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Synthesis, Characterization and Biological Screening of Mutual Prodrug of Etodolac-Dextran: An Approach to Reduce Toxicity

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Abstract –

Objective: The project was aimed at synthesizing and characterizing conjugate of Etodolac (ETD) that is expected to enhance solubility without affecting permeability and is capable of delivering ETD to colon without significant reversion of prodrug in gastrointestinal conditions.

Methods: Thus, Etodolac- dextran (ETD-DEX) conjugate was pre-pared by conventional coupling method and the prodrug was characterized by FTIR, FAB mass and elemental analysis. The conjugate was then subjected to selected pharmaceutical preformulation studies like aqueous solubility analysis and pH partition studies.

Results: These studies established 1.24 folds higher solubility of the ETD-DEX over ETD in phosphate buffer pH 6.8 without compromising its partitioning ability. The in vitro stability studies suggested its potential of safe transit to colon where the moiety is capable of reverting to 90.52 % ETD after 48 hrs of the experiment. In vivo evaluation of ETD-DEX in an experimentally induced colitis established its efficacy an anti-inflammatory prodrug moiety that was supported by histological studies. In addition to its ability to control colonic ulcers ETD-DEX demonstrated insignificant (P >0.05) gastric ulcerogenic potential. Colonic MPO activity for ETD-DEX in mU/100 mg tissue was found to be 54.97 which were much less than plain ETD (85.54).

Conclusion: Conclusively, the conjugate when suitably formulated can be considered as therapeutically efficacious drug delivery system with fewer pharmaceutical limitations.

Keywords: Colon Specific Prodrug, Etodolac, Synthesis of ETD-DEX Prodrug, Characterization of Prodrug.

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INTRODUCTION

Although the prodrug technique is frequently used primarily when the prototype encounters unforeseen challenges, it is a flexible approach to drug development that should not be viewed as a last resort. Drug inventors and designers can adjust drug pharmacokinetics, pharmacodynamics, and toxicity using the prodrug technique [1]. A prodrug is a weakly active or inert product containing the parent drug that undergoes biotransformation in vivo by chemical or enzymatic cleavage, allowing the active molecule to be delivered at effective quantities [2,3,4]. Prodrugs are traditionally divided into two categories: carrier-linked and bioprecursors. Bipartite prodrugs, in which the carrier is attached directly to the parent drug, and tripartite prodrugs, in which a spacer joins the carrier to the parent drug, are the two types of carrier-linked prodrugs [5]. Chemical groups such as ester, amide, carbamate, carbonate, ether, imine, and phosphate are often used to attach carriers [1,6,7]. Mutual prodrugs are a sort of carrier-linked prodrug in which two active molecules are linked together, with one serving as the carrier for the other. Synergistic activity has boosted the efficacy of these prodrugs [5,8]. The macromolecular prodrug is another form of carrier-linked prodrug; these prodrugs employ polymeric backbones as carriers.

Etodolac is a non-selective cyclooxygenase (COX) inhibitor with analgesic and anti-inflammatory properties derived from racemic acetic acid. Osteoarthritis, gout, rheumatoid arthritis, and

severe damage can all be treated with it. Oral use of a non-selective COX inhibitor induces GI adverse effects such as nausea, vomiting, dyspepsia, gastric irritation, peptic ulcers, and bleeding [9]. NSAIDs commonly cause gastrointestinal damage and increase the risk of ulcer complications due to these typical gastrointestinal side effects. Some evidence suggests that, like other NSAIDs, etodolac causes gastrointestinal adverse effects [10,11]. NSAIDs cause GI side effects either through direct contact or by an indirect action on the GI mucosa. The acidic nature of NSAIDs, as well as their ion trapping action and suppression of cytoprotective prostaglandins, are the main causes of GI side effects [6-8]. Chemical coupling of a polymeric, biodegradable carrier with drugs to form a polymeric or macromolecular prodrug [9-12] may be a useful approach to improve physicochemical properties and clinical acceptance of drugs, according to the literature. The parent drug is then released in vivo via biotransformation. Because of its higher physiological uptake and reduced toxicity, dextran, a polysaccharide macromolecule, has been employed in therapeutic practise. It possesses outstanding physicochemical properties, including strong aqueous solubility, many hydroxyl groups for drug conjugation, and availability across a wide range of molecular weights [13, 14]. Many synthetic dextran conjugates demonstrated enhanced physicochemical qualities, targeted drug administration, and colon specificity, according to the literature [15-19]. The goal of this study is to synthesise etodolac dextran conjugates and assess their potential for usage as a polymeric prodrug for oral drug administration. In addition, in vivo animal studies were carried out to analyse the drugs' pharmacological effects and gastrointestinal toxicity.

MATERIALS AND METHODS

Materials

Fleming Laboratories Limited, Telangana, India, generously donated ETD. Fluka Biochemika in Switzerland provided the N, N-carbonyl diimidazole (CDI). Hi-Media Laboratories Pvt. Ltd. in India provided Dextran. The rest of the compounds were of a synthetic kind. S. D. Fine Chem Ltd., Mumbai, India, provided the triethyl amine, benzene, methanol, dimethyl sulfoxide (DMSO), and sodium bicarbonate. Merck, Mumbai, India, provided diethylether, HPLC grade methanol, acetonitrile, and water. Throughout the investigation, distilled water was utilised.

Synthesis of mutual prodrug

The mutual prodrug (ETD DEX) was synthesised in two phases (figure 1). The first step involves utilising N, N-carbonyl diimidazole (CDI) (10 mol/l) to activate the carboxylic acid functional group of ETD (10 mol/ml), resulting in a reactive intermediate ETD acylimidazole. ETD acylimidazole was treated with dextran and evaluated using thin layer chromatography (TLC) with n-hexane, water, ethyl acetate, and glacial acetic acid (30: 10: 10: 2.5) as the stationary phase and n-hexane, water, ethyl acetate, and glacial acetic acid (30: 10: 10: 2.5) as the mobile phase. After vacuum evaporation of the ether layer, further extraction with ether was performed, and crude product was obtained. Methanol was used to recrystallize the product, which was then dried under vacuum [12].

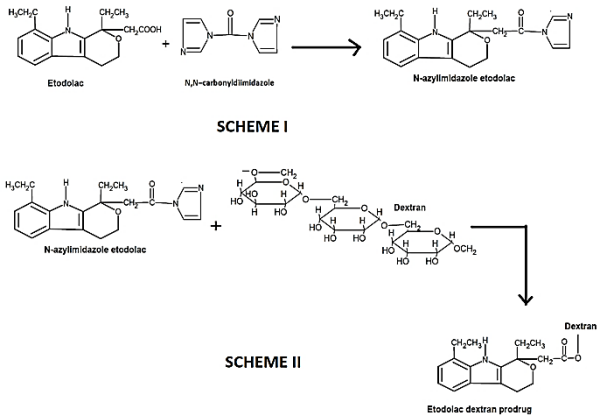


Figure 1. Synthesis of ETD-DEX Prodrug

Characterization and preformulation studies of the synthesized prodrug

Protein binding study

The protein binding of ETD-DEX is investigated using a cellophane membrane. In phosphate buffer (PBS) at pH 6.8, a prodrug solution (100 g/ml) was prepared. After washing with PBS, the cellophane membrane was attached to the opening end of a dialysis tube with 5% egg albumin. The whole assembly was then immersed in the drug solution and spun at 100 rpm at 37° on a magnetic stirrer. The medication solution was sampled at regular intervals. The withdrawn material was diluted further with phosphate buffer and UV spectroscopy at 280 nm was used to monitor it [16].

X-ray diffraction study (XRD)

IIT Kanpur used a Bruker D8 Advance X-ray diffractometer to perform qualitative powder x-ray diffraction of ETD and ETD-DEX prodrug. For the generator, a voltage of 40 kV and a current of 30 mA were utilised, with copper as the tube anode material. Copper potassium alpha radiation was applied to the materials at two different theta angles ranging from 30 to 400 degrees [17].

FT-IR spectroscopy

On a Shimadzu -8400 S FTIR spectrophotometer at PSIT, Kanpur, the FT-IR spectra of ETD and

ETD-DEX prodrug was recorded in potassium bromide (anhydrous grade) pellets to determine the presence of organic functional groups [18].

Mass Fab spectroscopy

On a JEOL SX 102/DA-6000 mass spectrometer (JEOL, USA), the mass spectrum of ETD and ETD-DEX prodrug were recorded to prove the correct mass or m/z ratio at IIT Kanpur, Uttar Pradesh, India [16, 18].

Aqueous solubility determination

The aqueous solubilities of the ETD and ETD-DEX prodrug were determined by adding excess amounts of the solutes in HCl buffer pH 1.2, acid phthalate buffer pH 4.0, phosphate buffer pH 6.8 and 6.8 and equilibrating them at 25^{0}C on water bath shaker (Hicon, New Delhi). After 72 hours the samples were withdrawn, filtered diluted with phosphate buffer pH 6.8 for ETD and with distilled water for ETD-DEX prodrug and analyzed by UV spectrophotometer at 247 nm and 217.5 nm respectively [19].

pH partition study of ETD and ETD-DEX prodrug

Preparation of calibration curve of ETD-DEX prodrug in n-octanol

In n-octanol, a calibration curve for the ETD-DEX prodrug was created. Appropriate aliquots of ETD-DEX prodrug (100g/ml) were pipette out into a succession of 10 ml volumetric flask and diluted with n-octanol to get final concentrations in the range of 5-20 g/ml. The absorbances of the resulting solutions were measured with a UV spectrophotometer at an appropriate maximum [19].

Determination of partition coefficient

Between 10 mL n-octanol and 10 mL buffer of various pH, the partition coefficient was calculated using the Hansch technique (1.2, 4.0, 6.8 and 6.8). In the separating funnel, buffers and n-octanol were introduced. For 60 minutes, both phases were soaked with occasional shaking. ETD and ETD-DEX prodrugs were weighed and put to distinct separating funnels, which were then agitated for another 30 minutes. In both phases, medication distribution must be achieved. After 5 minutes, the separating funnel was removed. The organic and aqueous layers were separated. For ETD-DEX prodrug, the organic layer was properly diluted and examined using a UV spectrophotometer. The aqueous layer was separated, diluted with phosphate buffer pH 6.8, and ETD was determined using a UV spectrophotometer [19].

RESULTS AND DISCUSSIONS

Protein binding for the ETD-DEX prodrug was 41% lower than ETD, indicating that the ETD-DEX prodrug will be more available for hydrolysis in plasma and that the needed dose will be lower, minimising the dose-related effect on prostaglandin production. The crystalline structure of ETD (figure 2) is confirmed by X-ray diffraction, but the amorphous structure of ETD-DEX prodrug is confirmed by the result, which also supports the prodrug's enhanced solubility over ETD [15].

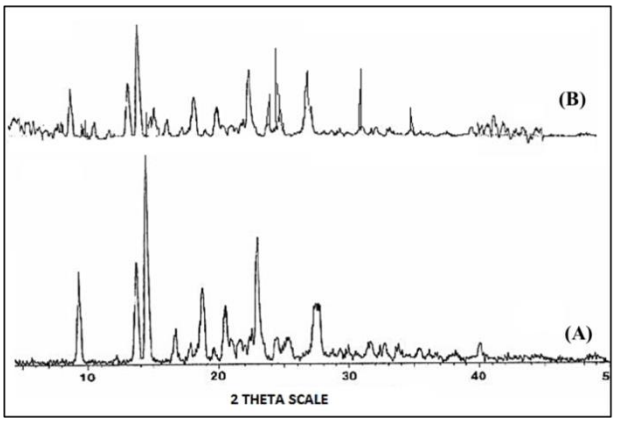


Figure 2. Powder x -ray diffraction pattern of ETD-DEX prodrug

ETD and ETD-DEX retention times (Rt values) were determined to be 1.992 and 1.01 minutes, respectively. The ETD-DEX synthesis is confirmed by the difference in retention time values between ETD and ETD-DEX (Figure 3). FT-IR spectrum of ETD-DEX conjugate (figure 4) showed absorption band at 2969 cm^{-1} (N-H stretching of amide), 1738 cm^{-1} (C=O stretching of ester), 1650 cm^{-1} (C=O stretching of amide), 1535 cm^{-1} (N-H bending of amide), 1261 cm^{-1} (C- N stretching), 1032 cm^{-1} (C-O stretching of ester), are the characteristic peaks of ester and amides and confirmed the presence of ester group and formation of amide bond of ETD-DEX conjugate. FT-IR spectrum of ETD showed absorption band at 3300-2500 cm^{-1} (O-H stretching of COOH group), 1406 cm^{-1} (O-H bending of COOH group), 1361 cm^{-1} (C-O bending of COOH group), those confirmed the acidic nature of ETD [20].

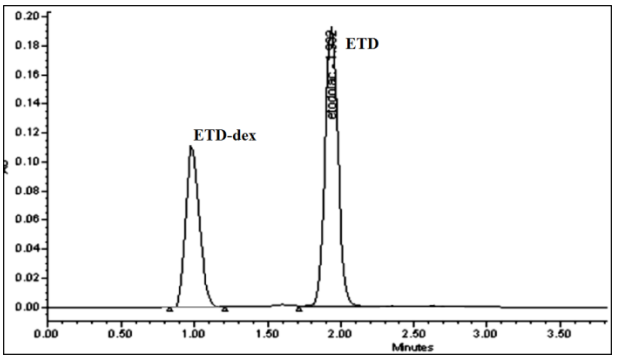


Figure 3. HPLC of ETD and ETD-DEX

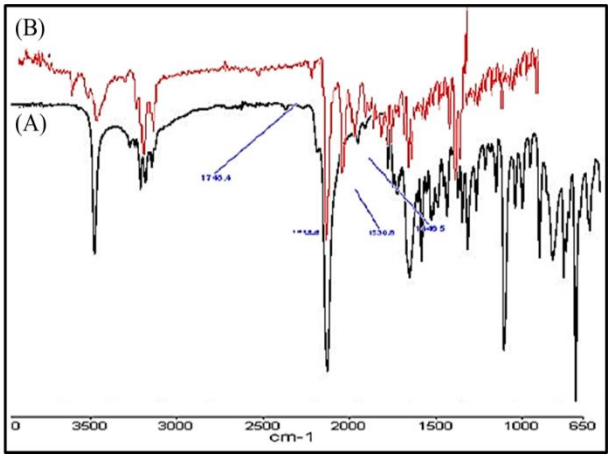


Figure 4. FT-IR spectrum of (A) ETD and (B) ETD-DEX conjugate

The parent (M+) peak was found at 287 and 351 in the mass spectra of ETD and ETD-DEX prodrugs (figure 5), indicating the molecular weight of ETD and ETD-DEX prodrug, respectively. Thus, mass spectroscopy verifies the structure and molecular weight of ETD-DEX prodrug [21]. The aqueous solubility of ETD and ETD-DEX prodrugs rises with increasing pH, according to the findings (figure 6). This behaviour might be explained by an increase in compound ionization when pH rises, resulting in enhanced water solubility. The inclusion of a carboxylic group in the parent medication, which experienced a greater degree of ionization with increased pH, caused ETD's solubility to increase significantly across the pH range. Because glycine includes polar groups like –NH2 and –COOH, the enhanced solubility of ETD-DEX prodrug in comparison to ETD was most likely owing to increased polarity [21].

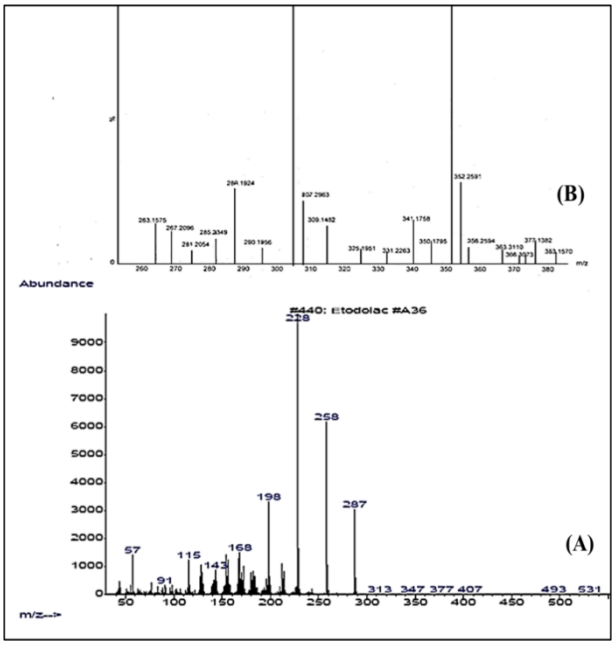


Figure 5. Mass spectra of (A) ETD and (B) ETD-DEX conjugate

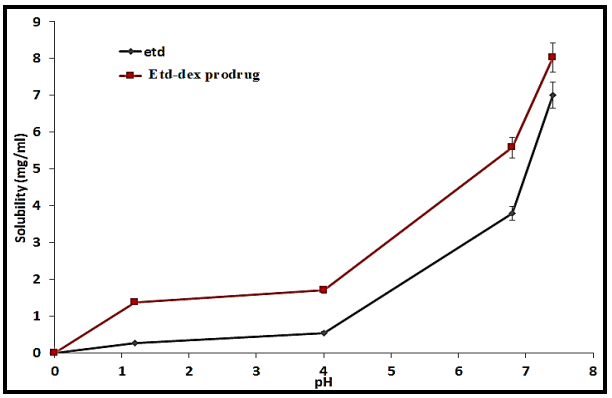


Figure 6. Comparative pH solubility profile of ETD and ETD-DEX prodrug

pH PARTITION PROFILE

The absorbance values (mean of three measurements SD) of the dilutions generated in the concentration range of 5-30 mg/ml in n-octanol were determined to be 274 nm. The data were plotted without a standard deviation, and the resulting calibration curve (figure 7) followed Beer's–Lambert law with a r2 value of 0.9997. The log P value of drug and prodrug drops when pH rises (figure 8). This might be owing to greater solubility of drug candidates at higher pH, resulting in more drug dispersion in the aqueous phase. The non-ionic structure of the prodrug (ETD-DEX prodrug) molecule, as opposed to the ionic nature of Glycine, may explain the increase in log P values for prodrug compared to ETD. As a result, a prodrug of this sort should increase ETD drug delivery. The log P value of ETD and ETD-DEX prodrugs did not differ significantly (t-test, 95 percent threshold of significance) [19].

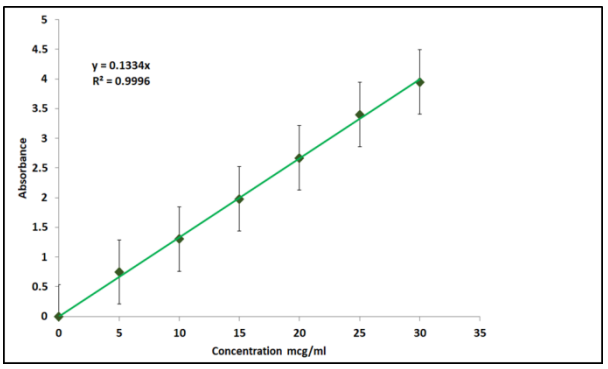


Figure 7: Calibration curve of ETD-dex prodrug in n-octanol

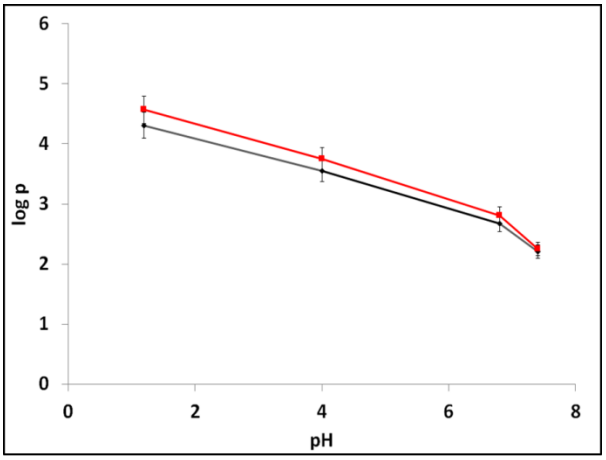


Figure 8: Comparative pH partition studies of ETD and ETD-dex prodrug

STABILITY TESTING

Physical stability at elevated temperatures did not show any significant difference, indicating that neither the temperature nor the humidity had a profound effect on *in vitro* release rate (Table 1, 2) [18].

Table 3.7. Physical stability study at elevated temperatures

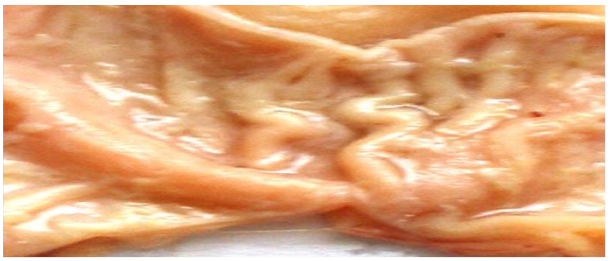
Days	Caking				Liquefaction				Odour				Discolouration			
	CT	40°C	50°C	60°C	CT	40°C	50°C	60°C	CT	40°C	50°C	60°C	CT	40°C	50°C	60°C
15	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+
30	-	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+
60	-	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+

Table 3.8. % Drug remaining in stability study at elevated temperatures

Days	Sample at 40°C			Sample at 50°C		
	Absorbance	Concentration (mcg/ml)	% Drug remaining	Absorbance	Concentration (mcg/ml)	%Drug remaining
15	0.901	9.03	99.23	0.911	9.99	99.09
30	0.912	9.11	99.92	0.933	9.91	99.42
60	0.911	9.37	99.34	0.987	9.89	99.74
90	0.921	9.41	99.41	0.99	9.87	99.54

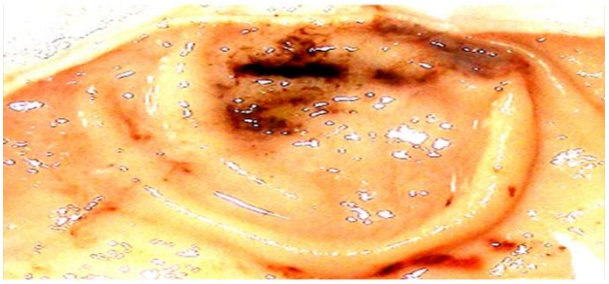
Biological evaluation

The 2,4,6-trini- trobenzene sulphonic acid (TNBS)-induced experimental colitis model was used to assess the practicality of orally given ETD-DEX prodrug for targeted drug delivery to the inflamed colon in IBD. The clinical activity score, colon to body weight ratio, and myeloperoxidase activity were used to determine the severity of inflammation. The intensity of inflammation and these parameters have a direct relationship, i.e., the more severe the inflammation, the higher the values for these parameters and the lower the moderating impact of the prodrugs. The mice were kept without therapy for the following three days after being infected with experimental colitis in order to sustain the development of a complete IBD model. The clinical activity score for all groups grew consistently throughout the course of these three days. After a 24-48-hour lag, all drug-receiving groups demonstrated a reduction in inflammation severity. On the seventh day, a significant difference was seen between the drug-treated groups and the colitis control group. The ulcer index (median range) was 2 1 for control animals, 66.2 ± 4.1 for ETD animals, and 17.0 ± 1.2 for ETD-DEX prodrug animals, demonstrating significant differences in ulcerogenic activity between the prodrug and ETD ($P < 3.234$) [18, 19]. No haemorrhagic or red spots were found on the stomach walls of control animals (Figure 9(A)). Animals given the ETD-DEX prodrug had haemorrhagic and red patches in their stomachs, but no necrosis of the cells (Figure 9(B)). Severe congestion, many haemorrhagic spots, streaks, erosion of the gastric mucosa, profound ulceration, and necrotic cells were seen on the stomach walls of ETD-treated animals (Figure 9(C)). When the histology of the stomachs of control rats (Figure 10(A)), those treated with prodrug (Figure 10(B)), and those treated with drug (Figure 10(C) was compared, the drug group had more severe bleeding, ulcers, and necrosis than the prodrug group. The animals were euthanized on day 11 (24 hours after the medication was administered) and the colon/body weight ratio was calculated to assess inflammation. When compared to the colitis control group, the ETD-DEX prodrug (0.0074 0.00043) treated group demonstrated a significant reduction in the colon/body weight ratio (Table 3). In vivo therapy with the ETD-DEX prodrug reduced the degree and severity of colonic injury significantly [20]. Its histological findings clearly revealed that therapy with ETD-DEX prodrug addressed the morphological abnormalities associated with TNBS administration. There were no deleterious effects on the liver or symptoms of pancreatitis in histological sections of prodrug-treated rat livers and pancreas. It may be concluded from these data that synthesised prodrugs have a better safety profile than ETD. One-way ANOVA was used to quantify statistical differences between the groups, followed by Dunnett's post hoc test. In comparison to control, differences were assessed at a P value of 0.05 [21].



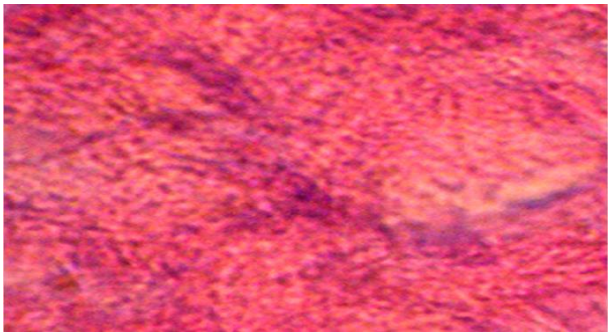
(a)

(b)

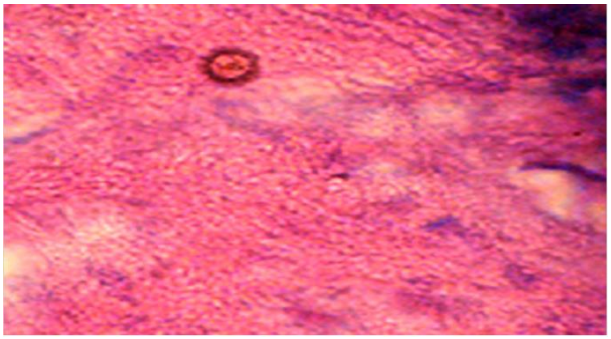


(c)

Figure 9. Stomach of albino rats of ulcerogenic activity (a) Control group, (b) ETD-DEX group, (c) ETD group



(a)



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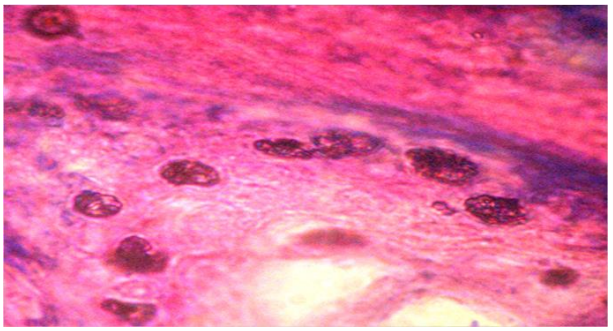

(c)

Figure 10. Histopathology of stomach of albino rats of ulcerogenic activity (a) Control group, (b) ETD-DEX group, (c) ETD group

Table 3. Weight of the albino rats during the acute toxicity study

Animal group	Weight in g ± S.D.			% Increase in weight
	On 0 day	On 14th day	Weight increased	
Control	161±11.31	173±12.72	12±1.41	7.45
Drug	173.5±16.26	184±18.38	10.5±2.12	6.05
Prodrug	171.5±14.84	183±16.97	11.5±2.13	6.70

CONCLUSION

Finally, compared to the parent drug ETD, the synthesized prodrug demonstrated enhanced solubility, synergistic anti-inflammatory and antiarthritic action, decreased toxicity, and less ulcerogenic activity. As a result, this prodrug method not only overcomes the ETD formulation problem (reduced water solubility, BCS class II drug), but it also minimizes stomach side effects.

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