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Design, Synthesis and biological evaluation of some novel indole derivatives as selective COX-2 inhibitors

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ABSTRACT

A new group of (4-substitutedphenyl)(3-((2-(4- substitutedphenyl)hydrazono)methyl)-1H-indol-1-yl)methanone derivatives **13a-f** as indomethacin analogs was synthesized through *N*-benzoylation of indole-3-cabaldehyde with the appropriate benzoyl fragment followed by reaction with substituted phenylhydrazine. All the synthesized compounds were evaluated *in vitro* for COX-1/COX-2 inhibitory activity and *in vivo* for their anti-inflammatory activity in comparison with the parent drug indomethacin. Compounds **13a,b,d,e** which contain SO_2Me or SO_2NH_2 group as a pharmacophore of COX-2, exhibited the most anti-inflammatory and selectivity actives so, they were more evaluated by calculating their ED50% doses and ulcerogenic indices to ensure their gastric safety margin relative to indomethacin.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutics. Through their anti-inflammatory, anti-pyretic and analgesic activities, they represent a choice treatment in various inflammatory diseases, especially arthritis, as well as relieving the pains of everyday life (Abuo-Rahma *et al*., 2014, Abdellatif *et al.*, 2015). Their activity usually arises from inhibition of cyclooxygenase (COX) enzyme which mediates the bioconversion of arachidonic acid to inflammatory prostaglandins (PGs) and thromboxanes (TXs) (Zebardast *et al.*, 2009, Abdelazeem *et al.*, 2014). Cyclooxygenase enzyme exists in two distinct isoforms, a constitutive form (COX-1) and an inducible one (COX-2). The constitutive COX-1 isozyme is produced normally in a variety of tissues and is important to perform physiological functions such as gastro protection and vascular homeostasis. In contrast, COX-2 is induced during pathological processes such as inflammation and various cancer types (Rathish *et al.*, 2009, Al-Hourani *et al.*, 2011, Hassan *et al.*, 2014). Despite of their activity, many of NSAIDs, such as aspirin (**1**), ibuprofen (**2**) and indomethacin (**3**), have pronounced side effects such as gastrointestinal and renal toxicity resulting from the inhibition of gastro protective PGs synthesized through COX-1pathway (Abdellatif *et al.*, 2009, Abdelazeem *et al.*, 2015). Thus, it was though those more selective COX-2 inhibitors would have reduced side effects. Based upon a number of selective COX-2 inhibitors such as celecoxib (4), rofecoxib (5) and valdecoxib (6) were developed as safer NSAIDs with improved gastric safety profile. However, the recent market removal of some COXIBs such as rofecoxib and valdecoxib due to their adverse cardiovascular side effects clearly encourages the researchers to explore and evaluate alternative templates with COX-2 inhibitory activity (Dogné *et al.*, 2005, Chowdhury *et al.*, 2010, Huang *et al.*, 2010).

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A large number of studies (Hu *et al.*, 2003, Kalgutkar *et al.*, 2005, Khanna *et al.*, 2006) have used the indole ring based NSAIDs as in Indomethacin (**3**), as a target to improve their COX-2 selectivity and reduce their ulcerogenic side effects attributed to their high COX-1 selectivity and the acidic properties of the drug. Also, in recent studies, novel series of indomethacin analogs **7a-f** (Abdellatif *et al.*, 2016) and **8a-h** (Lamie *et al.*, 2016) were synthesized which were approved as good COX-2 selective inhibitors. So, these results encouraged us to continue the research on such type of compounds. Our strategy in this research is based on maintaining the potency of the indomethacin by keeping the main scaffold of the drug with trials to increase COX-2 selectivity via the modifications of the side groups. We now describe the synthesis, *in vitro* evaluation as COX-1/COX-2 inhibitors, *in vivo* anti-inflammatory(AI) activity, and ulcerogenic liability for a new series of N-substituted indole derivatives as indomethacin analogs **13a-f** in which, (i) the $-CH_2COOH$ moiety in position 3 of indomethacin was replaced with an aromatic moiety containing phenyl hydrazine substituted with COX-2 pharmacophore, SO_2Me in **11a,d** or $SO2NH_2$ in **11b,e** or with methyl group in **11c,f** to evaluate the effect of these groups on COX selectivity and anti-inflammatory activity, (ii) the chlorobenzoyl moiety of indomethacin in position 1, which is important for anti-inflammatory activity, is maintained in **11d, 11e, 11f** or replaced with benzoyl in **10a, 10b, 10c**, and (iii) methyl group at position 2 and methoxy group at position 5 was removed in all compounds.

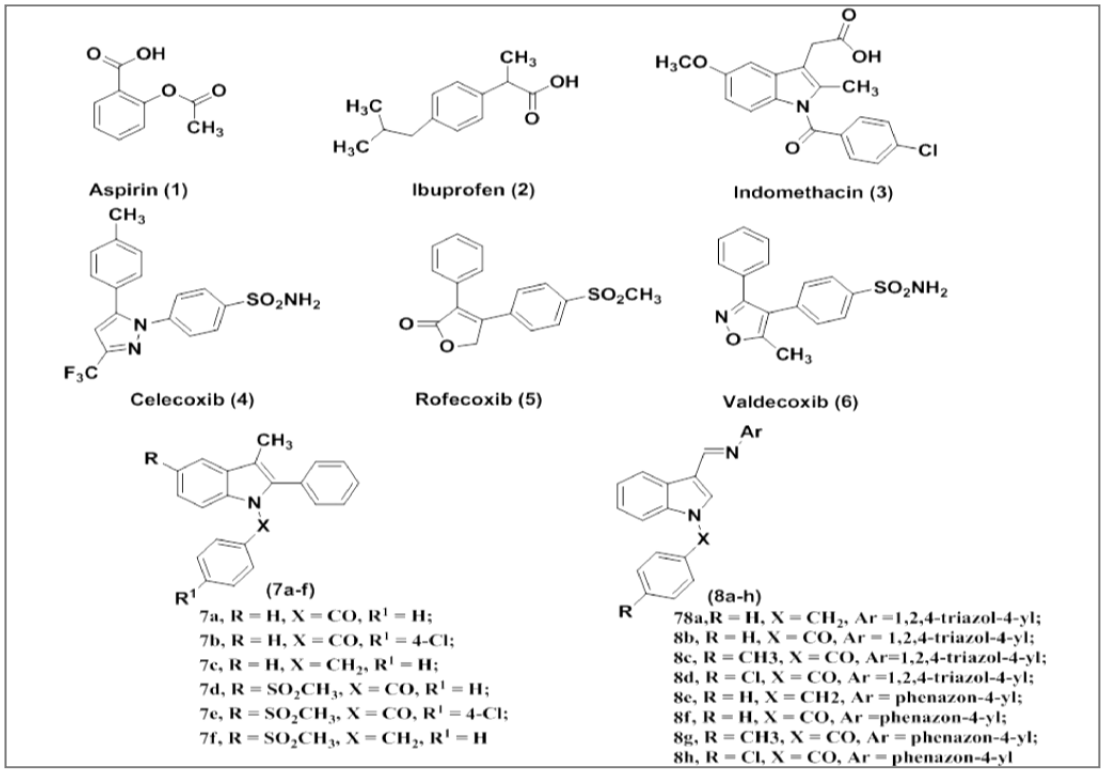


Fig. 1: Chemical structures of some traditional NSAIDs (**1-3**), some selective COX-2 inhibitor drugs (**4-6**) and reported indomethacin analogs (**7a-f** and **8a-h**).

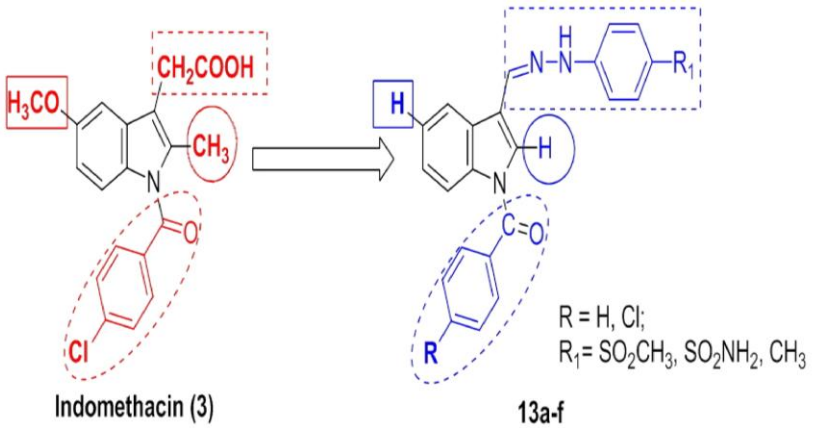


Fig. 2: Chemical structures of indomethacin (**3**) and the designed N-substituted indole derivatives **13a-f**.

MATERIALS AND METHODS

Instrument and reagents

Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded as films on KBr plates using a Nicolet 550 Series II Magna FT-IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker Avance III 400 MHz spectrophotometer, Faculty of Pharmacy, Beni-Suef University, Egypt in DMSO-d_6 with TMS as the internal standard, where *J* (coupling constant) values are estimated in Hertz (Hz) and chemical shifts were recorded in ppm on δ scale. Mass spectra (MS) were recorded on Hewlett Packard 5988 spectrometer. Microanalyses for C, H and N were carried out on Perkin-Elmer 2400 analyzer (Perkin-Elmer, Norwalk, CT, USA) at the regional center for mycology and Biotechnology, Al-Azhar University, Egypt. Silica gel column chromatography was performed using Merck silica gel 60 ASTM (70-120 mesh). All other reagents, indole (**9**) and p-tolylhydrazine hydrochloride (**12c**) were purchased from the Aldrich Chemical Company (Milwaukee, WI), were used without further purification.

Chemistry

Indole-3-carboxaldehyde (**10**)(Guillon *et al.*, 2011, Kumar *et al.*, 2012); 1-benzoyl-1*H*-indole-3-carbaldhyde (**11a**) (Wang *et al.*, 2012); 1-(4-chlorobenzoyl)-1*H*-indole-3-carbaldhyde (**11b**)(Singh *et al.*, 2010); (4-methylsulphonylphenyl) hydrazine hydrochloride (**12a**)(Abdellatif *et al.*, 2008) and (4-aminosulphonylphenyl) hydrazine hydrochloride (**12b**)(Abdellatif *et al.*, 2008) were prepared according to the reported procedures.

General procedure for the synthesis of (4-substitutedphenyl)(3-((2-(4- substitutedphenyl)hydrazono)methyl)-1H-indol-1-yl)methanone 13a-f

A mixture of **11a** or **11b** (0.3gm, 1 mmol) and the appropriate phenyl hydrazine hydrochloride derivative **12a-c** (1.2 mmol) in absolute ethanol (10 mL) and glacial acetic acid (1ml) was refluxed for 5-7h (monitored by TLC). The precipitate that formed on hot was filtered off, dried and recrystallized from 95% ethanol to afford **13a-f**

(3-((2-(4-(methylsulfonyl)phenyl)hydrazono)methyl)-1H-indol-1-yl)(phenyl)- methanone (13a)

Yellow solid; Yield 72%; mp 191-193 °C; IR (KBr) 3298 (NH), 3059, 3024 (CH aromatic), 2924, 2854 (CH aliphatic), 1685 (C=O), 1597 (C=N), 1323, 1138 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.12 (s, 3H, SO_2CH_3), 7.22 (d, 2H, *J* = 8.8Hz, phenyl H-3, H-5), 7.48 (d, 2H, *J* = 9.2Hz, phenyl H-2, H-6), 7.64 (d, 2H, *J* = 8Hz, indole H-5, H-6), 7.71 (s, 1H, indole H-2), 7.76 (d, 2H, *J* = 8.8Hz, benzoyl H-3, H-5), 7.81-7.86 (m, 3H, benzoyl H-2, H-4, H-6), 8.17 (s, 1H, CH=N), 8.33 (d, 1H, *J* = 8.8Hz, indole H-7), 8.46 (d, 1H, *J* = 8.8Hz, indole H-4), 10.93 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 44.78 (SO_2CH_3), 111.78, 116.35, 118.28, 122.94, 125.19, 126.16, 127.70, 129.31, 129.49, 129.61, 129.66, 130.06, 132.75, 134.24, 136.25, 136.86, 149.62(CH=N), 168.58(*C*=O); MS *m/z* (ES+) 417.48 (M^+). Anal. Calcd for $C_{23}H_{19}N_3O_3S$: C, 66.17; H, 4.59; N, 10.07. Found: C, 65.89; H, 4.71; N, 10.34.

4-(2-((1-benzoyl-1H-indol-3-yl) methylene) hydrazinyl) benzenesulfonamide (13b)

Brown solid; Yield 65%; mp 211-213 °C; IR (KBr) 3429, 3321 (NH2), 3290 (NH), 3116, 3062 (CH aromatic), 2924, 2854 (CH aliphatic), 1662 (C=O), 1597 (C=N), 1327, 1153 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.08 (s, 2H, NH_2, D_2O exchangeable), 7.16 (d, 2H, *J* = 8.8Hz, phenyl H-2, H-6), 7.35 (d, 2H, *J* = 7.6Hz, phenyl H-3, H-5), 7.44-7.62 (m, 5H, indole H-2, H-5, H-6, benzoyl H-3, H-5), 7.71-7.77 (m, 3H, benzoyl H-2, H-4, H-6), 8.14 (s, 1H, CH=N), 8.32 (d, 1H, *J* = 9.2Hz, indole H-7), 8.44 (d, 1H, *J* = 9.2Hz, indole H-4), 10.74 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 44.78 (SO_2CH_3), 111.35, 116.29, 118.25, 122.96, 125.14, 126.18, 126.74, 128.01, 129.36, 129.48, 129.74, 131.60, 133.07, 133.64, 135.14, 136.19, 148.12(CH=N), 167.59(*C*=O); MS *m/z* (ES+) 418.47 (M^+). Anal. Calcd for $C_{22}H_{18}N_4O_3S$: C, 63.14; H, 4.34; N, 13.39. Found: C, 63.25; H, 4.46; N, 13.61.

phenyl(3-((2-(p-tolyl)hydrazono) methyl)- 1H-indol-1-yl) methanone (13c)

Yellow solid; Yield 80%; mp 146-148 °C; IR (KBr) 3298 (NH), 3028 (CH aromatic), 2954, 2912, 2854 (CH aliphatic), 1662 (C=O), 1543 (C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.23 (s, 3H, CH3), 7.00 (d, 2H, *J* = 7.6Hz, phenyl H-2, H-6), 7.08 (d, 2H, *J* = 8Hz, phenyl H-3, H-5), 7.47 (d, 2H, *J* = 7.6Hz, indole H-5, H-6), 7.63 (d, 2H, *J* = 7.6Hz, benzoyl H-3, H-5), 7.71-7.77 (m, 2H, benzoyl H-4, indole H-2), 7.80 (d, 2H, *J* = 7.6Hz, benzoyl H-2, H-6), 8.02 (s, 1H, CH=N), 8.32 (d, 1H, *J* = 6.8Hz, indole H-7), 8.45 (d, 1H, *J* = 6.8Hz, indole H-4), 10.19 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 20.75 (CH_3), 112.24, 116.34, 119.06, 122.98, 125.00, 125.99, 127.46, 128.04, 128.14, 129.26, 129.59, 130.10, 132.03, 132.60, 134.34, 136.84, 143.65(CH=N), 168.49(*C*=O); MS *m/z* (ES+) 353.42 (M^+). Anal. Calcd for $C_{23}H_{19}N_3O$: C, 78.16; H, 5.42; N, 11.89. Found: C, 77.89; H, 5.86; N, 12.04.

(4-chlorophenyl)(3-((2-(4-(methylsulfonyl) phenyl) hydrazono) methyl)-1H-indol-1-yl)methanone (13d)

Buff powder; Yield 81%; mp 202-204 °C; IR (KBr) 3302 (NH), 3093, 3051 (CH aromatic), 2924, 2839 (CH aliphatic), 1678 (C=O), 1593 (C=N), 1327, 1091 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.12 (s, 3H, SO_2CH_3), 7.22 (d, 2H, *J* = 8.8Hz, phenyl H-3, H-5), 7.49 (d, 2H, *J* = 9.2Hz, phenyl H-2, H-6), 7.70 (d, 2H, *J* = 8.4Hz, indole H-5, H-6), 7.76 (d, 2H, *J* = 8.8Hz, benzoyl H-3, H-5), 7.84-7.89 (m, 3H, benzoyl H-2, H-6, indole H-2), 8.16 (s, 1H, CH=N), 8.31 (d, 1H, *J* = 8.8Hz, indole H-7), 8.42 (d, 1H, *J* = 8.8Hz, indole H-4), 10.95 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 44.82 (SO_2CH_3), 111.80, 116.62, 118.40, 123.05, 125.27, 126.54, 127.91, 129.45, 129.58, 129.87, 130.00, 130.14, 132.95,

134.52, 136.28, 137.00, 149.65(CH=N), 168.78(*C*=O); MS *m/z* (ES+) 451.93 (M^+). Anal. Calcd for $C_{23}H_{18}ClN_3O_3S$: C, 61.13; H, 4.01; N, 9.30. Found: C, 61.42; H, 4.18; N, 9.56.

4-(2-((1-(4-chlorobenzoyl)-1H-indol-3-yl)methylene) hydrazinyl) benzene-sulfonamide (13e)

Reddish brown solid; Yield 78%; mp 140-142 °C; IR (KBr) 3436, 3332 (NH2), 3294 (NH), 3089, 3051 (CH aromatic), 2924, 2850 (CH aliphatic), 1678 (C=O), 1593 (C=N), 1329, 1153 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.08 (s, 2H, NH_2, D_2O exchangeable), 7.16 (d, 2H, *J* = 8.8Hz, phenyl H-2, H-6), 7.49 (d, 2H, *J* = 8.8Hz, phenyl H-3, H-5), 7.69-7.72 (m, 3H, indole H-2, H-5, H-6), 7.84-7.87 (m, 4H, benzoyl H-2, H-3, H-5, H-6), 8.13 (s, 1H, CH=N), 8.33 (d, 1H, *J* = 9.2Hz, indole H-7), 8.43 (d, 1H, *J* = 9.2Hz, indole H-4), 10.77 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 111.47, 116.37, 118.42, 123.02, 125.38, 126.24, 126.85, 128.05, 129.40, 129.52, 129.88, 131.60, 133.12, 133.71, 135.23, 136.22, 148.27(CH=N), 167.82(*C*=O); MS *m/z* (ES+) 452.91 (M^+). Anal. Calcd for $C_{22}H_{17}ClN_4O_3S$: C, 58.34; H, 3.78; N, 12.37. Found: C, 58.62; H, 3.91; N, 12.48.

(4-chlorophenyl)(3-((2-(p-tolyl)hydrazono)methyl)-1H-indol-1-yl)methanone (13f)

Yellow solid; Yield 76%; mp 192-194 °C; IR (KBr) 3286 (NH), 3032 (CH aromatic), 2939, 2912, 2854 (CH aliphatic), 1654 (C=O), 1543 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.23 (s, 3H, CH3), 7.00 (d, 2H, *J* = 8Hz, phenyl H-2, H-6), 7.07 (d, 2H, *J* = 8Hz, phenyl H-3, H-5), 7.46 (d, 2H, *J* = 7.6Hz, indole H-5, H-6), 7.68-7.73 (m, 3H, benzoyl H-3, H-5, indoleH-2), 7.83 (d, 2H, *J* = 8.4Hz, benzoyl H-2, H-6), 8.00 (s, 1H, CH=N), 8.34 (d, 1H, *J* = 6.8Hz, indole H-7), 8.45 (d, 1H, *J* = 7.2Hz, indole H-4), 10.22 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 20.75 (CH_3), 112.24, 116.37, 119.25, 123.01, 125.10, 126.06, 127.49, 128.06, 128.14, 129.37, 130.11, 131.55, 131.94, 133.23, 136.84, 137.36, 143.61(CH=N), 167.53(*C*=O); MS *m/z* (ES+) 387.86 (M^+). Anal. Calcd for $C_{23}H_{18}ClN_3O$: C, 71.22; H, 4.68; N, 10.83. Found: C, 70.96; H, 4.60; N, 11.09.

Biological evaluation

Animals

Adult male Wister albino rats (120-150 g) were obtained from the animal house, (Nahda University, Beni-Suef, Egypt) were used throughout the study and were kept at controlled conditions (temperature 27 ± 2 °C, humidity 60 ± 10%) and a 12/12 h light/dark cycle. The animals were housed in stainless steel cages, divided into groups of four animals each and deprived of food not water 24 h before the experiment. All procedures relating to animal care and treatments were conducted in accordance with protocols approved by the Research Ethical Committee of Faculty of Pharmacy Beni-Suef University (2017-Beni-Suef, Egypt).

COX-1/COX-2 inhibition colorimetric assay

The ability of tested compounds listed in Table 1 was measured using colorimetric COX (ovine) Inhibitor Screening Assay Kit (catalog no.560131, Cayman Chemical, Ann Arbor, MI, USA) according to the previous reported method(Roschek Jr *et al.*, 2009). This assay directly measures $PGF_{2\alpha}$ that was produced by stannous chloride reduction of COX derived PGH_2 by enzyme immunoassay.

Carrageenan-induced rat paw edema assay

The anti-inflammatory activity of newly synthesized indomethacin derivatives was evaluated by using carrageenan-induced rat paw edema test (El-Nezhawy *et al.*, 2013). Rats were divided into 9 groups (4 animals per each group) then, they were administered with a suspension of vehicle, tested compounds or indomethacin in 10% DMSO at a dose of 10 mg/kg orally (one group per one compound). After 30 min, the rats received 100 µL of carrageenan (1% in saline) subcutaneously on the sub plantar region of the left hind paw. The left paw thickness was measured after 1, 3 and 6 h after carrageenan injection. The right hind paw served as a reference of non-inflamed paw for comparison. Results are expressed as percentage decrease in edema thickness induced by carrageenan. Compounds **13a**, **13b**, **13d**, **13e** and indomethacin were experimented for calculating ED_{50} values by using at least three doses and the paw thickness was measured after 3 h after carrageenan injection.

Ulcerogenic liability

The most potent Compounds **13a, 13b, 13d, 13e** and indomethacin were experimented for their ulcerogenic liability according to the reported method (Abdellatif *et al.*, 2015). Rats were divided into 6 groups of 5 animals each, and then were fasted for about 18h before drug administration. The 4 tested compounds and indomethacin as a reference drug were given orally at a dose of 10 mg/kg suspended in 10% DMSO while, remaining group received DMSO as a control negative group. Treatment was continued once daily for 3 successive days in all groups. At fourth day, one hour after the last dose, animals were sacrificed under general anesthesia and stomachs were removed, collected, opened along the greater curvature, washed with distilled water and rinsed with saline. The gastric mucosa of each stomach was examined for the presence of lesions by using magnifying lens (10X). The number of mucosal lesions which appeared as red spots was counted, and their severity was determined and graded from 0-4.

The following parameters were calculated:

1- % Incidence /10 = [Number of rats showing ulcer of any grade divided by total number of rats in the group x 100]/10
2- Average number of ulcer = Number of ulcer in the group/ total number of rats in the group
3- Average severity =∑ [each ulcer multiplied by its score of severity/ number of ulcer in the group.
4- Ulcer index = the sum of the above three parameters. (% Incidence /10 + Average number of ulcer + Average severity)

Ulcer index value was compared to that of indomethacin**.**

Molecular Docking

The 3D crystal structure of valdecoxib bound at the COX-2 (PDB:ID 2AW1) active sites (Abdellatif et al., 2008), obtained from protein data bank at research collaboration for structural Bioinformatics (RSCB) protein database [PDB]. Preparation of the synthesized compounds 13d and 13e for docking was achieved via their 3D structure built by Molecular Operating Environment (MOE, Version 2014.09, Chemical Computing Group Inc., Montreal, Quebec, Canada). They were 3D protonated and subjected to energy minimization using MMFF94 _ force field with 0.05 gradient. Preparation of the enzyme for docking was achieved as follows: (1) The Co-crystallized ligand and water molecules were removed. (2) The enzyme was 3D protonated, in which hydrogen atoms were added to their standard geometry, the partial charges were computed and the system was optimized. The conformers generated were docked into the COX-2 receptor with MOE-DOCK using the triangle matcher placement method and the London dG scoring function. A molecular mechanics force field refinement was carried out on the top 100 poses generated. Docking for the synthesized compounds was applied. Amino acid interactions and the hydrogen bond lengths were summarized in (Table 4).

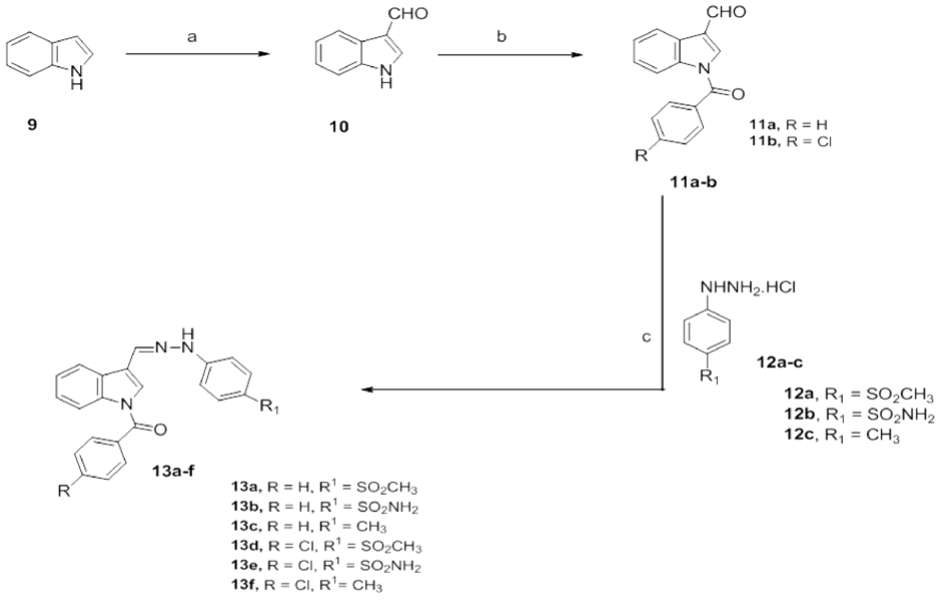
RESULTS AND DISCUSSIONS

Chemistry

The synthesis of the new compounds (4-substitutedphenyl)(3-((2-(4- substitutedphenyl)hydrazono)methyl)-1H-indol-1-yl)methanone derivatives was achieved through using the reaction sequence illustrated in Scheme 1. The starting material indole-3-carbaldehyde 10 was prepared in a good yield (70%) via vilsmeier reaction, and then reacted with benzoyl or p- chloro benzoyl chloride in dry DMF under basic condition using NaH to give compounds 11a-b. Compounds 11a-b were allowed to react with 4-methylsulfonyl- phenylhydrazine hydrochloride 12a, 4-aminosulphonylphenylhydrazine hydrochloride 12b or 4-methylphenylhydrazine hydrochloride 12c in absolute ethanol under reflux conditions to give target compounds 13a-f in good yields (65-80%).

All the newly synthesized compounds 13a-f has been characterized by IR, 1HNMR, 13CNMR, mass spectra, and elemental analyses. The IR spectra of these compounds showed the appearance of a sharp singlet peak at 3302-3286 cm-1 corresponding to NH group, two sharp peaks at 1654-1685 and 1543-1597 cm-1 corresponding to C=O and C=N respectively. While, compounds such as 13a,b,d,e exhibited two sharp peaks at 1323-1329 and 1138-1153 cm-1 corresponding to SO2, in addition to a forked peak at 3436-3429 and 3332-3321 cm-1 corresponding to NH2 for compounds 13b,e.

Also, 1HNMR spectra for indole derivatives showed singlet peak at δ 3.12 or 7.08 or 2.23 corresponding to SO2CH3 for compounds 13a,d or SO2NH2 for compounds 13b,e or CH3 for compounds 13c,f. Additionally, all compounds exhibited two singlet peaks at δ 8.00-8.17 and 10.19-10.95 corresponding to CH=N and NH respectively. Finally, 13CNMR spectra showed peak at δ 44.78-44.82 corresponding to SO2CH3 for compounds 13a,d, peak at δ 20.75 for CH3 for compounds 13b,e and absence of aliphatic carbons for compounds 13c,f. Two other peaks appeared at δ 143.61-149.65 and 167.53-168.78 corresponding to CH=N and C=O for all final compounds.



Reagents and reaction conditions: (a) $POCl_3$, dry DMF, reflux, 8 h; **(b)** appropriate acid chloride, NaH, DMF, RT, overnight; **(c)** abs.EtOH, gl.acetic acid, refux, 5-7 h.

Biological evaluation

***In vitro* cyclooxygenase (COX) inhibition assay**

The *in vitro* COX-1/COX-2 isozyme inhibition studies for the new indomethacin analogs **13a-f** revealed a reversal of COX selectivity profile compared to indomethacin. The newly synthesized compounds showed relatively weak inhibition of COX-1 subtype with IC_{50} values 6.7 – 10.1 μM while, they were highly potent inhibitors of COX-2 subtype with IC_{50} values 0.19 - 0.53 μM consequently compounds **13a-f** were highly COX-2 selective with COX-2 selectivity indexes (S.I. 12.64 – 53.16) in comparison with standard indomethacin (COX-1 IC_{50} = 0.039 μM, COX-2 IC_{50} = 0.49 μM and COX-2 S.I. = 0.079) (Table 1). Data from Table 1 revealed that i) all tested compounds **13a-f** exhibited more potent inhibition for COX-2 than COX-1, ii) compounds having the SO_2Me or SO_2NH_2 as COX-2 pharmacophore (**13a,d** with S.I. 37.83 and 51.11 respectively and **13b,e** with S.I. 35.42 and 53.16 respectively) were more potent inhibitors of COX-2 than the corresponding analogs containing CH_3 (**13c-f** with S.I. 12.64 and 14.79 respectively) and that confirms the importance of SO_2Me for COX-2 selectivity, iii) compounds having chloro benzoyl moiety at indole N (**13d,e,f** with S.I. 51.11, 53.16, 14.79 respectively) exhibited higher potency for COX-2 than that having benzoyl one (**13a,b,c** with S.I. 37.83, 35.43, 12.64 respectively) and iv) within all compounds **13a-f**, compounds **13e** was the most potent COX-2 inhibitors and the most COX-2 selective and it was about 650 folds more COX-2 selective than indomethacin (COX-2 IC_{50} = 0.49 μM, S.I. = 0.079).

Table 1: In vitro COX-1 and COX-2 inhibition for compounds **13a-f,** and reference drug (Indomethacin).

Compounds	COX Inhibition (IC_{50} μM)[a]		Selectivity Index[b]
	COX-1	COX-2	
13a	8.7	0.23	37.83
13b	8.5	0.24	35.42
13c	6.7	0.53	12.64
13d	9.2	0.18	51.11
13e	10.1	0.19	53.16
13f	7.1	0.48	14.79
Indomethacin	0.039	0.49	0.079

[a] The concentration of test compound produce 50% inhibition of COX-1, COX-2 enzyme, the results are the mean of two value obtained by assay of enzyme kits obtained from (Cayman Chemicals Inc., AnnArbor, MI, USA) where the deviation from the mean is < 10% of the mean value.
[b] Selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).

***In vivo* anti-inflammatory activity**

The anti-inflammatory activity of the prepared indomethacin derivatives **13a-f** was evaluated using carrageen-induced rat paw edema test in comparison to indomethacin as a reference drug. Each compound was administered orally (10 mg/kg) immediately prior to induction of inflammation by carrageenan subcutaneous injection. The anti-inflammatory activity was then calculated based on paw-thickness changes at 1, 3 and 6 hours after carrageenan injection as presented in Table 2.

A comparable study of the anti-inflammatory activity of the test compounds relative to indomethacin as a reference drug at different time intervals indicated that; after 1 h, the indomethacin derivatives (**13a-f**) showed an intermediate edema inhibition activity between 41.6–58.7% and compounds **13d,e** were the most potent derivatives (56.7, 58.7% edema inhibition for **13d** and **13e**) in comparison with indomethacin (56% edema inhibition). After 3 h, **13a-f** showed a remarkable increase in edema inhibition percentage activities 60.6 – 87.2% and compound **13e** was also the most potent derivatives (87.2% edema) in comparison with indomethacin (86.7% edema inhibition). After 6 h, all compounds showed a little increase in edema inhibition percentage activities 65.8 – 91.5%, while indomethacin showed a much increase in edema inhibition percentage activity 95.1%.

The results, seen in (Table 2**),** were consistent with the *in vitro* results and in a similar manner to *in vitro* data, the *in vivo* data indicated the same conclusions; i) the presence SO_2Me or SO_2NH_2 moiety (**13a,d** and **13b,e)** increases the anti-inflammatory activity for this class of compounds, ii) 4-chlorobenzoyl is favorable over unsubstituted benzoyl for substitution at indole N, iii) also, within all compounds **13a-f**, the most potent COX-2 inhibitor and the most COX-2 selective (**13e**) was the most potent anti-inflammatory derivative after 3 h of carrageenan injection (91.5% edema inhibition) in comparison with indomethacin (86.7% edema inhibition).

Moreover, ED_{50} values for the most four potent derivatives (**13a**, **13b, 13d** and **13e**) were calculated after three hours from drug administration in comparison with reference drug indomethacin. The four derivatives (**13a, 13b, 13d and 13e**) showed good anti-inflammatory activities (ED_{50} = 0.6, 1.05, 0.48 and 0.22 mg/kg respectively) in comparison with indomethacin (ED_{50} = 0.4 mg/kg). The most COX-2 selective derivative (**13e**, about 650 folds more COX-2 selective than indomethacin) was the most potent anti-inflammatory derivative (ED_{50} = 0.22 mg/kg = approximately 1.8 x potency of indomethacin).

Table 2: Percentage Inhibition of tested compounds (**13a-f)** at 1, 3, and 6 h after carrageenan injection in comparison with indomethacin.

Comp	Oedema thickness (mm) ± SEM (oedma inhibition %)[a]			ED50[b] (mg/kg)
	1 h (% inhibition)	3h (% inhibition)	6 h (% inhibition)	
Control	2.118 ± 0.025	2.215 ± 0.028	1.878 ± 0.029	…….
Indomethacin	0.933 ± 0.027 (55.96%)	0.295 ± 0.033 (86.68%)	0.050 ± 0.015 (95.07%)	0.40
13a	0.960 ± 0.014 (54.67%)	0.408 ± 0.023 (81.58%)	0.260 ± 0.027 (86.16%)	0.60
13b	1.013 ± 0.025 (52.17%)	0.400 ± 0.021 (81.94%)	0.215 ± 0.028 (88.55%)	1.05
13c	1.238 ± 0.024 (41.55%)	0.878 ± 0.026 (60.63%)	0.643 ± 0.026 (65.76%)	NDc
13d	0.918 ± 0.017 (56.66%)	0.300 ± 0.012 (86.46%)	0.160 ± 0.014 (91.48%)	0.48
13e	0.875 ± 0.012 (58.69%)	0.280 ± 0.019 (87.24%)	0.176 ± 0.010 (90.63%)	0.22
13f	1.200 ± 0.017 (43.34%)	0.818 ± 0.021 (63.07%)	0.573 ± 0.034 (69.49%)	ND[c]

[a]Data analyzed by one way ANOVA, (n = 4), P < 0.05, all were significant from control. [b]ED50 values are determined at 3 h after oral administration of compounds and expressed in mg/Kg. [c]ND = Not Determined.

Ulcerogenic liability test

The most potent anti-inflammatory compounds (**13a**, **13b, 13d** and **13e**) were tested for their ulcerogenic liability in comparison with indomethacin (Table 3). The results revealed that, all tested compounds exhibited lower ulcerogenic liability (ulcer index = 8.87, 6.40, 6.97 and 3.9 respectively) in comparison with indomethacin (Ulcer Index = 20.2). **13e (**the most COX-2 selective derivative with about 650 folds more COX-2 selective than indomethacin and the most potent derivative has approximately 1.8 x potency of indomethacin) was also the least ulcerogenic derivative (Ulcer Index = 3.9) which approximately one fifth ulcerogenic liability of indomethacin). The tested compounds (**13a**, **13b, 13d** and **13e**) were characterized by the presence of a SO_2Me or SO_2NH_2 moiety (COX-2 pharmacophore) and absence of an acidic center, in contrast to indomethacin which having an acidic center and devoid of a COX-2 pharmcophore moiety. Consequently, these compounds possess more selectivity to COX-2 isozyme and exhibited an excellent gastric safety profile compared to indomethacin which caused a great damage on gastric membrane that could be attributed to the high affinity to COX-1 over COX-2.

Table 3: Ulcer index of tested compounds (**13a,b,d,e)** in comparison with indomethacin as a reference drug.

Compound No	% Incidence	Average no of ulcer	Average severity	ulcer index
13a	6	1.2	1.67	8.87[a]
13b	4	1.0	1.4	6.4[a]
13d	4	1.4	1.57	6.97[a]
13e	2	0.6	1.30	3.9[a]
Indomethacin	8	9.2	3	20.2

Molecular Docking

With the aim to understand the protein-inhibitor interaction of the synthesized compounds **13d,e** within the COX-2 isozyme, molecular docking experiments were performed using X-ray crystal structure data for COX-2 obtained from the protein data bank (Kurumbail *et al.*, 1996, Di Fiore *et al.*, 2006). Valdecoxib (**6**) was used as a ligand for COX-2 isoform. The interaction of valdecoxib with COX-2 isozyme afforded three hydrogen bonding interactions and one hydrophobic interaction ; i) NH_2 with His96 (3.31 A^o), ii) NH_2 with Thr199 (2.94 A^o), iii) SO_2 with Thr199 (2.92 A^o) and iv) phenyl ring with Asn67 (4.73 A^o).

In this work the docking results including the energy associated with intermolecular interactions (affinity in Kcal/mol) obtained upon computational docking for all compounds (**13d,e** and valdecoxib) within COX-2 active site and the hydrogen bonding interactions between the amino acid residues and functional groups of the compounds are listed in Table 4. With COX-2, compounds **13d,e** showed excellent binding interactions (affinity in Kcal/mol ranges from -6.7235 to -7.0539 with three or four hydrogen bonding interactions) in comparison with valdecoxib (-6.7084 with 3 hydrogen bonding interactions). The docking results were consistent with the *in vitro* inhibitory activity and suggested that compounds (**13d**) and (**13e**) good selectivity against COX-2 isozyme similar to valdecoxib (**6**) which both of them contain pharmacophore of COX-2 (SO_2Me or SO_2NH_2) (Fig. 3-5).

Table 4: Molecular modeling data for compounds **13d,e** and valdecoxib during docking in COX-2 (PDB:ID 2AW1) active site.

Compound	COX-2 Affinity Kcal/mol	Affinity Kcal/mol	Distance (in A^o) from main residue		Functional group	Interaction
13d	-7.0539	-2.8	2.82	Thr199	$-SO_2$	H-acceptor
		-0.7	3.28	His119	$-SO_2$	H-acceptor
		-2.0	3.32	His96	$-SO_2$	H-acceptor
		-3.6	2.99	Asn67	=N-	H-acceptor
13e	-6.7235	-3.9	2.94	Thr199	$-SO_2$	H-acceptor
		-1.6	3.11	Thr199	$-NH_2$	H-donor
		-0.7	3.29	His96	$-NH_2$	H-acceptor
Valdecoxib	-6.7084	-3.7	2.92	Thr199	$-SO_2$	H-acceptor
		-3.5	2.94	Thr199	$-NH_2$	H-donor
		-0.7	3.31	His96	$-NH_2$	H-acceptor
		-0.8	4.73	Asn67	-Ph-ring	pi-H

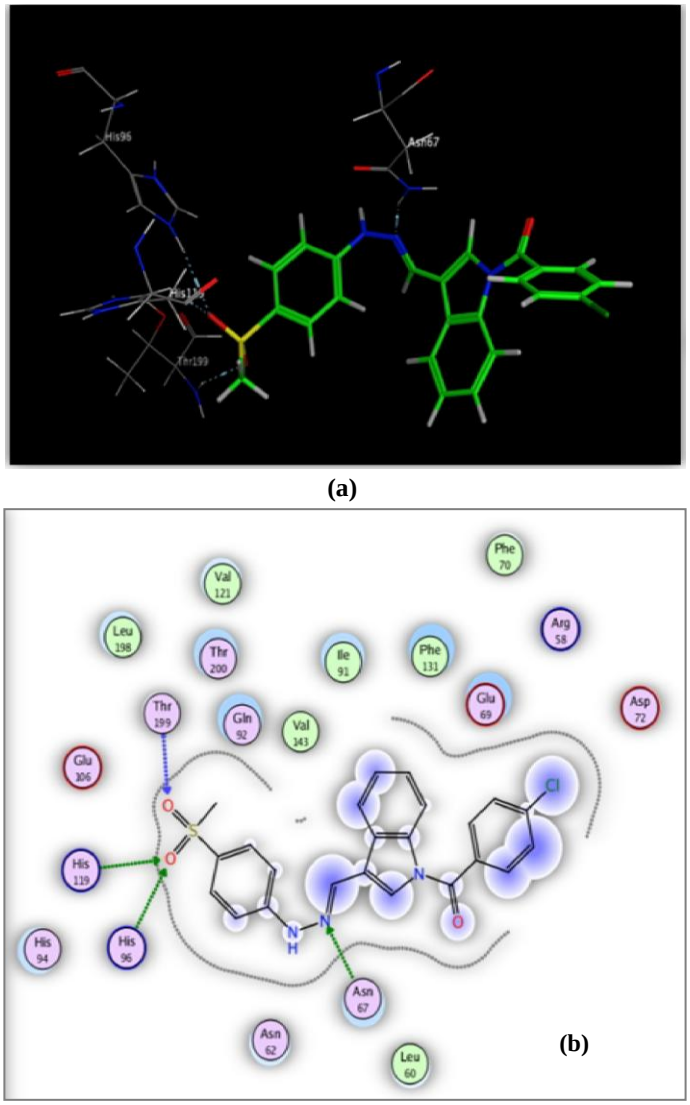
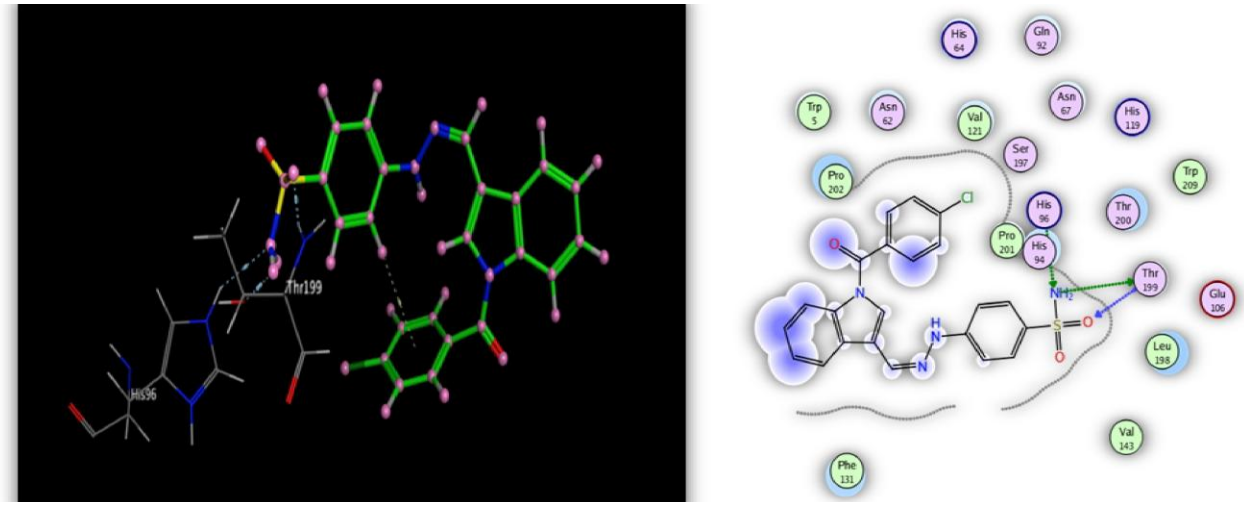
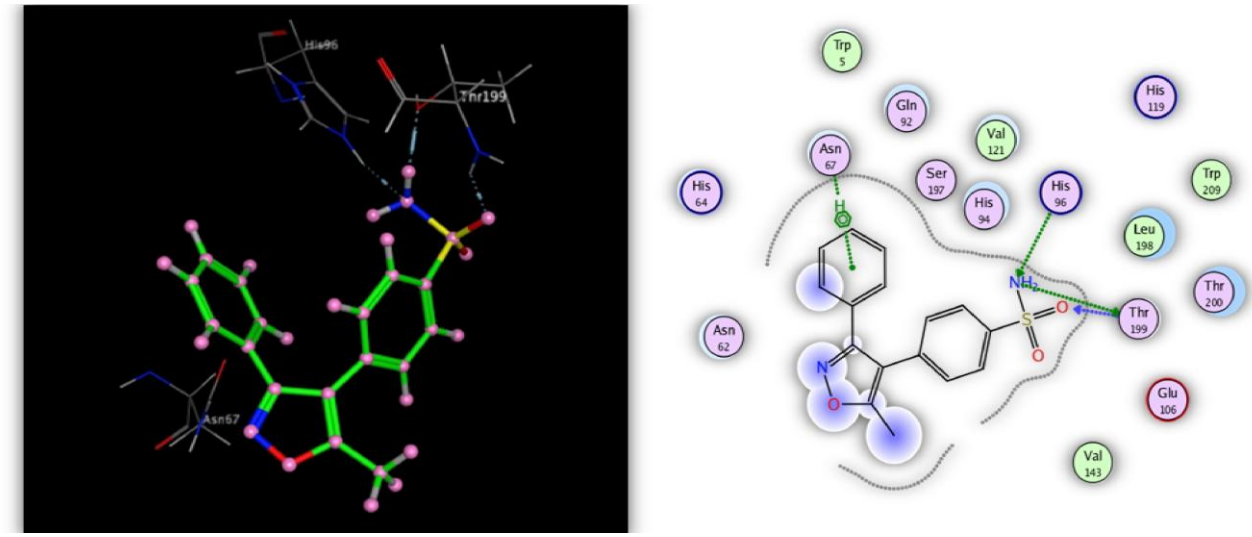


Fig. 3: Binding of the compound **13d** inside COX-2 active site. **a)** The 3D proposed binding mode inside the active site of COX-2 resulting from docking, the most important amino acids are shown together with their respective numbers. **b)** 2D interaction.



(a) **(b)**

Fig. 4: Binding of the compound **13e** inside COX-2 active site. **a)** The 3D proposed binding mode inside the active site of COX-2 resulting from docking, the most important amino acids are shown together with their respective numbers. **b)** 2D interaction.



(a) **(b)**

Fig. 5: Binding of the valdecoxib inside COX-2 active site. **a)** The 3D proposed binding mode inside the active site of COX-2 resulting from docking, the most important amino acids are shown together with their respective numbers. **b)** 2D interaction.

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