more hydrophobic so they can interact with a hydrophobic side pocket unique to COX-2, further enhancing selective binding.

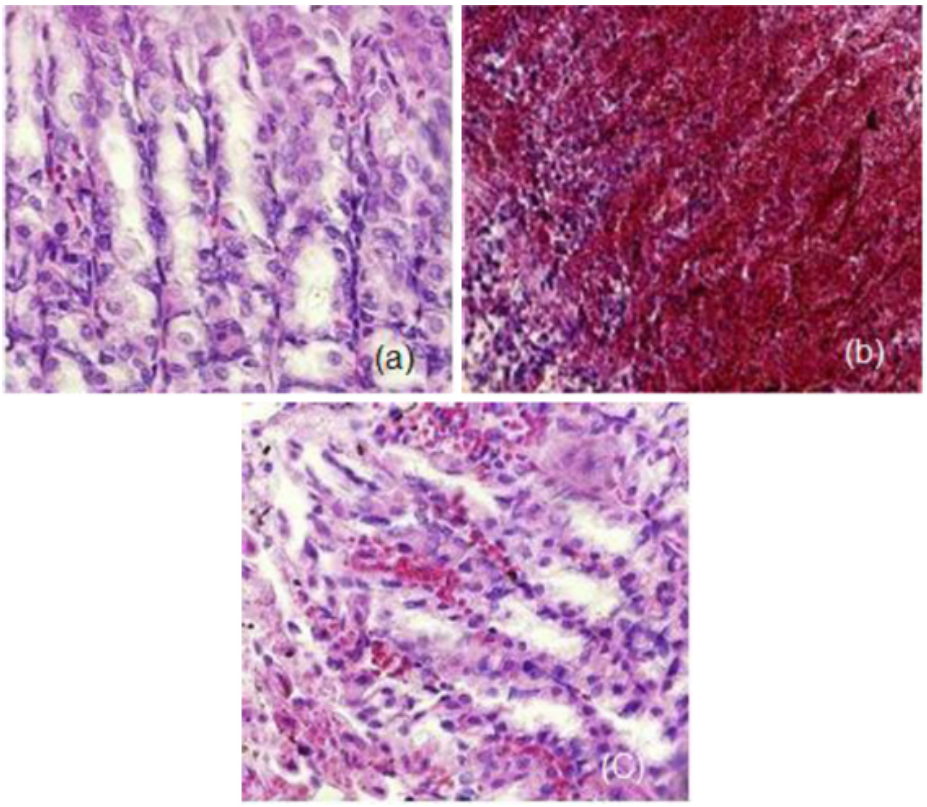


Fig. (11). Micrographs showing histological conditions of gastric mucosa: normal (**a**), treated with diclofenac sodium (**b**) and diclofenac-chitosan (**c**). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. IC_{50} values (mean ± SD, n = 3) of the compounds.

Compound	IC_{50} (μM)	
	COX-1	COX-2
Diclofenac-glucosamine	27.3±1.0	2.4±0.4
Diclofenac-chitosan	28.1±1.2	2.0±0.3
Physical mixture of diclofenac and glucosamine	3.7±0.3	0.9±0.1
Physical mixture of diclofenac and chitosan	3.4±0.2	0.8±0.1
Diclofenac sodium	3.5±0.2	0.8±0.1
Celecoxib	27.2±1.3	2.3±0.2

Thus, the findings of this study suggest that the Diclofenac-glucosamine and Diclofenac-chitosan conjugates are potential candidates for clinical evaluation as selective COX-2 ulcer-safe anti-inflammatory drugs, as demonstrated through *in vitro* and *in vivo* biological testing.

3.7. Pharmacokinetics

The plasma concentration-time curves are shown in Fig. (**12**), and the pharmacokinetic parameters (t_{max}, $t_{1/2}$, Cl, C_{max}, AUC_{0-t}) are listed in Table **2**. Each of the three curves differs from the others, suggesting distinct pharmacokinetics for the conjugates compared to standard diclofenac sodium. Notably, the conjugates do not exhibit burst release *in vivo*; instead, they release the drug gradually over an extended period. Diclofenac-chitosan showed a more prolonged release than diclofenac-glucosamine, likely due to the stability of the amide linkage between diclofenac and glucosamine or chitosan, which resists hydrolysis. These results suggest that the conjugates function as long-acting anti-inflammatory agents.

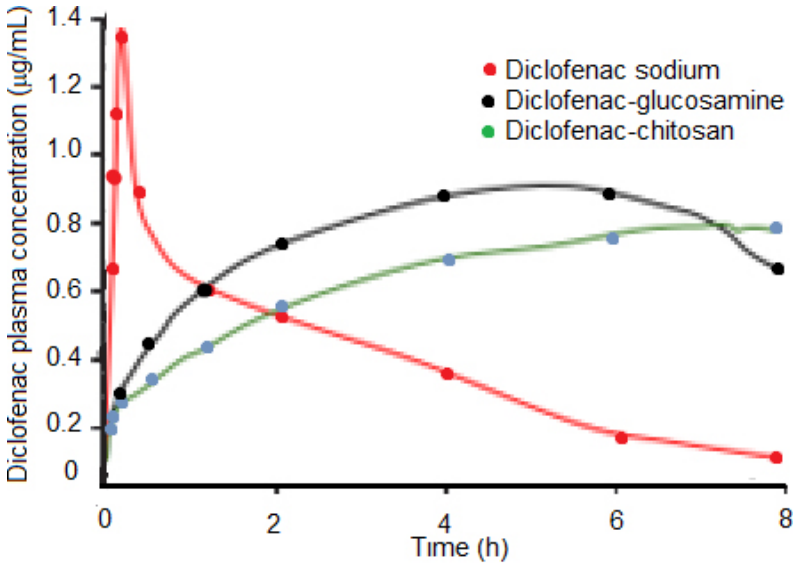


Fig. (12). Plasma concentration with time after oral administration of diclofenac sodium and its conjugates. The dose was 2 mg kg^{-1} equivalent to diclofenac. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Pharmacokinetic data of diclofenac sodium, Diclofenac-glucosamine and Diclofenac-chitosan.

Parameter	Value (mean ± SD)		
	Diclofenac-Na	Diclofenac-glucosamine	Diclofenac-chitosan
t_{max} (h)	0.21±0.10	3.20±0.13	7.51±0.32
$t_{1/2}$ (h)	1.20±0.20	7.48±0.21	> 8
Cl (L h^{-1})	0.75±0.20	0.15±0.03	0.10±0.02
C_{max} (μg mL^{-1})	1.38±0.20	0.85±0.14	0.74±0.11
AUC_{0-t}	2511±250	3801±201	4902±285

3.8. Electrocardiography

A normal ECG for healthy rats was observed in the case of animals treated with the conjugates. Typical features were: i) a rapid heart rate around 250-450 bpm, ii) clear P waves, narrow QRS complexes, and T waves, with consistent morphology, iii) stable intervals (PR, QT), and iv) absence of arrhythmias. In rats with thrombosis (such as coronary or pulmonary thrombotic events), ECG differences may include: elevation or depression in the ST segment suggesting ischemia, especially in coronary thrombosis, T-wave inversion or flattening indicating ischemic stress in the myocardium. In cases of pulmonary embolism, signs of right heart strain might appear, such as right-axis deviation or changes in the QRS complex due to increased right ventricular workload.

These results suggest that the conjugates under investigation may be a better choice than, for example, rofecoxib — a selective COX-2 inhibitor known to cause serious cardiovascular complications [44].

CONCLUSION

In this work, diclofenac, a non-steroidal anti-inflammatory drug, was derivatized *via* amidation with unacetylated glucosamine and chitosan using mechanochemical synthesis. The objective was to mitigate the drug's ulcerogenic effect and enhance its COX-2 selectivity by masking the carboxylic group through derivatization. The products, Diclofenac-glucosamine and Diclofenac-chitosan conjugates were obtained with yields exceeding 95%. Accelerated stability studies indicated that these conjugates would remain stable for three years in solid form under normal storage conditions and for three months under physiological conditions. *in vitro* and *in vivo* biological tests demonstrated that the conjugates have greater anti-inflammatory activity, enhanced COX-2 selectivity, and improved cardiovascular safety. Additionally, they showed reduced ulcerogenic effects compared to standard diclofenac sodium. Both conjugates exhibited distinct pharmacokinetic profiles from diclofenac sodium, indicating their potential as long-acting, ulcer-safe anti-inflammatory agents. Consequently, these synthesized conjugates may be considered promising candidates for clinical trials.

AUTHORS' CONTRIBUTIONS

The authors confirm their contribution to the paper as follows: study conception and design: M.S.I., A.H.K.; data collection, analysis and interpretation of results and draft manuscript: S.A. All authors reviewed the results and approved the final version of the manuscript.

LIST OF ABBREVIATIONS

NSAIDs	=	Non-Steroidal Anti-Inflammatory Drugs
COX	=	Cyclooxygenase
CV	=	Cardiovascular
FT-IR	=	Fourier-Transform Infrared
TGA	=	Thermogravimetric Analysis

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol used in this work was approved by the Institutional Review Board of Forman Christian College, Lahore (vide #IRB-412/03-2023 dated March 20, 2023).

HUMAN AND ANIMAL RIGHTS

This study adheres to internationally accepted standards for animal research, following the 3Rs principle. The ARRIVE guidelines were employed for reporting experiments involving live animals, promoting ethical research practices.

The animal experimentation was conducted according to the Guide for the Care and Use of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supports the findings that are available in the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors are thankful to Dr Laiba Arshad of the Department of Pharmacy, FC College, for help in animal handling.

SUPPLEMENTARY MATERIAL

Supplementary material, along with the published article, is available on the publisher's website.

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