Synthesis and biological evaluation of novel enol carboxamide derivatives of aspirin and diflunisal

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ABSTRACT

A series of enol carboxamide derivatives (4, 7, 12 and 15) were synthesized from aspirin and diflunisal that conjugated with 2-amino-5 - (ethylthio)-1, 3, 4-thiadiazole, and 2-amino-6- (trifluoromethyl) benzothiazole using dicyclohexylcarbodimide (DCC) as coupling agent and their structures were confirmed by IR and ^{1}H NMR spectra. The preliminary evaluation indicate that all tested compounds produced significant reduction of paw edema compared to propylene glycol 50% (control group) and also had shown lesser gastrotoxicity than their parent drugs. In addition, all tested compounds exhibited the maximal anti-inflammatory activity compared to their parent drugs (except for compound (12) which was less potent than diflunisal). Moreover, compound (15) showed the highest anti-inflammatory activity and showed least ulcerogenic effect. The result of this study indicates that the incorporation of the trifluoromethyl benzothiazole or ethylthio-1, 3, 4- thiadiazole pharmacophores into aspirin and diflunisal maintained or increased their anti-inflammatory activity and may increase selectivity towards COX-2 enzyme.

Keywords*: Enol carboxamide, Aspirin, Diflunisal, anti-inflammatory activity, Gastric ulcer.*
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INTRODUCTION

Inflammation is body's response to disturbed homeostasis caused by infection, injury or trauma resulting in systemic and local effects [1]. It is characterized by five cardinal signs: pain, heat, redness, swelling and loss of function [2].

Prostaglandins (PGs) are potent lipid mediators for numerous homeostatic biological functions and plays an important role in the inflammations [3]. They are produced by the most cells and also present in most tissues, this explain their broad spectrum of biological responses [4].

Cyclooxygenase (COX) enzymes are responsible for catalyzing an important intermediate step in the synthesis of PGs and thromboxanes from arachidonic acid [5]. COX exists in three isoforms, COX-1, COX-2 and COX-3 [6]. COX-1 has constitutive form (housekeeping) and responsible producing PG that help regulate normal kidney, stomach functions and vascular homeostasis, whereas COX-2 is induced during inflammation and produces PGs mediators of inflammation [7].

NSAIDs are chemically heterogeneous compounds, although most are organic acids [8].

Their strong anti-inflammatory and analgesic properties have made them the most widely utilized classes and the first-line therapy for osteoarthritis and rheumatoid arthritis [8].

NSAIDs inhibits PG biosynthesis by acting as reversible (excluding aspirin) and competitive inhibitors of COX activity with a varying degree of selectivity [9].

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Traditional NSAIDs such as aspirin and diflunisal mainly inhibit COX-1 over COX-2 and thus their long term used lead to gastrointestinal (GI) hemorrhage and ulceration [10], while selective COX-2 inhibitors (Rofecoxib, Celecoxib) exert their effect with less GI toxicity than traditional NSAIDs but the drugs with higher selectivity for COX-2 tend to induce cardiovascular disease [11].

Preferentially selective COX-2 Inhibitors (e.g. Meloxicam) are novel NSAID acting by preferential inhibition of COX-2 over COX-1 [12].

Selective COX-2 inhibitors are heterogenic and lacking a carboxylic group, thus effecting COX-2 affinity by a different orientation within the enzyme without formation of a salt bridge in the hydrophobic channel of the enzyme [13]. Although COX-1 and COX-2 have slightly differences in structures, the different binding sites of the both COX isoforms contribute to the understanding of the pharmacological activity and selectivity as well as aid in the design of more potent and selective inhibitors of the two isoenzymes [14, 15].

An increasing in the number of studies indicate that the structural modification of traditional NSAIDs, lead to improve their selectivity for COX–2 such as piroxicam, which converted to preferential selective COX–2 inhibitor (meloxicam) due to the presence of the enol–carboxamide and heterocyclic ring with methyl substituent at 5 position [16].

The objective of this study, to synthesis and preliminary evaluation of new aspirin and diflunisal derivatives in the area of oxicam derivatives that are class of enolic acid derivatives to give more potent NSAIDs with less GIT side effect and may exhibit certain selectivity as COX-2 inhibitors.

EXPERIMENTAL SECTION

2.1. Materials:
2.1.1. Chemicals
Diflunisal powder was obtained as a gift from Rameda pharmaceutical company in Egypt.

Meloxicam Aspirin and Diclofenac Na powder was obtained as a gift from Yemen Egyptian Pharma Company, Yemen. 2-amino-6- (trifluoromethyl) benzothiazole, 2-amino–5-(ethylthio)-1,3,4 thiadiazole , N, N-Dicyclohexylcarbodiimide (DCC) and Dimethyl sulfoxide (DMSO) were purchased from Apollo Scientific Chem. Co. U.K.
All others chemicals were purchased from Scharlau Chemics S.A. European Union.

2.1.2. Equipments:
(^{1}H–NMR) spectra were carried out on, JEOL 500 MHz- spectrometer (δ ppm), USA, using tetramethylsilane as the internal reference in DMSO.d$_6$. IR spectra were recorded using PerkinElmer FT-IR spectrometer (USA) using KBr pellets. Melting points were determined by using a calibrated STUART SMP11 (U.K.) melting apparatus. Rotary evaporator (R-210 V-700 V-850, Buchi, Switzerl). Kieselgel GF$_{254}$ (type 60) for TLC was supplied by E. Merck AG, Germany. The chromatographic spots were eluted by Ethyl acetate: Hexane (4:6) and was revealed by UV Lamb.

2.2. Methods
2.2.1. Chemical Synthesis [17, 18, and 19]
2.2.1.1. Synthesis of N–(5–ethylthio–2–(1, 3, 4–thiadiazole) Salicylamide (4)
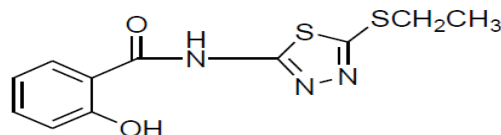
Aspirin, (10 g, 55.5 mmol) was dissolved in (160 ml) dichloromethane and dicyclohexylcarbodimide (DCC), (5.72 g, 27.7 mmol) was added. The reaction mixture was stirred at room temperature for 3 hrs. A white precipitate of dicyclohexylurea was formed and removed by filtration.

The solvent was evaporated under vacuum to yield the aspirin anhydride **(1)**.

Aspirin anhydride **(1)**, (2.5 g, 7.30 mmol), 2-amino–5-(ethylthio-1,3,4) thiadiazole (1.17gm , 7.30 mmol), zinc dust (0.005 g), glacial acetic acid (0.7 ml, 12.24 mmol), dioxane (30 ml) were placed in round bottom flask. The reaction

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mixture was refluxed gently with stirring for 2 hrs, the solvent was evaporated under vacuum, and the residue was dissolved in ethyl acetate, washed with 20 ml of $NaHCO_3$ (10%) 3 times , 20 ml of HCl (IN) 3 times and 3 times with 20 ml of distilled water, and filtered over anhydrous magnesium sulfate. The filtrate was evaporated under vacuum to give a crude product. The recrystallization was carried out using ethyl acetate-petroleum ether (60-80°C) mixture to produce compound (2) in 44.35% yield as a white powder.

Mp. 180-182. IR, (KBr, Cm^{-1}) 3250 (NH, amide), 3050 (ArH), 1740 (C=O ester), 1630 (C=O, amide), 1610, 1560, 1450 (C=C, Ar).

Compound (2), (1.61 g, 5 mmol) was dissolved in volume of ethanol (95%). The solution was cooled to 18 °C, and then sodium hydroxide (3 ml, 6 mmol, 2 N) was added dropwise, with stirring at 18 °C to produce compound (3) which was acidified with HCl (3 ml, 6 mmol, 2 N), excess of cold water was added and the crude phenolic compound was precipitated. The recrystallization was carried out by using ethanol 95% to yield compound (4) in 35% yield as a faint white crystals.

Mp. 214-216, R_f = 0.46. IR, (KBr, Cm-1) 3250 (NH, amide), 1604, 1541, 1405 (C=C, Ar), 1676 (-NH-C=O, carbonyl). ^{1}H – NMR (DMSO.d$_6$) δ pmm: 7.8-7.9 (1H, d, at 6 positions, ArH), 6.9-7.0 (3H, m, at 3 ,4,5 positions, ArH), 3.21-3.68 (2H,m, S-CH$_2$- thiadiazolyl), 1.31-1.41 (3H,m, CH$_3$, thiadiazolyl), 7.4 (s,1H, CONH,H exchangeable with D$_2$O), 12.16 (1H,s, OH, H exchangeable with D$_2$O).

2.2.1.2. Synthesis of N–(6'-trifluoromethyl –2'–benzothiazole) salicylamide (7)
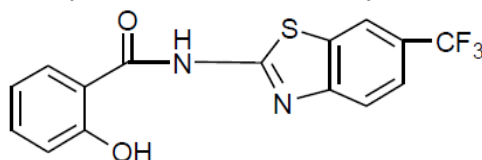

Aspirin anhydride (1), (2.5 g, 7.30 mmol), 2-amino–6-(trifluoromethyl) benzothiazole (1.59 g , 7.30 mmol), zinc dust (0.005 g), glacial acetic acid (0.7 ml, 12.24 mmol), dioxane (30 ml) were prepared as described before in compound (2), to generate compound (5) in yield of 52% as white powder.

Mp. 185-187. IR, (KBr, Cm^{-1}) 3341, 3248 (NH, amide), 3073 (ArH), 1765 (C=O ester), 1677 (C=O, amide), 1607, 1536, 1452 (Ar).

Compound (5), (1.90 g, 5 mmol) was treated as described in synthesis compound (3) to yield compound (6) which was treated as described in compound (4) afforded compound (7) in 43 % yields as a faint white crystals.

Mp. 282 decomposed, R_f = 0.26. IR, (KBr, Cm^{-1}) 3243 (NH, amide), 1609, 1501, 1415 (C=C, Ar), 1677 (-NH-C=O, carbonyl). ^{1}H – NMR (DMSO.d$_6$) δ pmm: 8.52 (1H, s, at 7' position, ArH), 7.95-7.96 (1H, d, at 6 position , ArH), 7.74-7.76 (2H, m, at 4' and 5' positions ArH), 6.9-7.0 (3H, m, at 3 ,4,5 positions, ArH), 7.4 (s,1H, CONH, H exchangeable with D$_2$O), 12.24 (1H,s, OH, H exchangeable with D$_2$O).

2.2.1.3. Synthesis of 5–(2', 4'–Difluorophenyl)–N–(5–(Ethylthio)–2–(1, 3, 4–thiadiazole) Salicylamide (12)
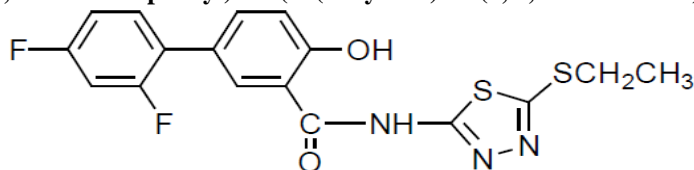
A dry Diflunisal, (10 g, 40 mmol) was placed in 200 ml round conical flask. Acetic anhydride (25 ml, 262 mmol) was added, and 5 drops of concentrated sulfuric acid was added dropwise, mixing the contents by rotating the conical flask, warm in water bath to about 50-60 °C, with stirring for 30 minutes. The reaction mixture was allowed to cool with occasionally stirring, then cold distilled water was added slowly to destroy the excess of acetic anhydride until formation of the precipitate, and filtered by using suction pump, washed with cold distilled water several times, and the crude product was collected. The recrystallization was carried out by using ethanol 95%, the precipitate was collected and dried to obtain acetyl diflunisal (8) in 82 % yield as a white crystals.

Acetyl diflunisal (8), (10 g, 34.22 mmol) was treated as described in synthesis compound (1) to yield diflunisal anhydride (9). Diflunisal anhydride (9), (2.5 g, 4.41 mmol), 2–amino-5-(ethylthio)–1,3,4–thiadiazole (0,71 g , 4.41
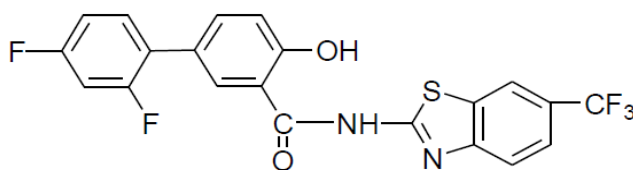
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mmol), zinc dust (0.004 g), glacial acetic acid (0.43 ml, 7.432), dioxane (40 ml), were prepared as previously described in synthesis compound (2), afforded compound (10) in 42% yield as faint white powder.

Mp. 115-118. IR, (KBr, Cm-1) 3331 (NH, amide), 3067 (ArH), 1767 (C=O ester), 1676 (C=O, amide), 1607, 1536, 1452 (Ar).

Compound (10), (2.17 g, 5 mmol) was treated as described in preparation compound (3) to liberate compound (11) which was treated as described in compound (4) to generate compound (12) in 29% yields as a white crystals.

Mp. 234-236, R_f = 0.38. IR, (KBr, Cm^{-1}) 3227 (NH, amide), 3075, 2974, 2926 (Ar), 1615, 1500, 1450 (C=C, Ar), 1680 ((-NH-C=O, carbonyl). ^{1}H – NMR (DMSO.d$_6$) δ pmm: 8.04 (1H,s, at 3'position ArH) 7.57-7.63 (2H, m, at 5'& 6' positions, ArH), 7.09-7.17 (3H, m, at 3 ,4,6 positions, ArH), 3.21-3.34 (2H,m, S-CH$_2$- thiadiazolyl), 1.31-1.34 (3H,m, CH$_3$, thiadiazolyl), 7.3 (s,1H, CONH, H exchangeable with D$_2$O), 12.51 (1H,s, OH, H exchangeable with D$_2$O).

2.2.1.4. Synthesis of 5–(2', 4'–Difluorophenyl) – N–(6''-trifluoromethyl) –2''–benzothiazole) salicylamide (15)

Diflunisal anhydride (**7**), (2.5 g, 4.41 mmol), 2-amino–6-(trifluoromethyl) benzothiazole (0.96 g , 4.41 mmol), zinc dust (0.004 g), glacial acetic acid (0.43 ml, 7.432), dioxane (40 ml), were prepared as described before in compound (**2**) to give compound (**13**) in 39% yield as a white powder.

Mp. 170-172°C. The IR (KBr, Cm^{-1}): 3233 (NH, amide), 3001, 3050 (ArH), 1745 (C=O, ester), 1630 (C=O, amide), 1605, 1560, 1450 (Ar).

Compound (**13**), (2.46 g, 5 mmol) was treated as described in compound (**3**) to generate compound (**14**) which was treated as described for compound (**12**) to generate compound (**15**) in 32% yields as a white powder.

Mp. 290-292, R_f = 0.37. IR, (KBr, Cm-1) 3233 (NH, amide), 1616, 1550, 1450 (C=C, Ar), 1686 (-NH-C=O, carbonyl). ^{1}H – NMR (DMSO.d$_6$) δ pmm: 8.52 (1H, s, at 7'' position, ArH), 8.1(1H,s,at 3' position, ArH) 7.88 (1H, d, at 6 position , ArH), 7.76 (2H, m, at 4'' and 5'' positions ArH), 7.66 (2H,m, at 5' and 6' positions), 7.18 (2H, m, at 3 ,4 positions, ArH), 7.32 (s,1H, CONH, H exchangeable with D$_2$O), 12.35 (1H,s, OH, H exchangeable with D$_2$O).

2.2.2. Preliminary Pharmacological Evaluations:
The acute anti-inflammatory activity of the newly synthesized compounds was evaluated in comparison with standard potent anti-inflammatory drug (Diclofenac Na), and with their parent drugs (Aspirin & Diflunisal). In addition, the acute ulcerogenicity of these compounds were evaluated in comparison with preferential selective COX-2 inhibitor (Meloxicam), and with COX-1 inhibitors (Aspirin & Diflunisal).

2.2.2.1. Experimental animals
The healthy albino rats weighing (200-250 g) were housed under standardized laboratory conditions of light and temperature (12 light-12 dark cycle at 23± 2 °C) for 7 days for adaptation and allowed free access to water and standard diet. The rats were fasted overnight prior to the experiment but allowed free access to water. All the animal experiments were performed by following the approval of study protocols by the Research Animals Ethics Committee, UST.

2.2.2.2. Evaluation of the acute anti-inflammatory activity
The acute anti-inflammatory activity in vivo model of the tested compounds were evaluated by utilizing undiluted fresh egg albumin-induced paw edema method [20]. The animals were randomly divided into eight different groups, each containing sex rats. The aspirin, diflunisal and diclofenac Na were suspended in propylene glycol 50% v/v and injected intraperitoneal (I.P) in different dose levels of (100, 50 and 10 mg/kg body weight) respectively. The control group received vehicle only (2 ml/kg propylene glycol 50% v/v) [21,22,23]. The tested compounds were given (I.P) in doses of equivalent to parent drugs doses after they dissolved in propylene glycol 50% v/v [24].

Thirty minutes post-treatment, the acute inflammation was induced by injecting 0.05ml of fresh egg albumin into the sub planter side of the left hind paw of each rat. Two hour after phlogistic agent injection, the rats were anaesthetized with ether, and the both left and right hind paws each rat were cut identically at the ankle joint and were weighed [25].

The edema weight difference between left and right paws was calculated. The average weight (mean) are determined and evaluated statistically. The percentage of inhibition of edema were calculated for control, diclofenac Na, aspirin, diflunisal and tested compounds by:

% Inhibition of edema = $(EW_C - EWt / EWc)$ x 100

Where: EWt and EWc: mean change in paw weight in test and control group, respectively [26].

2.2.2.3. Determination the gastric ulcerogenic effects
The gastric ulcerogenic effects in animal model was investigated by detection of possible ulcerogenic activity for tested compounds that exhibited marked anti-inflammatory activity compared with (Meloxicam), and with (Aspirin & Diflunisal). The animals were randomly divided into eight differences groups, each containing sex rats; they were fasted 20 hrs before vehicle and drug administration. The control group received vehicle only (DMSO). The aspirin, diflunisal and meloxicam were given orally in doses of (100,100, and 1.2 mg/kg body weight) respectively.

The tested compounds were given orally in doses of equivalent to diflunisal dose after they dissolved in DMSO. After that, rats were fasted for 2hrs, allowed to feed for 2 hrs, then fasted for another
20 hrs and given another doses in the second and third days. In the fourth day, animals were anaesthetized with ether, sacrificed, the stomach removed, opened along with the greater curvature and rinsed with water and 0.9% saline.

Under a dissecting microscope (10x), the number of mucosal damage were counted for each stomach from 0 to 4 according to the following score assignment system [27]: Zero = for normal (no injury), 1= latent small red spot, 2= wide red spot, 3= slight injury, and 4= sever injury.

% ulceration, average severity and ulcer index of test compounds were calculated according to [28]:
• % ulceration = No. of rats showing ulcer of any grade /total No. of rats in the group x 100
• % Incidence/10 = % ulceration /10.
• Average number of ulcers = No. of ulcers in the group/total No. of rats in the group.
• Average severity = Σ [each ulcer * by its score of severity]/number of ulcers in the group.
• Ulcer index = Σ [% Incidence/10 + Average number of ulcers + Average severity].

2.2.3. Statistical Analysis
Statistical processing of the result by using the test of analysis of variance (ANOVA test) to show the differences among all groups if it is present, the highly significance is considerable, in which $(p < 0.01)$. To conform that the result obtained by ANOVA test using T-test, in which highly significance if $(p < 0.01)$.

RESULTS AND DISCUSSION

3.1. Chemistry
The enol-carboxamide derivatives of aspirin and diflunisal (4, 7, 12 and 15) have been synthesized successfully according to the synthetic pathways shown in schemes (1 & 2), and their structures were confirmed, using ^{1}HNMR, IR spectra and their purity was confirmed by their physical data (melting points and R_f values).

The phenolic group in diflunisal was protected with acetic anhydride to prevent the interference of the phenolic group in subsequent reactions and allow to carboxyl group to react, as in scheme (2). This reaction is irreversible and accelerated by adding drops of conc. H_2SO_4 to produce acetyl diflunisal (8) [29].

The acetyl diflunisal and aspirin were converted to their reactive intermediates anhydride (1 & 9) respectively in presence (DCC) as coupling reagent and acidified methylene dichloride. Introduction of 2-amino-5-ethylthio-1,3,4-thiadiazole or 2-amino-6- (trifluoromethyl) benzothiazole to aspirin anhydride and acylated diflunisal anhydride resulted in formation of carboxamides compounds (2 & 5) and (10 &13) respectively in slightly acidic media (glacial acetic acid) and in the presence of Zn dust as catalyst to accelerate the coupling reaction .

This reaction is a nucleophilic reaction in which the nucleophile (–NH$_2$) is added to carbonyl carbon of anhydride. Finally, the removal of the acetate group in these acetylsalicylamide compounds by alkaline hydrolysis at 18^0 C, under acid catalysis, provided the novel enol-carboxamide derivatives of aspirin and diflunisal (4,7,12 and 15) [17, 18, and 19].
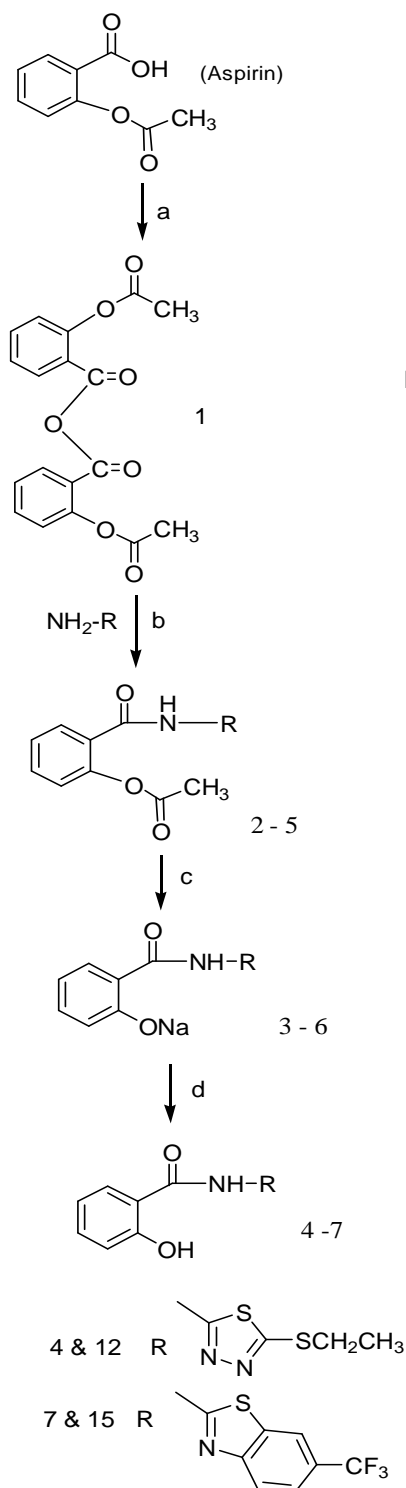
Scheme (1):
Synthesis of compounds (4 & 7)
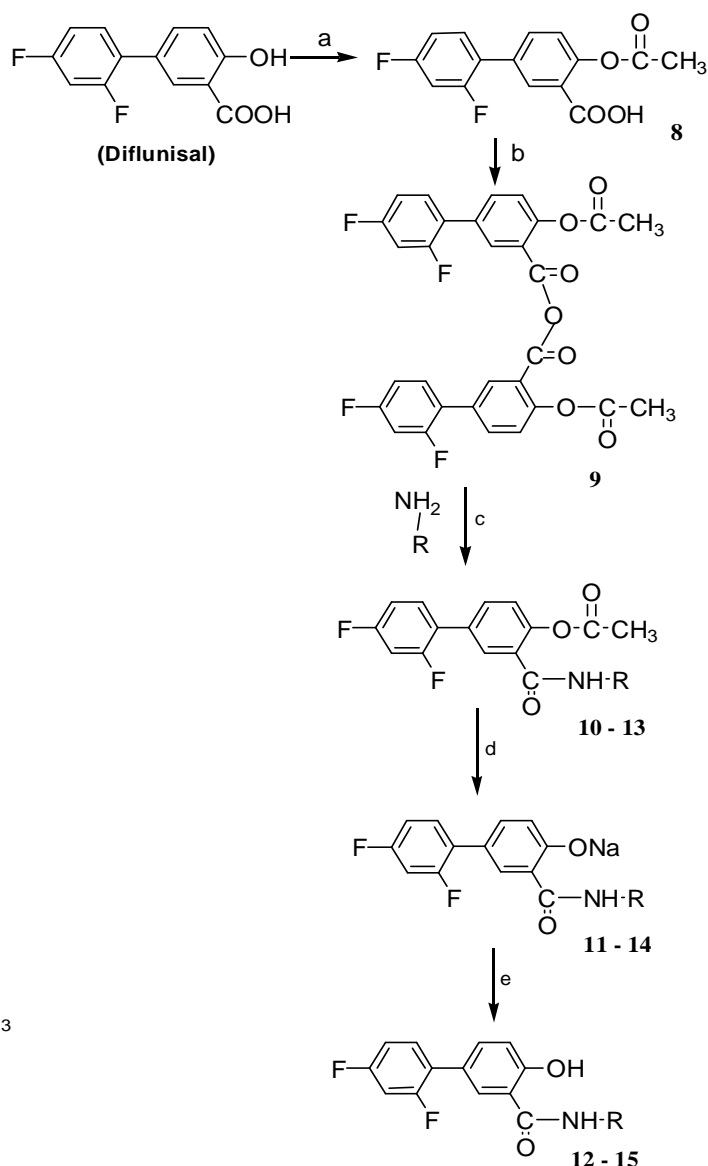
Scheme (2):
**Synthesis of compounds (12 & 15)**

3.2. Pharmacology

The acute anti-inflammatory activity of the test compounds were evaluated in rat using an egg-white induced edema model.

The differences in mean paw weights between the control (was 0.486) and diclofenac Na as standard (was 0.243) indicates that the method used in this test is a valid method and can effectively be used for the evaluation of the anti-inflammatory effect of the newly synthesized compounds.

As shown in (Fig.1), all tested compounds (4,7,12 and 15) showed significant anti-inflammatory activity (39.09, 48.81, 34.77 &53.32 %, respectively) as compared with the control group.

In addition, compound (15) showed high potent compared to diclofenac followed by compound (7) which showed a comparable effect to that of diclofenac, while compounds (4 & 12) showed less
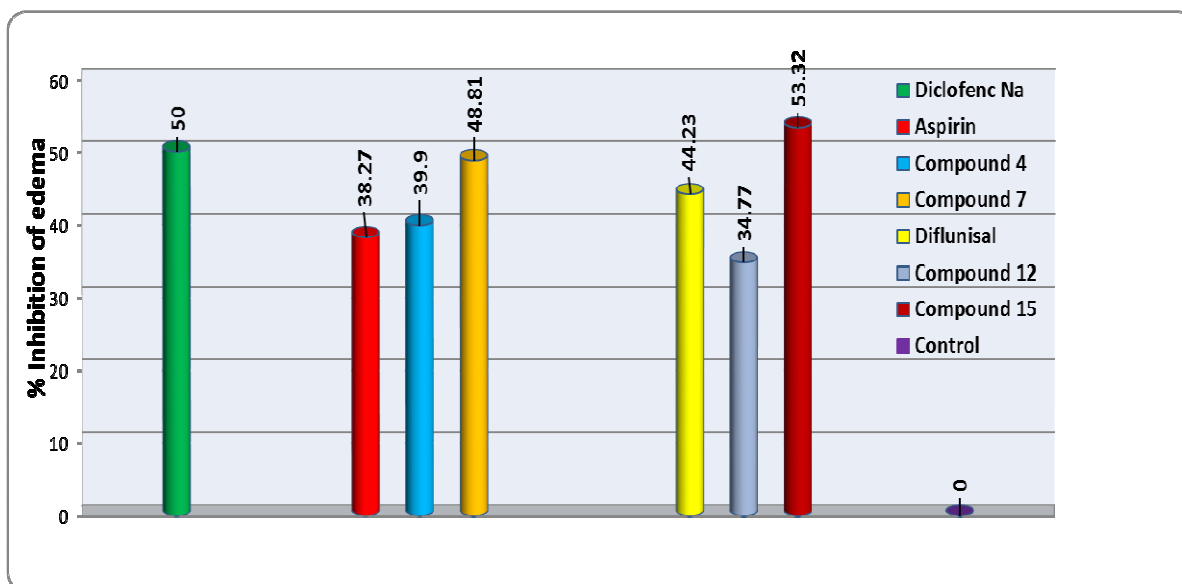
However, all tested compounds exhibited the most potent anti-inflammatory activity compared to their parent drugs (except for compound (12) which was less potent than diflunisal).

The ulcerogenic potential of tested compounds were evaluated.

COX-1 inhibitors (Aspirin & Diflunisal) showed the highest index (14.7 & 14) respectively, while preferentially selective COX-2 inhibitors (Meloxicam) showed the least index (4.5).

The all tested compounds (4, 7, 12 and 15) had shown lesser gastrotoxicity than their parent drugs and their ulcer index values were10.8, 9.5, 7.3 and 6.8, respectively, as showed in (Fig.2).

This improvement in anti-inflammatory and gastro protection effects of tested compounds compared to parent drugs might due to the masking of carboxylic groups of aspirin and diflunisal with antioxidants and anti-inflammatory heterocycles, such as thiadiazole and benzothiazole [30].

The compound (15) exhibited the highest anti-inflammatory activity among the all tested compounds, diclofenac sodium and its parent drug (diflunisal) with 53.32% edema reduction.

The compound (15) also had much weaker ulcerogenic effect than all tested compounds with 6.8 ulcer index. In addition, compound (12) achieved low ulcerogenic despite their lower anti-inflammatory activity as compared to diclofenac and diflunisal.

The presence of a difluorophenyl substituent in compounds (12 & 15) as well as the presence of trifluoromethyl moiety of the benzothiazole ring in compound (15) might play a role in incorporating this compound into the side pocket of COX-2 enzyme, so, achieved a good anti-inflammatory activity toward COX-2 inhibition with less GIT side effect [31,32].

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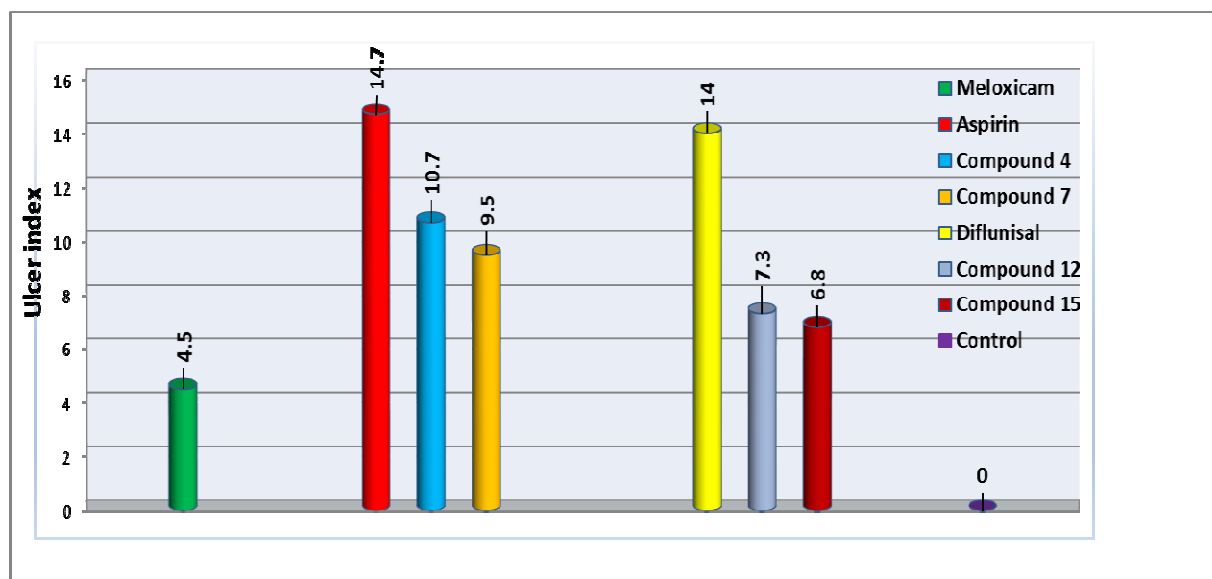
CONCLUSION

A series of enol carboxamide derivatives of aspirin and diflunisal (4,7,12 and 15) were synthesized in order to obtain new compounds as a potential anti-inflammatory agents with expected selectivity against COX-2 enzyme.

All tested compounds produced significant reduction of paw edema compared to the propylene glycol 50% v/v (control group) and had shown lesser gastrotoxicity than their parent drugs.

In addition, all tested compounds exhibited the most potent anti-inflammatory activity compared to their parent drugs (except for compound (12) which was less potent than diflunisal). Moreover, compound (15) showed maximal anti-inflammatory activity and least ulcerogenic effect.

The result of this study indicates that the incorporation of the trifluoromethyl benzothiazole or ethylthio thiadiazole pharmacophore into aspirin and diflunisal maintained or increased their anti-inflammatory activity and may increase selectivity towards the COX-2 enzyme, which will be confirmed in the future by assessing COX-2: COX-1 inhibitory ratios using a whole blood assay.

Acknowledgment

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