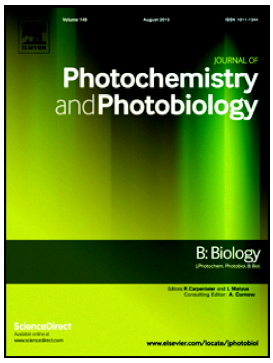

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Anti-inflammatory, analgesic and COX-2 inhibitory activity of novel thiadiazoles in irradiated rats.

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Abstract
In this work, novel series of pyran, thiophene and thienopyrimidine derivatives based on 2-acetamide-thiadiazole scaffold were designed and synthesized for evaluation as selective COX-2 inhibitors *in-vitro* and investigated *in-vivo* as anti-inflammatory and analgesic agents against carrageenan-induced rat paw oedema model in irradiated rats, since its well-known that ionizing radiation plays an important role in exaggerating the inflammatory responses and in enhancing the release of inflammatory mediators in experimental animals. Toxicological studies were carried out to evaluate the ulcerogenic activity, acute toxicity and kidney and liver functions for the most potent compounds. In order to understand the binding mode of the synthesized compounds into the active site of COX-2, docking study was performed. Most of the tested compounds showed high inhibitory ability to COX-2. Among them, thiadiazole derivatives bearing thiophene and thienopyrimidine moieties were the most active derivatives, compound **26** showed extremely high selectivity index (SI) of >555.5 µM which is nearly two folds better than celecoxib (>277.7 µM), in addition to compounds **3**, **16, 17, 21** and **26** with SI in the range of >308.6- >384.6 µM. The 4-chlorothieno[2,3-d]pyrimidine derivative of thiadiazole **21** showed the highest anti-inflammatory activity in this study having 24.49% of oedema compared to celecoxib (18.61%) in addition to compounds **17** and **26** with 24.70 and 25.40 % of oedema, respectively, while the thiadiazol-2-acetamide derivative **2** was the most potent analgesic compound with the highest nociceptive threshold (85.72g) very close to that of celecoxib (90.23g). These compounds showed high safety margin on gastric mucosa with no ulceration effect. Also the most active *in-vivo* anti-inflammatory compounds **17, 21** and **26** were found to be non-toxic in experimental rats with normal kidney and liver functions. Docking study of the synthesized compounds showed similar orientation as celecoxib within the active site of COX-2 enzyme and similar ability to emerge deeply in the additional pocket and binding with Arg513 and His90 the key amino acids responsible for selectivity.

Keywords: Thiadiazoles, COX-2 inhibitors, anti-inflammatory, analgesic, toxicological studies, irradiated rats, docking.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) continue to be widely used group of therapeutic agents, which inhibit both COX-1 and COX-2 with an extremely varying levels of selectivity [1, 2]. Their clinical use as analgesics and anti-inflammatory agents is always accompanied with adverse gastrointestinal disorders and the design of novel NSAIDs with an advanced safety profile on GIT is a challenge in pharmaceutical industry. Since the discovery

celecoxib, , researchers have focused on the synthesis of novel derivatives of this class which reduce inflammation with fewer side effects [3, 4].

Inflammation is a complex biological response to harmful stimulus which may vary from a localized response to a generalized one and is mediated by prostaglandins (PGs) [5]. The biosynthesis of (PGs) is carried out by the bifunctional enzyme prostaglandin H2 synthase (PGHS or cyclooxygenase, COX), which exhibits both cyclooxygenase and peroxidase activities. There are three distinct COX isoforms: COX-1, the constitutive which is involved in the regulation of physiological functions and production of cycloprotective prostaglandin in GIT and maintaining platelet aggregation by production of proaggregatory thromboxane. COX-2, the inducible form which is released in inflammatory cells in response to cytokines such as tumor necrosis factor-α (TNF-α), interleukines, growth factors, and other inflammatory mediators. COX-3, a third full active isoform, and two partial isoforms, pCOX1a and b recently reported to be detected in the cerebral cortex and in human heart [6-8].

Most studies on new anti-inflammatories have been focused on healthy population. However, inflammatory processes have particular relevance in the context of cancer, as inflammation is increasingly recognized as a contributor to cancer development and progression especially in breast and prostate cancer [9]. Moreover, radiation therapy activates pro-inflammatory cytokine production as part of a coordinated response designed to control damage and promote tissue repair [10]. Pain management and controlling of inflammatory responses should be concluded in protocols before starting of radiation treatment [11].

Carrageenan-induced paw oedema is an acute inflammatory model commonly used in experimental rats [12]. Ionizing radiation has been shown to exaggerate the inflammatory responses induced by this model due to enhancement of release of inflammatory mediators through the cyclooxygenase and lipoxygenase pathways and also through the release of reactive oxygen metabolites resulting from the interaction between radiation and water from the cellular environment [13-15].

The common structure of COX-2 inhibitors includes two classes: tricyclic and non-tricyclic compounds [16, 17], the tricyclic group consists of two aryl rings linked to a central homocyclic, heterocyclic or fused ring moieties such as, thiophene, pyrazole, furanone, isoxazole, cyclopentene and fused heterocyclics. One of the two aryl rings carries a sulfonamide moiety which is deeply immerged into the additional hydrophilic pocket of COX-2 enzyme and is capable of binding to the key amino acids His90 and Arg513 responsible for selectivity [16, 17]. In the non-tricyclics, the cyclic core is replaced by acyclic centre such as olefinic, iminic, azo, urea, and a,b-unsaturated structures [18-20]. This common pharmacophore presents a wide framework which allows medicinal chemists to design novel selective COX-2 inhibitors with varying structures.

Pyran derivatives have recently attracted considerable attention due to their wide spectrum ofbiological activity [21-24], Hyup et al. [25] introduced 2,3-diaryl benzopyrans as a part of the vicinal diaryl heterocyclic family as a promising lead structure for selective COX-2 inhibition. Caturla et al [26] reported a new class of 2-phenylpyran-4-ones as selective COX-2 inhibitors. Moreover, the anti-inflammatory activity of thiophene and thienopyrimidine derivatives is well-documented in addition to their array of pharmacological activities [27-30]. In 1995, Gierse et al.[31] introduced a new generation of selective COX-2 inhibitors that include 5-bromo-2-(4-fluorophenyl)-3-(methylsulfonyl) thiophene (DuP-697). This new class binds tightly to the COX-2 active cite and dissociate slowly showing a long lasting action. Hence, different series of thiophene and thienopyrimidines have been synthesized with optimal COX-2 inhibition [32].

The 1,3,4-thiadiazole ring is endowed with relatively high aromaticity and weak basicity due to its sulfur inducible effect, the electron withdrawing effect of its nitrogen atoms are

responsible for its electron deficiency and susceptibility to nucleophilic attack, it is relatively stable in aqueous acid solutions but can undergo ring cleavage with aqueous base. Thus, with these structural properties, 1,3,4-thiadiazole derivatives are widely applied in medicinal chemistry and displayed significant biological activities [33, 34].

Based on these facts, we decided to synthesize novel series of non-tricyclic COX-2 inhibitors possessing a central azo acyclic core, the two aromatic rings are replaced by 1,3,4-thiadiazole, and by either pyran, thiophene or thienopyrimidine. By using the structural features of these biologically active moieties and by diverting the substituents, novel inhibitors were synthesized. The aim is to study the SAR and the effect of thiadiazole ring on the orientation and binding mode of pyran, thiophene and thienopyrimidine within COX-2 active site in order to reach the best inhibitory activity with higher selectivity index which could lead to the discovery of new class of COX-2 inhibitors (Figure 1). These new derivatives were screened using *in-vitro* COX inhibitory assay, the most potent candidates were subjected to anti-inflammatory and analgesic testing. Toxicological studies were performed by evaluation of their ulcerogenic activity, acute toxicity, and kidney and liver functions in experimental rats. Molecular docking was carried out for all the synthesized compounds into the active site of COX-2 to explore their binding interactions and their accessibility to the additional pocket responsible for selectivity.

Figure 1. The designed synthesized compounds.

2. Material and Methods

2.1.Instruments

The melting points were taken in an open capillary tube on a Stuart melting point apparatus (Stuart Scientific, Redhill, UK) and are uncorrected. The IR spectra of the compounds were recorded on FT-IR Shimadzu spectrometer (Shimadzu, Tokyo, Japan). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC-500 Ultra Shield NMR spectrometer (Bruker, Flawil, Switzerland) at 500 MHz using TMS as an internal Standard and DMSO-d_6 as solvent. Mass spectra were run on HP Model MS-5988 (Hewlett Packard, Palo, Alto, California, USA). Microanalyses were obtained on a Carlo Erba 1108 Elemental Analyzer (Heraeus, Hanau, Germany), all values were within±0.4% of the theoretical values. The purity of the compounds was checked by TLC on pre-coated SiO_2 gel (HF_{254}, 200 mesh) aluminum plates (Merk, Darmstadt, Germany). A developing solvent system of chloroform/methanol (8:2) was used and the spots were visualized in UV light. IR, ^{1}H-NMR, ^{13}C-NMR, Mass and elemental analysis were consistent with the assigned structures. All reagents used were of AR grade and were purchased from Sigma (St. Louis, MO).

2.2.Chemistry

*General procedure for the synthesis of compounds **3-5**.*

A mixture of 2-acetamide thiadiazole **2** (0.7 g, 0.004 mol), activated nitrile such as malononitrile, ethyl cyanoacetate and/or ethyl acetoacetate (0.004 mol), sulphur (0.13 g,

0.004 mol), ethanol (50 mL) and a catalytic amount of triethylamine (0.2 mL) were refluxed for 6h. The reaction mixture was cooled, poured onto ice water and the precipitated solid was collected by filtration, dried, and recrystallized from ethanol to give **3-5**, respectively.

2-Amino-4-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)thiophene-3-carbonitrile ***(3).***
Yield, 85 %, mp 265–267^{o} C. IR (KBr, cm^{-1}): 3330, 3229, 3200 (NH, NH_2), 2200 (C≡N), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.41 (s, 1H, NH, exchangeable with D_2O), 5.31 (s, 1H, CH-thiophene), 8.32, 9.71 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm):79.51 (C-CN), 104.21 (CH-thiophene), 115.32 (CN), 125.72 (C-thiophene), 151.41 (C-NH_2), 156.64 (C-thiadiazole), 181.71 (C=S). MS m/z: 255 (M^+). Analysis calculated for $C_7H_5N_5S_3$: C, 32.93; H, 1.97; N, 27.43, found: C, 32.83; H, 1.87; N, 27.33.

Ethyl 2-amino-4-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)thiophene-3-carboxylate ***(4).***
Yield, 75 %, mp 199–201^{o} C. IR (KBr, cm^{-1}): 3320, 3230, 3200 (NH, NH_2), 2929, 2870 (CH aliph.), 1720 (C=O), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 1.23 (t, 3H, *J*= 3.5Hz, CH_3 ethyl), 2.41 (s, 1H, NH, exchangeable with D_2O), 4.32(q, 2H, *J*= 4.5Hz,CH_2 ethyl), 5.71 (s, 1H, CH thiophene), 8.61, 10.70 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 14.3 (CH_3 ethyl), 60.8 (CH_2 ethyl), 103.8 (CH-thiophene), 124.1(C-thiophene), 124.6(C-thiophene), 156.6 (C-thiadiazole), 159.8 (C=O), 161.6 (C-NH_2), 181.7 (C=S). MS m/z: 302 (M^+). Analysis calculated for $C_9H_{10}N_4O_2S_3$: C, 35.75; H, 3.33; N, 18.53, found: C, 35.65; H, 3.23; N, 18.43.

2-Methyl-4-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)thiophene-3-ethylcarboxylate ***(5)****.*

Yield, 90 %, mp 200–202^{o} C. IR (KBr, cm^{-1}): 3227, 3200 (NH), 2939, 2870 (CH aliph.), 1700 (C=O), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 1.21 (t, 3H, *J*= 2.5Hz, CH_3 ethyl), 2.32 (s, 3H, CH_3), 2.51 (s, 1H, NH, exchangeable with D_2O), 4.21(q, 2H, *J*= 4.5Hz, CH_2 ethyl), 5.35 (s, 1H, CH-thiophene), 8.32 (s, 1H, NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 14.12 (CH_3), 14.70 (CH_3 ethyl), 60.82 (CH_2 ethyl), 105.81 (CH-thiophene), 123.13(C-thiophene), 124.61(C-thiophene), 148.50 (C-CH_3), 156.62(C-thiadiazole), 160.61 (C=O), 181.70 (C=S). MS m/z: 301 (M^+). Analysis calculated for $C_{10}H_{11}N_3O_2S_3$: C, 39.85; H, 3.68; N, 13.94. found: C, 39.65; H, 3.88; N,13.64.

General procedure for the preparation of Ethyl 2-amino-4-(4-substituted phenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-4H-pyran-3-carboxylate ***(7-10)*** *and 2-Amino-4-(4-substituted phenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-4H-pyran-3-carbonitrile* ***(11-14).***

A mixture of 2-acetamide thiadiazole **2** (0.7 g, 0.004 mol), 2-(4-substituted benzylidene) cyanoacetate and/or 2-(4-substituted benzylidene) malononitrile **6a-h** (0.004 mol) in ethanol

(50 mL) and a catalytic amount of triethylamine (0.2 mL) were refluxed for 6h. The reaction mixture was cooled, poured onto ice water and the precipitated solid was collected by filtration, dried, and recrystallized from methanol to give **7-14**, respectively.

Ethyl-2-amino-4-(4-chlorophenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-4H-pyran-3-carboxylate ***(7).***

Yield, 78 %, mp 270–272^{o} C. IR (KBr, cm^{-1}): 3320, 3230, 3200 (NH, NH_2), 3058 (CH arom.), 2939, 2880 (CH aliph.), 1680 (C=O), 1626 (C=N), 1230 (C=S), 835 (C-Cl). ^{1}H-NMR (DMSO-d_6, ppm): 1.32 (t, 3H, *J*= 5.2Hz, CH_3 ethyl), 2.50 (s, 1H, NH, exchangeable with D_2O), 3.61 (d, 1H, *J*= 6.5Hz, C<u>H</u>-pyran), 4.14(q, 2H, *J*= 5.5Hz, CH_2 ethyl), 4.71 (d, 1H, *J*= 5.1Hz, C<u>H</u>-pyran), 7.21,7.82 (2d, 4H, *J*= 9.2Hz, Ar-H), 8.72, 9.71 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 14.31 (CH_3 ethyl), , 36.82 (<u>C</u>H-pyran), 61.84 (CH_2 ethyl), 74.61 (<u>C</u>H- pyran), 75.80 (<u>C</u>- pyran), 128.13 (2C), 130.62 (2C), 142.22, 131.54 (<u>C</u>-Cl), 144.80 (C-thiadiazole), 162.17(<u>C</u>-pyran), 162.23 (<u>C</u>-NH_2), 167.86 (C=O), 181.78 (C=S). MS m/z: 410 (M^+), 412 (M+2). Analysis calculated for $C_{16}H_{15}ClN_4O_3S_2$: C, 46.77; H, 3.68; N, 13.64, found: C, 46.67; H, 3.58; N, 13.74.

Ethyl 2-amino-4-(4-methoxyphenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-4H-pyran-3-carboxylate ***(8).***

Yield, 85 %, mp 159–161^{o} C. IR (KBr, cm^{-1}): 3330, 3220, 3200 (NH, NH_2), 3048 (CH arom.), 2949, 2880 (CH aliph.), 1720 (C=O), 1627 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 1.32 (t, 3H, *J*= 6.2Hz, C<u>H</u>$_3$ ethyl), 2.51 (s, 1H, NH, exchangeable with D_2O), 3.54 (d, 1H, *J*= 5.5Hz, C<u>H</u>-pyran),3.75 (s, 3H, OC<u>H</u>$_3$), 4.12(q, 2H, *J*= 5.5Hz, C<u>H</u>$_2$ ethyl), 4.85 (d, 1H, *J*= 6.1Hz, C<u>H</u>-pyran), 6.82,7.75 (2d, 4H, *J*= 7.5Hz, Ar-H), 8.12, 8.91 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 13.91 (CH_3 ethyl), 36.72 (<u>C</u>H-pyran), 56.12 (O<u>C</u>H$_3$), 61.92 (CH_2 ethyl), , 73.63(<u>C</u>H- pyran), 75.81(<u>C</u>- pyran), 114.12 (2C), 130.60 (2C), 136.22, 156.81 (<u>C</u>-OCH$_3$), 144.86 (C-thiadiazole), 161.91(<u>C</u>-pyran), 162.44 (<u>C</u>-NH_2), 169.72 (C=O), 181.70 (C=S). MS m/z: 406 (M^+). Analysis calculated for $C_{17}H_{18}N_4O_4S_2$: C, 50.23; H, 4.46; N, 13.78, found: C, 50.13; H, 4.36; N, 13.68.

Ethyl-2-amino-4-(4-nitrophenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-4H-pyran-3-carboxylate ***(9).***

Yield, 85 %, mp 210–212^{o} C. IR (KBr, cm^{-1}): 3320, 3230, 3200 (NH, NH_2), 3058 (CH arom.), 2939, 2880 (CH aliph.), 1690 (C=O), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 1.32 (t, 3H, *J*= 5.7Hz, C<u>H</u>$_3$ ethyl), 2.52 (s, 1H, NH, exchangeable with D_2O), 3.31 (d, 1H, *J*= 5.5Hz, C<u>H</u>-pyran), 4.26 (q, 2H, *J*= 6.5Hz, C<u>H</u>$_2$ ethyl), 4.62 (d, 1H, *J*= 6.3Hz, C<u>H</u>-pyran), 7.12,7.70 (2d, 4H, *J*= 9.5Hz, Ar-H), 8.12, 8.92 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 14.12 (CH_3 ethyl), 36.42 (<u>C</u>H-pyran), 61.80 (CH_2 ethyl), 74.62(<u>C</u>H-pyran), 75.81(<u>C</u>-pyran), 123.72 (2C), 127.61 (2C), 150.26,144.50 (<u>C</u>-NO_2), 144.83

(C-thiadiazole), 162.22(C-pyran), 162.40 (C-NH_2), 167.70 (C=O), 181.71 (C=S). MS m/z: 421 (M^+). Analysis calculated for $C_{16}H_{15}N_5O_5S_2$: C, 45.60; H, 3.59; N, 16.62, found: C, 45.70; H, 3.49; N, 16.52.

Ethyl-2-amino-4-(4-(dimethylamino)phenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-4H-pyran-3-carboxylate ***(10).***

Yield, 79 %, mp 230–232° C. IR (KBr, cm^{-1}): 3320, 3220, 3200 (NH, NH_2), 3048 (CH arom.), 2929, 2870 (CH aliph.), 1720 (C=O), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 1.32 (t, 3H, *J*= 2.5Hz, CH_3 ethyl), 2.51 (s, 1H, NH, exchangeable with D_2O), 3.24 (s, 6H, 2CH_3), 3.71 (d, 1H, *J*= 6.5Hz, CH-pyran), 4.22 (q, 2H, *J*= 5.9Hz CH_2 ethyl), 4.81 (d, 1H, *J*= 6.7Hz, CH-pyran), 6.82,7.84 (2d, 4H, *J*= 8.5Hz, Ar-H), 8.21, 8.93 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 14.12 (CH_3 ethyl), 36.72 (CH-pyran), 41.30 (N-2CH_3), 61.92 (CH_2 ethyl), 73.62(CH- pyran), 75.82(C-pyran), 112.12 (2C), 126.61 (2C), 133.20, 148.51 (C-N(CH_3)$_2$), 144.84 (C-thiadiazole), 162.22(C-pyran), 162.43 (C-NH_2), 167.73 (C=O), 181.72 (C=S). MS m/z: 419 (M^+). Analysis calculated for $C_{18}H_{21}N_5O_3S_2$: C, 51.53; H, 5.05; N, 16.69, found: C, 51.43; H, 5.15; N, 16.59.

2-Amino-4-(4-chlorophenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-yl amino)-4H-pyran-3-carbonitrile ***(11).***

Yield, 90 %, mp 230–232° C. IR (KBr, cm^{-1}): 3320, 3220, 3200 (NH, NH_2), 3048 (CH arom.), 2200 (C≡N), 1626 (C=N), 1230 (C=S), 825 (C-Cl). ^{1}H-NMR (DMSO-d_6, ppm): 2.56 (s, 1H, NH, exchangeable with D_2O), 3.82 (d, 1H, *J*= 5.5Hz, CH pyran), 4.82 (d, 1H, *J*= 5.8Hz, CH-pyran), 7.26,7.83 (2d, 4H, *J*= 8.6Hz, Ar-H), 8.22, 8.91 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 26.70 (CH-pyran), 58.42(C-pyran), 74.62(CH-pyran), 119.26 (CN), 128.21 (2C), 130.62 (2C), 140.52, 131.72 (C-Cl), 144.84 (C-thiadiazole), 159.61 (C-NH_2), 162.73 (C-pyran), 181.72 (C=S). MS m/z: 363 (M^+), 365 (M+2). Analysis calculated for $C_{14}H_{10}ClN_5OS_2$: C, 46.21; H, 2.77; N, 19.25, found: C, 46.11; H, 2.87; N, 19.15.

2-Amino-4-(4-methoxyphenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-4H-pyran-3-carbonitrile ***(12).***

Yield, 79 %, mp 250–252° C. IR (KBr, cm^{-1}): 3330, 3230, 3200 (NH, NH_2), 3048 (CH arom.), 2939, 2870 (CH aliph.), 2190 (C≡N), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.51 (s, 1H, NH, exchangeable with D_2O), 3.73 (s, 3H, OCH_3), 3.92 (d, 1H, *J*= 5.8Hz, CH- pyran), 4.72 (d, 1H, *J*= 5.2Hz, CH-pyran), 7.32,7.91 (2d, 4H, *J*= 9.8Hz, Ar-H), 8.32, 8.91 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 26.81 (CH-pyran), 55.30 (OCH_3), 58.42(C-pyran), 74.85 (CH- pyran), 119.51 (CN), 114.22 (2C), 130.66 (2C), 134.52, 157.71 (C-OCH_3), 144.82 (C-thiadiazole), 159.91 (C-NH_2), 162.94 (C-pyran), 181.71 (C=S). MS m/z: 359 (M^+). Analysis calculated for $C_{15}H_{13}N_5O_2S_2$: C, 50.12; H, 3.65; N, 19.48, found: C, 50.22; H, 3.55; N, 19.38.

2-Amino-4-(4-nitrophenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-yl amino) -4H-pyran-3-carbonitrile ***(13).***

Yield, 88 %, mp 200–202^{o} C. IR (KBr, cm^{-1}): 3320, 3220, 3200 (NH, NH_2), 3038 (CH arom.), 2200 (C≡N), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.51 (s, 1H, NH, exchangeable with D_2O), 3.72 (d, 1H, *J*= 5.8Hz, C<u>H</u>-pyran), 4.82 (d, 1H, *J*= 5.2Hz, C<u>H</u>-pyran), 7.22,7.81 (2d, 4H, *J*= 8.8Hz, Ar-H), 8.41, 8.81 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 26.71 (<u>C</u>H-pyran), 58.82(<u>C</u>-pyran), 75.62(<u>C</u>H-pyran), 119.81 (CN), 123.61 (2C), 126.62 (2C), 148.52, 144.54 (C-thiadiazole), 144.91 (C-NO_2), 159.65 (<u>C</u>-NH_2), 162.70 (<u>C</u>-pyran), 181.72 (C=S). MS m/z: 374 (M^+). Analysis calculated for $C_{14}H_{10}N_6O_3S_2$: C, 44.91; H, 2.69; N, 22.45, found: C, 44.81; H, 2.59; N, 22.35.

2-Amino-4-(4-(dimethylamino)phenyl)-6-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol -2-ylamino)-4H-pyran-3-carbonitrile ***(14).***

Yield, 92 %, mp 240–242^{o} C. IR (KBr, cm^{-1}): 3330, 3220, 3200 (NH, NH_2), 3048 (CH arom.), 2939, 2870 (CH aliph.), 2190 (C≡N), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.52 (s, 1H, NH, exchangeable with D_2O), 3.24 (s, 6H, $2CH_3$), 3.81 (d, 1H, *J*= 5.2Hz, C<u>H</u>-pyran), 4.93 (d, 1H, *J*= 5.7Hz, C<u>H</u>-pyran), 6.82,7.41 (2d, 4H, *J*= 9.8Hz, Ar-H), 8.12, 8.82 (2s, 3H, NH, NH_2, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 26.81 (<u>C</u>H-pyran), 41.72 ($2CH_3$), 58.41(<u>C</u>-pyran), 74.62(<u>C</u>H-pyran), 119.70 (CN), 112.22 (2C), 128.61 (2C), 132.55, 144.82 (C-thiadiazole), 148.71 (<u>C</u>-$N(CH_3)_2$), 154.22 (<u>C</u>-NH_2), 163.80 (<u>C</u>-pyran), 181.71 (C=S). MS m/z: 372 (M^+). Analysis calculated for $C_{16}H_{16}N_6OS_2$: C, 51.59; H, 4.33; N, 22.56, found: C, 51.49; H, 4.23; N, 22.46.

2-Chloro-N-(3-cyano-4-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino) thiophen-2-yl)acetamide ***(15).***

A mixture of compound **3** (1.02 g, 0.004 mol) and chloroacetylchloride (0.9 mL, 0.008 mol) in dimethylformamide was stirred for 8 h at room temperature, the reaction mixture was poured onto ice water. The obtained solid was filtered, dried and recrystallized from ethanol to give **15**. Yield, 76 %, mp 200–202^{o} C. IR (KBr, cm^{-1}): 3229, 3200 (NH), 2939, 2870 (CH aliph.), 2200 (C≡N), 1680 (C=O), 1626 (C=N), 1230 (C=S), 825 (C-Cl). ^{1}H-NMR (DMSO-d_6, ppm): 2.51 (s, 1H, NH, exchangeable with D_2O), 4.22 (s, 2H, C<u>H</u>$_2$-Cl), 5.72 (s, 1H, C<u>H</u>-thiophene), 8.31, 8.92 (2s, 2H, 2NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm):42.91 (<u>C</u>H$_2$-Cl), 65.51(<u>C</u>-CN), 94.82 (<u>C</u>H-thiophene), 115.32 (CN), 125.72 (<u>C</u>-thiophene), 162.81 (C-NH_2), 156.73 (C-thiadiazole), 165.63 (C=O), 181.73 (C=S). MS m/z: 331 (M^+), 333 (M+2). Analysis calculated for $C_9H_6ClN_5OS_3$: C, 32.58; H, 1.82; N, 21.11, found: C, 32.48; H, 1.72; N, 21.21.

Ethyl-N-3-cyano-4-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-thiophen-2-yl-formimidate ***(16).***

A solution of compound **3** (1.02 g, 0.004mol) in triethylortho formate (30 mL) containing 3 drops of acetic anhydride was refluxed for 8 h, the reaction mixture was cooled and then poured onto ice water, the obtained solid was filtered, dried and recrystallized from methanol to give **16.** Yield, 86 %, mp 180–182^{o} C. IR (KBr, cm^{-1}): 3240, 3200 (NH), 2949, 2870 (CH aliph.), 2220 (C≡N), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d$_6$, ppm): 1.25 (t, 3H, *J*= 6.1Hz, CH_3 ethyl), 2.62 (s, 1H, NH, exchangeable with D_2O), 3.71 (q, 2H, *J*= 6.6Hz, CH_2 ethyl), 5.62 (s, 1H, C<u>H</u>-thiophene), 7.82 (s, 1H, N=C<u>H</u>), 8.61 (s, 1H, NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d$_6$, ppm): 16.72 (CH_3 ethyl), 61.81 (CH_2 ethyl), 64.51(<u>C</u>-CN), 102.82 (<u>C</u>H-thiophene), 115.62 (CN), 124.71 (<u>C</u>-thiophene), 160.83 (C-NH_2), 156.70 (C-thiadiazole), 159.15(N=<u>C</u>H), 181.71 (C=S). MS m/z: 311 (M^+). Analysis calculated for $C_{10}H_9N_5OS_3$: C, 38.57; H, 2.91; N, 22.49, found: C, 38.47; H, 2.81; N, 22.39.

5-(4-Imino-3-(phenylamino)-3,4-dihydrothieno[2,3-d]pyrimidin-5-ylamino)-1,3,4-thiadiazole-2(3H)-thione ***(17).***

A mixture of **16** (1.2 g, 0.004mol) and phenyl hydrazine (0.004 mol) in ethanol (60 mL) was heated under reflux for 8 h. The solid product was filtered on hot, dried, and recrystallized from ethanol to give **17**. Yield, 84 %, mp 160–162^{o} C. IR (KBr, cm^{-1}): 3240, 3200 (NH), 3038 (CH arom.), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d$_6$, ppm): 2.54 (s, 1H, NH, exchangeable with D_2O), 5.32 (s, 1H, C<u>H</u>-thiophene), 7.32-7.87 (m, 5H, Ar-H), 8.22 (s, 1H, C<u>H</u>-pyrimidine), 8.52, 8.81, 10.85 (3s, 3H, 3NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d$_6$, ppm): 103.63 (<u>C</u>H-thiophene), 113.41 (2C), 123.13, 129.72 (2C), 147.81, 117.86, 123.61, 146.14 (3C-thiophene), 147.12 (<u>C</u>H-pyrimidine), 156.26 (<u>C</u>=NH), 156.71 (C-thiadiazole), 181.72 (C=S). MS m/z: 373 (M^+). Analysis calculated for $C_{14}H_{11}N_7S_3$: C, 45.02; H, 2.97; N, 26.25, found: C, 45.12; H, 2.77; N, 26.15.

*4-Imino-3-phenyl-5-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-3,4-dihydrothieno[2,3-d]pyrimidin-2(1H)-one (****18****).*

A mixture of compound **3** (1.02 g, 0.004 mol) and phenyl isocyanate (0.43 mL, 0.004 mol) in ethanol (20 mL) was refluxed for 5 h. The reaction mixture was cooled and then poured onto ice water, the solid obtained was filtered, dried and recrystallized from ethanol to give **18**. Yield, 74 %, mp 210–212^{o} C. IR (KBr, cm^{-1}): 3240, 3200 (NH), 3038 (CH arom.), 1680 (C=O), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d$_6$, ppm): 2.51 (s, 1H, NH, exchangeable with D_2O), 5.82 (s, 1H, C<u>H</u>-thiophene), 7.32-7.89 (m, 5H, Ar-H), 8.21, 8.85, 9.82 (3s, 3H, 3NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d$_6$, ppm): 97.63 (<u>C</u>H- thiophene), 128.12, 128.76 (2C), 129.14(2C), 132.82, 103.81, 123.64, 172.15 (3C-thiophene), 152.42 (C=O), 156.20 (<u>C</u>=NH), 156.71 (C-thiadiazole), 181.71 (C=S). MS m/z: 374 (M^+). Analysis calculated for $C_{14}H_{10}N_6OS_3$: C, 44.90; H, 2.69; N, 22.44, found: C, 44.70; H, 2.59; N, 22.34.

*5-(5-Thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)thieno[2,3-d]pyrimidin-4(3H)-one (****19****).*

A solution of compound **3** (1.02 g, 0.004 mol) in formic acid (20 mL) was refluxed for 6 h, the reaction mixture was cooled then poured onto ice water, the obtained solid was filtered, dried and recrystallized from dioxane to give **19**. Yield, 89 %, mp 198–200^{o} C. IR (KBr, cm^{-}

^{1}): 3240, 3200 (NH), 3038 (CH arom.), 1720 (C=O), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.52 (s, 1H, NH, exchangeable with D_2O), 5.81 (s, 1H, CH-thiophene), 8.14 (s, 1H, CH-pyrimidine), 8.57, 8.91 (2s, 2H, 2NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 103.62 (CH-thiophene), 122.81, 133.65, 157.14 (3C-thiophene), 146.12 (CH-pyrimidine), 156.90 (C-thiadiazole), 162.73 (C=O), 181.71 (C=S). MS m/z: 283 (M^+). Analysis calculated for $C_8H_5N_5OS_3$: C, 33.91; H, 1.78; N, 24.72, found: C, 33.71; H, 1.68; N, 24.52.

5-(5-Thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)thieno[2,3-d]pyrimidine-4(3H)-thione ***(20).***

A mixture of compound **19** (1.13 g, 0.004 mol) and phosphorus pentasulfide (0.43 mL, 0.004 mol) in pyridine (20 mL) was refluxed for 8 h, the reaction mixture was cooled, poured onto ice water, then acidified with dil HCl. The solid obtained was filtered, dried and recrystallized from ethanol to give **20**. Yield, 93 %, mp 222–224^{o} C. IR (KBr, cm^{-1}): 3240, 3200 (NH), 3038 (CH arom.), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.42 (s, 1H, NH, exchangeable with D_2O), 5.32 (s, 1H, CH-thiophene), 8.16 (s, 1H, CH-pyrimidine), 8.24, 9.12 (2s, 2H, 2NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 102.66 (CH-thiophene), 118.82, 124.61, 147.13 (3C-thiophene), 145.15 (CH-pyrimidine), 156.90 (C-thiadiazole), 180.14(C=S-pyrimidine), 181.76 (C=S-thiadiazole). MS m/z: 299 (M^+). Analysis calculated for $C_8H_5N_5S_4$: C, 32.09; H, 1.68; N, 23.39, found: C, 32.19; H, 1.78; N, 23.29.

5-(4-Chlorothieno[2,3-d]pyrimidin-5-ylamino)-1,3,4-thiadiazole-2(3H)-thione ***(21).***

A solution of compound **19** (1.13 g, 0.004 mol) in thionyl chloride (15 mL) was refluxed for 3 h, the thionyl chloride was then removed by distillation and the obtained solid was washed twice with methanol, dried and recrystallized from ethanol to give compound **21**. Yield, 89 %, mp 266–268^{o} C. IR (KBr, cm^{-1}): 3240, 3200 (NH), 3038 (CH arom.), 1626 (C=N), 1230 (C=S), 835 (C-Cl). ^{1}H-NMR (DMSO-d_6, ppm): 2.51 (s, 1H, NH, exchangeable with D_2O), 5.92 (s, 1H, CH-thiophene), 8.15 (s, 1H, CH-pyrimidine), 8.24 [s, 1H, NH, exchangeable with D_2O].^{13}C-NMR (DMSO-d_6, ppm): 103.62 (CH- thiophene), 128.81, 141.62, 142.15 (3C-thiophene), 151.40 (C-Cl), 156.92 (C-thiadiazole), 157.12 (CH-pyrimidine), 181.70 (C=S). MS m/z: 301 (M^+), 303(M+2). Analysis calculated for $C_8H_4ClN_5S_3$: C, 31.84; H, 1.34; N, 23.21, found: C, 31.74; H, 1.24; N, 23.31.

5-(4-Isothiocyanatothieno[2,3-d]pyrimidin-5-ylamino)-1,3,4-thiadiazole-2(3H)-thione ***(22).***

A mixture of compound **21** (1.2 g, 0.004 mol) and ammonium thiocyanate (0.3 g, 0.004 mol) was refluxed in dry acetone for 3 h. The reaction mixture was cooled and poured onto ice water. The solid obtained was filtered, dried and recrystallized from ethanol to give **22**. Yield, 90 %, mp 200–202^{o} C. IR (KBr, cm^{-1}): 3240, 3200 (NH), 3038 (CH arom.), 2019 (N=C=S), 1626 (C=N), 1230 (C=S). ^{1}H-NMR (DMSO-d_6, ppm): 2.45 (s, 1H, NH, exchangeable with D_2O), 5.91 (s, 1H, CH-thiophene), 8.15 (s, 1H, CH- pyrimidine), 8.35 (s, 1H, NH, exchangeable with D_2O).^{13}C-NMR (DMSO-d_6, ppm): 102.62 (CH-thiophene), 124.82,

141.61, 143.15 (3C-thiophene), 137.82 (N=C=S), 142.74 (C-NCS), 156.98 (C-thiadiazole), 158.15 (CH-pyrimidine), 181.71 (C=S). MS m/z: 324 (M^+). Analysis calculated for $C_9H_4N_6S_4$: C, 33.32; H, 1.24; N, 25.90, found: C, 33.22; H, 1.14; N, 25.70.

General procedure for the preparation of 4-(4-imino-2-thioxo-5-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-1,2-dihydrothieno[2,3-d]pyrimidin-3(4H)-yl) N-substituted benzenesulfonamide ***(24-26).***

A mixture of compound **3** (1.02 g, 0.004 mol) and the appropriate substituted *4-isothiocyanato-benzenesulfonamide* **23 a-c** (0.004 mol) in dimethylformamide (20 mL) and a catalytic amount of triethylamine (0.2 mL) was refluxed for 6h. The reaction mixture was cooled, poured onto ice water and the precipitated solid was collected by filtration, dried, and recrystallized from ethanol to give **24-26**, respectively.

4-(4-imino-2-thioxo-5-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-1,2-dihydrothieno[2,3-d]pyrimidin-3(4H)-yl)benzenesulfonamide ***(24).***
Yield, 92 %, mp 200–202^o C. IR (KBr, cm^{-1}): 3320, 3230, 3200 (NH, NH_2), 3038 (CH arom.), 1626 (C=N), 1230 (C=S), 1380, 1145 (SO_2). ^{1}H-NMR (DMSO-d_6, ppm): 2.51 (s, 1H, NH, exchangeable with D_2O), 5.92 (s, 1H, CH-thiophene), 6.81, 7.92 (2d, 4H, *J*= 8.5Hz, Ar-H), 8.15, 8.91, 9.24 (3s, 3H, 3NH, exchangeable with D_2O), 11.15 (s, 2H, SO_2NH_2, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6, ppm): 103.62 (CH-thiophene), 118.81, 127.60, 159.14 (3C-thiophene), 126.72 (2C), 129.81 (2C), 136.15, 138.92, 156.15 (C=NH), 156.94 (C-thiadiazole), 179.82 (C=S pyrimidine), 181.71 (C=S). MS m/z: 469 (M^+). Analysis calculated for $C_{14}H_{11}N_7O_2S_5$: C, 35.81; H, 2.36; N, 20.88, found: C, 35.71; H, 2.26; N, 20.68.

4-(4-imino-2-thioxo-5-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-1,2-dihydrothieno[2,3-d]pyrimidin-3(4H)-yl)-N-(pyridin-2-yl)benzenesulfonamide ***(25).***
Yield, 86 %, mp 220–222^o C. IR (KBr, cm^{-1}): 3230, 3200 (NH), 3048 (CH arom.), 1626 (C=N), 1230 (C=S), 1387, 1145 (SO_2). ^{1}H-NMR (DMSO-d_6, ppm): 2.51 (s, 1H, NH, exchangeable with D_2O), 5.82 (s, 1H, CH-thiophene), 6.72, 7.24 (2d, 4H, *J*= 7.9Hz, Ar-H), 7.34-7.91 (m, 4H, CH pyridine), 8.15, 8.91, 9.32, 10.75 (4s, 4H, 4NH, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6, ppm): 102.61 (CH-thiophene), 108.15, 117.56, 137.81, 148.82, 153.53 (CH-pyridine), 122.82, 145.64, 159.15 (3C-thiophene), 126.70 (2C), 128.83 (2C), 135.17, 138.90, 156.42 (C=NH), 156.61 (C-thiadiazole), 179.80 (C=S pyrimidine), 181.73 (C=S). MS m/z: 546 (M^+). Analysis calculated for $C_{19}H_{14}N_8O_2S_5$: C, 41.74; H, 2.58; N, 20.50, found: C, 41.64; H, 2.48; N, 20.30.

4-(4-imino-2-thioxo-5-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylamino)-1,2-dihydrothieno[2,3-d]pyrimidin-3(4H)-yl)-N-(quinoxalin-2-yl)benzene sulfonamide ***(26).***
Yield, 84 %, mp 190–192^o C. IR (KBr, cm^{-1}): 3240, 3200 (NH), 3038 (CH arom.), 1626 (C=N), 1230 (C=S), 1387, 1145 (SO_2). ^{1}H-NMR (DMSO-d_6, ppm): 2.51 (s, 1H, NH,

exchangeable with D_2O), 5.84 (s, 1H, CH-thiophene), 6.88,7.25 (2d, 4H, *J*= 9.8Hz, Ar-H), 7.31-7.92 (m, 4H, CH-quinoxaline), 8.15 (s, 1H, CH-quinoxaline), 8.54, 8.91, 9.15, 10.72 (4s, 4H, 4NH, exchangeable with D_2O). ^{13}C-NMR (DMSO-d_6, ppm): 103.61 (CH-thiophene), 117.82, 123.61, 159.15 (3C-thiophene), 124.70 (2C), 126.73 (2C), 135.16, 138.18, 125.80 (2C), 127.24 (2C), 135.15(2C), 137.13, 162.18(C, CH-quinoxaline), 156.42 (C=NH), 156.62 (C-thiadiazole), 179.80 (C=S-pyrimidine), 181.71 (C=S). MS m/z: 597 (M^+). Analysis calculated for $C_{22}H_{15}N_9O_2S_5$: C, 44.21; H, 2.53; N, 21.09, found: C, 44.11; H, 2.33; N, 21.19.

2.3.Biological Evaluation

2.3.1. Materials and Animals

Male Wistar rats (150-180 g) aged (2-3 months) (used for paw oedema, ulcer and liver & kidney function tests) and adult male swiss albino mice (25-35 g) aged (3-4 months) (used for acute toxicity test) were purchased from the animal breeding unit of the National Research Centre, Giza, Egypt, and acclimatized in the animal facility of the National Centre for Radiation Research and Technology (NCRRT)-Atomic Energy Authority, Cairo, Egypt, for one week before being used. Animals were housed at a temperature of 25 ± 5ºC, humidity of 60 ± 5% and 12/12-hour light-dark cycle. They were fed standard pellet diet obtained from the National Research Centre, Dokki, Cairo and allowed free access to water ad libitum. The study was conducted in accordance with the guidelines set by the European Economic Community (EEC) regulations (Revised Directive 86/609/EEC) and approved by the Ethics Committee at the Faculty of Pharmacy, Cairo University. An enzyme immunoassay (EIA) kit (catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA) was purchased and used as manufacturer's instructions.

2.3.2. Irradiation

Irradiation of animals was carried out using the facilities provided by National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt using the gamma Cell-40 biological irradiator furnished with a Caesium137 (Cs ^{137}) source produced by the Atomic Energy of Canada (dose rate= 0.47 G/min). Animals were pre-irradiated 24 hour before the experiment at a dose level of 4 Gy [35].

2.3.3. In-vitro COX-1/COX-2 enzyme inhibition assay

The *in-vitro* ability of the synthesized compounds and celecoxib to inhibit the COX-1 and COX-2 isozymes was carried out using Cayman colorimetric COX (ovine) inhibitor screening assay kit (kit catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Celecoxib was used as a reference drug in this screening assay. COX catalyzes the first step in the biosynthesis of arachidonic acid to PGH2. The PGF2α produced from PGH2 by reduction with stannous chloride is measured by enzyme immunoassay. All the synthesized compounds were dissolved in 100% DMSO to prepare a stock concentration of 10mg/mL. Briefly, the enzyme COX-1 and COX-2 (10 µL), heme (10 µL) and 10 µL of the tested drug solutions (100 µM) were added in duplicate to the

supplied reaction buffer solution (950 μL, 0.1 M Tris–HCl, pH 8 containing 5 mM ethylenediamine tetraacetate (EDTA) and 2 mM phenol).These solutions were incubated for a period of 5 min at 37◦C after which 10 μL of AA (100 μM) solution were added and the COX reaction was stopped by the addition of 50 μL of 1 M HCl after 2 min. This assay based on competition between PGs and PGs acetylcholinesterase (AChE) conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that it is able to bind to PG antiserum was inversely proportional to concentration of PG in the well since the concentration of PG tracer is held constant while the concentration of PGs varies. This screening assay directly measures PGF2α produced by $SnCl_2$ reduction of COX-derived PGH2. This antibody PG complex binds to a mouse anti-rabbit monoclonal antibody that had been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent, which contains the substrate to acetylcholine esterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow colour that absorbs at 410 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation: (Absorbance α [Bound PG Tracer] α1/PGs). Percent inhibition was calculated by the comparison of compound treated to various control incubations. The concentration of the test compound causing 50% inhibition (IC_{50}, μM) was calculated from the concentration-inhibition response curve.

2.3.4. Docking Study

All docking calculation and docking studies were carried out using Molecular Operating Environment MOE version 2008.10. For this purpose, crystal structure of COX-2/SC-558 (a selective inhibitor) complex (PDB codes: 1CX2) was obtained from the Protein Data Bank in order to prepare the protein for docking studies. Docking procedure was followed using the standard protocol implemented in MOE 2008.10 and the geometry of resulting complexes was studied using the MOE's Pose Viewer utility. The enzyme was prepared for docking as follows: 1) The Co-crystallized ligand and water molecules were removed. 2) The enzyme was 3D protonated, where hydrogen atoms were added at their standard geometry, the partial charges were computed and the system was optimized. Flexible ligand- rigid receptor docking of the most stable conformers was done with MOE-DOCK using triangle matcher as placement method and London dG as a scoring function. The obtained poses were subjected to force field refinement using the same scoring function. Thirty of the most stable docking models for each ligand were retained with the best scored conformation.

2.3.5. In-vivo anti-inflammatory activity against carrageenan-induced rat paw oedema pre-exposed to whole body gamma irradiation

The anti-inflammatory activity of twelve compounds of the most potent COX-2 inhibitors resulting from *in-vitro* enzyme inhibition assay, compounds **2, 3, 7, 11, 14, 16, 17, 19, 21, 22, 24** and **26** was evaluated using *in-vivo* carrageenan-induced paw oedema model as reported [36] using diclofenac and celecoxib as standards. A total of 90 Male Wister rats (150- 180 g body weight) and aged (2-3 months) were used in this experiment. Rats were randomly separated to 16 groups, each of 6 rats in labelled cages. Group 1 served as control

inflammation and rest groups were exposed to whole body γ-irradiation (4 Gy) [35] (24 h prior to carrageenan sub-plantar injection (0.1 ml of 1% soln.) of each rat. Each tested compound was dissolved in 5% tween 80 and administered intraperitoneally in a dose of 100 mg/kg.b.wt. 1 h before induction of oedema by carrageenan. The initial volume (Vi) of induced oedema was measured using a water digital plethysmometer, LE 7500 (Panlab, HARVARD Apparatus, Spain) by immersing the paw till the level of tibiotarsic articulation into the container of the plethysmometer and the displacement volume (in mL) was measured by two platinum electrodes introduced beforehand into the container. The rat's foot pad became oedematous soon after the injection of carrageenan and the paw volume (Vf) was measured again 3 h after carrageenan injection. The increase in paw volume was calculated as percentage of oedema compared to the basal paw volume according to the formula:

$$\% \text{ of oedema} = [(Vf - Vi) / Vi] \times 100$$

2.3.6. In-vivo analgesic activity against carrageenan-induced rat hyperalgesia pre-exposed to whole body gamma irradiation

Analgesic activity of the same twelve potent compounds **2, 3, 7, 11, 14, 16, 17, 19, 21, 22, 24** and **26** was evaluated in hyperalgesic rats using the Carrageenan-induced hyperalgesia method according to Randall and Selitto [37] using diclofenac and celecoxib as standards. A total of 96 Male Wister rats (150- 180 g body weight) and aged (2-3 months) were used in this experiment. Rats were randomly separated to 16 groups, each of 6 rats in labelled cages. Group 1 served as normal, group 2 served as control inflammation and groups from 3-16 were exposed to whole body γ-irradiation (4 Gy) [35] 24 h prior to carrageenan injection (0.1 ml of 1% soln.). Each tested compound was dissolved in 5% tween 80 and administered intraperitoneally in a dose of 100 mg/kg.b.wt. 1 h before carrageenan injection. The nociceptive threshold of the hind paw injected with carrageenan was quantified with an anlgesimeter (Ugo Basile, Comerio, Varese, Italy) 3 h after carrageenan injection. The force, in grams, applied to the paw was increased at a constant rate until the rat withdraws its paw. The pressure was immediately removed and the force required to elicit the end-point response was recorded.

2.4.Toxicological studies

2.4.1. Ulcerogenic liability

The ulcerogenic effect of the same twelve potent compounds having the highest anti-inflammatory activity and the standards diclofenac and celecoxib were evaluated by the reported method [38]. To measure gastric ulceration, a total of 90 adult male Wister rats (150- 180 gm body weight) and aged (2-3 months) were divided into 15 groups of six animals each. Animals were kept under standard laboratory conditions and fasted 24 h prior to administration of the tested compounds. The tested compounds were administered orally in a dose of 100 mg.kg.bwt. The animals sacrificed 4 h after drug treatment [38]. The stomachs were removed and examined with an eye lens for the presence of ulcers and erosions. Ulcer

index was calculated according to the method of Kulkarni [39] using the following scores involving number and severity of ulcers:

0.0= normal colored stomach 0.5= red coloration 1.0= spot ulcer 1.5= hemorrhagic streaks. 2.0= ulcers with area >3mm^2 but ≤5 mm^2. 3.0= ulcers with area >5mm^2.

Ulcer index (UI) = (UN+US+UP) * 10-1

Where, UN= average no. of ulcers per animal. US= average of severity scores. UP= percentage of animals with ulcers per group.

The presence of any one of these criteria was considered to be evidence of ulcerogenic activity.

2.4.2. Acute Toxicity Study

The approximate 50% lethal dose (ALD50) of the most representative promising compounds (**17**, **21** and **26**) was determined. Adult Swiss male albino mice (3-4 months) weighing 25- 35 g were obtained from the animal breeding unit of the National Research Centre, Giza, Egypt. The study was conducted in accordance with the guidelines set by the European Economic Community (EEC) regulations (Revised Directive 86/609/EEC) and approved by the Ethics Committee at the Faculty of Pharmacy, Cairo University. A total of 40 mice were taken and separated to 10 mice groups in labelled cages. The tested compounds were injected intraperitoneally (i.p.) at different dose levels (100, 200, 500 and 1000 mg/kg. b.wt) . Animals were kept under observation for 24- 48 h during which any mortality in each group was recorded. All the animals had free access to food and water after drug administration. After 24 hrs, they were sacrificed by cervical dislocation. From the data obtained, the ALD50 was calculated by the method of Smith [40].

2.4.3. Liver and kidney functions estimation

This study was carried on adult male wistar rats (150- 180g), 36 rats were randomly divided into 6 groups, 6 rats in each group, group 1 received vehicle (5% tween 80) and served as control group, groups 2 & 3 received diclofenac and celecoxib (50 mg/ kg. i.p.) and groups from 4 to 6 received the promising compounds (**17**, **21** and **26**; 100 mg/ kg. i.p.) [according to pilot studies]. All animals were injected with the selected vehicle or drug for 2 consecutive days [41]; blood samples were collected 4 hours from the last dose of vehicle or drug. Blood samples were allowed to clot for 45 min at room temperature and serum was separated by centrifugation at 3000 rpm for 15 min and analyzed for assessment of liver and kidney functions such as serum glutamic- pyruvic transaminase (SGPT) and serum glutamatic-oxaloacetic transaminase (SGOT) by reported method of Reitman and Frankel [42], and serum creatinine (SCr) by Schirmeister et. al. method [43]**,** respectively.

2.5.Statistical Analysis

All values were expressed as means ± S.E. Data was analyzed using one-way ANOVA followed by Tukey-Kramer multiple comparison test. The p value was considered significant at $P < 0.05$. Graphpad software instat (version 6) was used to carry out these statistical tests.

3. Results, discussion and SAR findings

3.1.Chemistry

The synthetic pathways adopted to obtain in good yields the target compounds **3-26** are outlined in schemes 1 and 2. For the exploration of structure activity relationship of 1,3,4-thiadiazole based compounds as selective COX-2 inhibitors, different synthetic strategies were done by varying the substituents on the reported 2-acetamide thiadiazole derivative **2** [44] which was obtained from monoacetylation of the reported 5-amino-3H-[1,3,4]thiadiazole-2-thione **1** [45] via simple synthetic methods.

Following Gewald's thiophene synthesis, the thiophene derivatives **3-5** were synthesized through reaction of **2** with activated nitrile (malononitrile, ethyl cyanoacetate and/or ethyl acetoacetate) in the presence of elemental sulfur [46]. Treatment of 2-acetamide thiadiazole **2** with the appropriate 2-(4-substituted benzylidene) cyanoacetate and/or 2-(4-substituted benzylidene) malononitrile [47] in ethanol yielded the corresponding pyran derivatives **7-14** following the reported method [48]. (scheme 1)**.**

Scheme 1. Synthetic pathways for compounds **3-5**, **7-14**.

2-Chloroacetamido thiophene derivative **15** was obtained by stirring compound **3** with chloroacetyl chloride in DMF. While, the ethyl formamidate thiophene derivative **16** was prepared by refluxing compound **3** in triethylorthoformate in the presence of a few drops of acetic anhydride, followed by cycloaddition of phenyl hydrazine yielding the 3-(phenylamino)-thieno[2,3-d]pyrimidine derivative **17**. Refluxing compound **3** with phenyl isocyanate in ethanol yielded the thieno[2,3-d]pyrimidin-2(1H)-one derivative **18** by nucleophilic substitution followed by intramolecular cyclization. (scheme 2)

Formylation of compound **3** was carried out by heating with formic acid to give the thieno[2,3-d]pyrimidin-4(3H)-one derivative **19**. Compound **19** was further subjected to thionation with phosphorus pentasulfide in pyridine to afford the thieno[2,3-d] pyrimidine-4(3H)-thione derivative **20**, and chlorination with thionyl chloride yielding the 4-chlorothieno[2,3-d]pyrimidine derivative **21**. Compound **21** was then converted to the 4-isothiocyanatothieno[2,3-d]pyrimidine derivative **22** through reaction with ammonium thiocyanate in dry acetone. On the other hand, refluxing compound **3** with the appropriate substituted 4-isothiocyanato benzenesulfonamide **23a-c** prepared by the reported method [49] in DMF yielded the thieno[2,3-d] pyrimidin-3(4H)-yl) N-substitutedbenzenesulfonamide derivatives **24-26,** respectively. (scheme 2).

The synthesized compounds were characterized by IR, ^{1}H-NMR,^{13}C-NMR and mass spectra and were in conformity with the assigned structures as listed in the material and methods section.

Scheme 2. Synthetic pathways for compounds **15-18**, **19-22** and **24-26**.

3.2.Biological activity

3.2.1. In-vitro COX-1/COX-2 enzyme inhibition assay

All the target compounds were evaluated for their ability to inhibit COX-1 and COX-2 using an ovine COX-1/COX-2 assay kit (Catalog No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA). The concentration causing 50% enzyme inhibition (IC_{50} µM) was determined as well as the selectivity index (SI values) which is defined as IC_{50} (COX-1)/IC_{50}(COX2). In the assay system, we selected two commercially available drugs, diclofenac, a potent non-selective COX inhibitor, and celecoxib, a potent selective COX-2 inhibitor as reference drugs. The IC_{50} values of diclofenac on COX-1 and COX-2 were 0.25 and 0.26 µM respectively, with low SI (0.96 µM). The IC_{50} values of celecoxib on COX-1 and COX-2 were determined to be >50 and 0.18 µM respectively, with high SI (>277.77 µM). The results showed that most of the synthesized compounds showed no activity on COX-1 with IC_{50} values >50 µM, among them only three compounds **2**, **11** and **19** were active on COX-1, with IC_{50} values 23.5, 40.1and 25.3 µM, respectively. With respect to activity on COX-2, some of the tested compounds showed potent COX-2 inhibition with IC_{50} values ranging from 0.09-0.36 µM) compared to celecoxib. The most potent compounds in this study were **3, 7, 14, 16, 17, 21, 22, 24** and **26** having similar or even higher SI than celecoxib ranging from 231.48-555.55 µM as listed in Table 1.

As aforementioned, the aim of the present study was to obtain selective COX-2 inhibitors, so it is worthy to understand the SAR of the synthesized compounds. The design of the target compounds relies on the synthesis of several distinct novel compounds based on 2-acetamido thiadiazole scaffold. The activity of unsubstituted 2-amino-thiadiazole ring was explored by testing compound **1** the starting material which has no activity on both COX enzymes, while, upon addition of acetamide group at position 2, significant COX-1/COX-2 activity was observed but with lower selectivity index as compound **2** (COX-1/IC_{50}= 23.5, COX-2/IC_{50}= 0.366; SI=64.2 µM).

Introduction of thiophene ring in compounds **3-5, 15** and **16** showed COX-2 inhibition depending on the substitution at position 2 and 3 of the thiophene ring, , the 2-amino-3-carbonitrile substitution resulted in potent COX-2 activity with high selectivity index in compound **3** (IC_{50}= 0.15; SI>333.3 µM), the 2-ethyl formamidate-3-carbonitrile substitution resulted in high COX-2 activity and high selectivity index (IC_{50}= 0.198; SI=384.61 µM) as in compound **16**, while, the 2-amino-3-ethylcarboxylate, the 2-methyl-3-ethyl-carboxylate and 2-chloracetamide-3-carbonitrile substitution in compounds **4**, **5** and **15** showed no activity on both COX enzymes.

The COX-2 inhibitory activity and the selectivity indices of the pyran derivatives **7-14** varies according to the substituent at position 3 of the pyran ring and the *para*-substitution on the aromatic ring at position 4. the *para*-chloro-ethylcarboxylate **7** (IC_{50}= 0.171; SI>292.3 µM) showed potent COX-2 inhibition with extremely high SI, while, the *para*-chloro-carbonitrile **11** derivative (IC_{50}= 0.257; SI=156.03 µM) showed similar COX-2 activity but with lower SI. In addition, *N*-dimethyl-carbonotrile **14** derivative (IC_{50}= 0.168; SI>297.6 µM) showed significant COX-2 activity with high SI. Other pyran derivatives have no activity on both COX enzymes.

Considering the thieno[2,3-d]pyrimidine derivatives **17-22**, SAR is discussed according to the type and position of the substituent on the pyrimidine ring., the 3-phenylamino-4-imino derivative **17** (IC_{50}= 0.151; SI>331.1 µM) showed increased COX-2 activity with high SI, while, the 2-oxo-3-*N*-phenyl-4-imino derivative **18** showed no activity on both COX enzymes. Upon removing the substituents at position 2 and 3 and only introducing carbonyl group at position 4 in compound **19**, the SI is highly affected and decreased to be 121.05 µM. The nature of the substituent at position 4 of the pyrimidine ring affected the COX-2 activity, where, the 4-thioxo derivative **20** displayed diminished activity on both enzymes. While, COX-2 activity is restored upon chlorination in compound **21** and slightly decreased in the 4-isothiocyanato derivative **22** (IC_{50}= 0.162, 0.216; SI=308.6, 231.48 µM, respectively). Introduction of sulfonamide group at position 3 of the pyrimidine was very successful especially for the sulfanilamide derivative **24** (IC_{50}= 0.156; SI>320.51 µM) and the sulfaquinoxaline derivative **26** (IC_{50}= 0.09; SI>555.55 µM) derivative which was the most potent and selective COX-2 inhibitors in this study. Generally, introduction of thieno[2,3-d]pyrimidine with sulfonamide moiety on thiadiazole ring led to the most potent and selective compounds in this study, also, we can observe the crucial inhibitory ability of either thiophene and pyran substituents on thiadiazole ring may be by allowing a better orientation of the compounds within the additional pocket responsible for selectivity of COX-2 enzyme.

Finally, it could be noted from the above results that 1,3,4-thiadiazole bearing thiophene, pyran or thieno[2,3-d]pyrimidine moieties are promising scaffolds to develop potent selective COX-2 inhibitors.

Table 1. *In-vitro* COX-1/COX-2 enzyme inhibition assay.

3.2.2. Docking Study

In order to further interpret the mechanism of interaction and the binding mode of the synthesized compounds within COX-2 active site, docking study was performed. Docking of all the new compounds together with the reference drugs celecoxib and diclofenac into the crystal structure of COX-2 enzyme catalytic domain in complex with the celecoxib analogue SC-558 [PDB ID code 1CX2] was performed using Molecular Operating Environment MOE version 2008.10. The most stable docking pose was selected according to the best scored conformation predicted by the MOE scoring function, the docking results are presented in

Table 2, where, the docking scores, amino acids interactions and bond lengths within the active site are listed for all the compounds.

The COX active site consists of three distinct sites, the carboxylate site at entrance which is composed of three hydrophilic residues Arg120, Tyr 355 and Glu524 arranged in a way to form H-bond network. This entrance leads to a long hydrophobic channel that deeply extends into the catalytic domain. The main difference between the two COX isoforms is that in COX-2, this hydrophobic channel forks to a primary hydrophobic pocket and side pocket. The primary pocket is defined by the amino acids Tyr385, Trp87, Phe518 and Ser530 which is the site of NSAIDS binding. While, the side pocket is located above Arg120 and this pocket is bordered by Val523 and contains the main residues responsible for COX-2 selectivity His90 and Arg513. This structural difference between the two COX isoform is the result of exchange of the relatively bulky isoleucine (Ile) at position of 523 in COX-1 with the less bulky Val residue at the same position in COX-2, this modification allows the access to an additional side pocket responsible for selectivity [6, 16, 18, 19].

To validate the docking protocol, the co-crystallized ligand was re-docked into COX-2 active site and the root mean square deviation (RMSD) is calculated and found to be 0.209 Å with docking score (S= -13.01 kcal/mol). Celecoxib was docked into the active site of COX-2, it showed similar orientations as reported [56], where, the toluene moiety is towards the primary hydrophobic pocket, the trifluoromethyl group is near Arg120, while, the phenyl sulfonamide moiety is inserted in the side pocket where the SO2 group H-bonded with His90 and Arg513 with bond lengths 2.90 and 1.56 Å, respectively and docking score (S= -11.03 kcal/mol). On the other hand, docking of diclofenac revealed that it could only access to the primary hydrophobic pocket, where, its carboxylate group binds to Ser530 and Tyr385 as reported (Table 2).

Considering docking of the synthesized compounds, there is strong correlation between the results of in-vitro binding assay with that of docking study. The most potent compounds **3**, **7**, **14**, **16**, **17**, **21**, **22**, **24** and **26** showed the best docking scores in the range of -11.49 to -9.54 kcal/mol. By closer observation of the binding mode and SAR of the synthesized compounds, in the thiophene containing compounds **3**-**5**, **15** and **16**, the 1,3,4-thiadiazole bearing thiophene-2-Amino-3-carbonitrile moiety **3** showed similar orientation as celecoxib, the introduced CN group was able to bind to Arg513 and His90, similarily, the 2-formamidate moiety of compound **16** was able to enter deeply into the selectivity pocket and binds to Arg513. On the other hand, no binding to the selectivity pocket was observed for compounds **4**, **5** and **15** which were inactive in the previous study.

In the pyran series **7**-**14**, the most selective candidates with higher SI were compounds **7** and **14**, the introduced *p*-chlorophenyl and the carboxylate group of **7** were able to interact with Arg513, the *p*-*N*-dimethylphenyl and carbonitrile moieities bonded with Arg513 in addition to another H-bond with Val523 in case of compound **14**, the bulky N-dimethyl group pushed the compound into the selectivity pocket. (Figures 2-4; Table 2). In case of compound **11**, the less bulky carbonitrile group failed to enter the selectivity pocket and resulted in a different orientation which explains the low SI in the previous experiment.

In the thieno[2,3-d]pyrimidine series **17**-**22**, the bulkiness and the position of substitutions on pyrimidine ring greatly affected the orientation of the compounds within the active site, the 3-phenylamino substitution in **17** was oriented in a way to bind through arene-cation interaction

with Arg513 in addition to binding to His90 through the thiadiazole ring, increasing the COX-2 activity. (Figure 5; Table 2). In compounds **21** and **22**, the 4-chloro and the 4-Isothiocyanate substituents bind to Arg513 through arene-cation interaction in addition to binding of the pyrimidine ring to His90 (Figures 6 and 7; Table 2). On the other hand, the selectivity of the sulfonamide derivatives **24** and **26** was explained by their higher binding affinity within the active site of COX-2 through SO_2 group which was pushed more deep within the side pocket resulting in stronger bonds with Arg513 and His90 with shorter bond length (Figures 8 and 9; Table 2).

Generally, it was interesting to note that the docking study helped to more understand the mechanism of binding of the synthesized compounds within the active site of COX-2. The most potent compounds showed similar orientation as celecoxib and showed high liability to enter the additional side pocket. The compounds that showed low SI were deeply immerged into the primary hydrophobic pocket in similar manner as traditional NSAIDS.

Table 2. Binding scores, amino acid interactions and bond length of the synthesized compounds within the active site of COX-2.

3.2.3. In-vivo anti-inflammatory activity against carrageenan-induced rat paw oedema pre-exposed to whole body gamma irradiation

The most potent COX-2 inhibitors resulting from *in-vitro* enzyme inhibition assay, compounds **2, 3, 7, 11, 14, 16, 17, 19, 21, 22, 24** and **26** were subjected to *in-vivo* anti-inflammatory assay using standard carrageenan-induced rat paw oedema model pre-exposed to 4 Gy of whole body gamma irradiation 24 h prior to carrageenan sub-plantar injection. However, cytokines production post irradiation is time-dependent and peaking usually achieved at 4–24 hrs after irradiation [50, 51]. The results are listed in Table **3** and are expressed as the percentage of oedema compared to basal paw volume. From the results we can conclude that the inflammatory response produced by carrageenan in irradiated rats was significantly higher than that induced in non-irradiated animals. This response is attributed to the increased levels of prostaglandins as well as lysosomal enzymes as a result of disruption of the cell membranes due to radiation exposure caused by direct interaction of cellular membranes with gamma-rays or by the action of the free radicals produced by ionizing radiation on the cellular membranes [52, 53]. Treatment of irradiated inflamed rats with the tested compounds showed significant decrease in the percentage of oedema in the range between 24.49-62.50%. The most potent compound in this study was the thiadiazole derivative bearing the 4-chlorothieno[2,3-d]pyrimidine moiety **21** having 24.49% of oedema compared to celecoxib (18.61%). Similarly, potent anti-inflammatory activity and non-significant difference from celecoxib was observed for the 3-phenylamino thieno[2,3-d]pyrimidine **17** and the sulfonamide derivative **26** with percentages of oedema of 24.70 and 25.40 %, respectively. Also compounds **14** and **16** showed potent anti-inflammatory activity and significant decrease in the percentage of oedema to 40.08-41.30%, respectively. These results were in agreement with the results of *in-vitro* enzyme inhibition assay indicating the crucial anti-inflammatory role of introduction of either thiophene and pyran moieties to the

thiadiazole scaffold, as well as incorporation of the bicyclic thieno[2,3-d]pyrimidine moiety and the sulfonamide substitution on the 1,3,4-thiadiazole ring.

3.2.4. In-vivo analgesic activity against carrageenan-induced rat hyperalgesia pre-exposed to whole body gamma irradiation

The most potent COX-2 inhibitors resulting from *in-vitro* enzyme inhibition assay, compounds **2, 3, 7, 11, 14, 16, 17, 19, 21, 22, 24** and **26** were subjected to *in-vivo* analgesic assay using standard carrageenan-induced hyperalgesia model pre-exposed to 4 Gy of whole body gamma irradiation 24 h prior to carrageenan sub-plantar injection. The nociceptive threshold defined as the maximum force, in grams, applied at a constant rate until the rat withdraws its paw, was quantified with an analgesimeter 3 h after carrageenan injection and presented in Table 3. Since whole body γ-irradiation led to significant increment in the paw volume and based on the sensitization theory of Randall and Selitto, the first to make use of the knowledge that inflammation increases sensitivity to pain [37], it was expected that upon exertion of mechanical hyperalgesia on the inflamed paw of irradiated rats, a significant decrement in the nociceptive threshold would be observed. In the present study the nociceptive threshold recorded in the inflamed irradiated rats did not differ significantly from that observed in the inflamed group. This was in accordance to the study of Kereskenyiova and Smajda who reported the possible underlying mechanism beyond this response showing that ionizing radiation would exert an analgesic effect mediated by the release of endogenous opioids [54]. The results showed that, celecoxib exhibited significantly high nociceptive threshold (90.23 g) when compared to the control group. All the tested compounds showed relatively high analgesic activities with nociceptive thresholds significantly different from control group in the range of 42.31-85.72 g. The most potent analgesic compound in this study was the thiadiazol-2-acetamide derivative **2** having the highest nociceptive threshold (85.72 g) very close to that of celecoxib. Also the pyran derivatives **11**and **14**, thiophene schiff's base derivative **16**, thieno[2,3-d]pyrimidine **19** and the sulfonamide derivative **24** showed potent analgesic activities with nociceptive thresholds significantly different from control group of 73.41, 62.12, 62.81, 61.50 and 61.21g, respectively. These results were also in agreement with the results of *in-vitro* enzyme inhibition assay underlining the analgesic activity of 1,3,4-thiadiazole-2-acetamide, sulfonamide substituted thiadiazole and 1,3,4-thiadiazole bearing pyran, thiophene or thieno[2,3-d]pyrimidine moieties.

Table 3. *In-vivo* anti-inflammatory, analgesic.

3.3.Toxicological studies

3.3.1. Ulcerogenic liability

The ulcerogenic potential of the most potent COX-2 inhibitors **2, 3, 7, 11, 14, 16, 17, 19, 21, 22, 24** and **26** was evaluated and the ulcer index was calculated as reported [38, 39]. The ulcerogenic liability was compared to celecoxib and the classical NSAID diclofenac. From the data listed in Table 4, it was observed that celecoxib as selective COX-2 inhibitor showed no ulceration effect (UI=0.0± 0.0), similarly, compounds **3, 7, 14, 16, 17, 21, 22** and **26**

showed extremely high safety margin on gastric mucosa with no ulceration effect (UI ranges 0.0-0.083), these results were in agreement with their high SI found in COX inhibitory assay. Furthermore, compounds **2, 11, 19** and **24** caused gastric ulceration effects of (UI= 0.50±0.033; 0.52±0.042; 0.45± 0.04 and 0.10± 0.004, respectively) in the experimental animals, these compounds showed low SI in the *in-vitro* binding assay due to non-selective inhibition of both COX enzymes. But interestingly, their observed ulcerogenic liabilities were less than that of diclofenac (UI=2.37± 0.09). In case of compound **24**, although it showed no activity on COX-1 and relatively high SI, slight ulcers were observed in experimental animals. This behavior could be interpreted from the docking study, the sulfonamide group was able to bind to Tyr355 in the carboxylate site where binds NSAIDS.

In conclusion, the potential value of the tested compounds as anti-inflammatory agents is their extremely high safety margin on gastric mucosa than diclofenac. These findings support the objective of the present work to present novel 1,3,4-thiadiazole bearing pyran, thiophene or thieno[2,3-d]pyrimidine moieties as selective COX-2 inhibitors with diminished gastric injuries.

Table 4. Ulcerogenic liability in rats.

3.3.2. Acute Toxicity Study

The most potent selective COX-2 inhibitors and anti-inflammatory compounds resulted from the previous experiments **17, 21** and **26** were selected to study their acute toxicity in rats. The approximate 50% lethal dose (ALD_{50}) was determined according to Smith's method as presented in Table 5. The results revealed that the tested compounds were relatively non-toxic in experimental rats showing ALD_{50}>1000 mg/kg.

3.3.3. Liver and kidney functions estimation

After determining the acute toxicity of the most potent compounds resulted from the previous experiments **17, 21** and **26.** They were further studied for their renal and hepatotoxic effect. Their effect on biochemical parameters (serum enzymes and serum creatinine) was studied. As shown in Table 5, activities of the liver enzymes serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) of compounds **17**, **21** and **26** almost remain the same with respect to the normal values. The histopathological studies of the liver samples of these compounds do not show any significant pathological changes in comparison to standard drug celecoxib. No hepatocyte necrosis or degeneration was seen in any of the samples.

On the other hand, serum creatinine (SCr) is the most sensitive biochemical marker employed in the diagnosis of renal damage. Therefore, marked increase in serum creatinine is an indication of functional damage to the kidney [55].

None of the tested compounds showed significant change in the mean values of creatinine in serum of rats when compared with the standard drug celecoxib. According to these indicators, the tested compounds **17, 21** and **26** are therefore, not nephrotoxic in rats.

Table5. *In-vivo* acute toxicity, kidney and liver Function Parameters.

4. Conclusion

The present study reported the design and synthesis of novel series of selective COX-2 inhibitors based on 1,3,4-thiadiazole moiety. The synthesized compounds were evaluated for their COX-1/COX-2 inhibitory activity *in-vitro*. Compounds **3, 7, 14, 16, 17, 21, 22, 24** and **26** were found to be potent and selective COX-2 inhibitors (IC_{50}= 0.09-0.366 µM) and were inactive against COX-1 (IC_{50}> 50 µM). SAR was discussed in terms of the enzyme inhibitory activity and was supported by molecular docking simulations and analysis of the binding modes of the new inhibitors within COX-2 active site. In addition, the most potent COX-2 inhibitors were assessed for their anti-inflammatory, analgesic activities and toxicological studies *in-vivo*. Interestingly, the most potent compounds in this study having the highest nociceptive threshold very close to that of celecoxib was the thiadiazol-2-acetamide derivative **2**, pyran derivatives **14** and **11** and the sulfonamide derivative **24**. While, the thiophene, thienopyrimidine derivatives **17** and **21** and the sulfonamide derivative **26** showed the highest anti-inflammatory activity compared to the reference drug celecoxib, they also had high safety margin on gastric mucosa with no ulceration effect and they were found to be non-toxic in experimental rats with normal kidney and liver function profiles. These results suggested a contributory role of 1,3,4-thiadiazole derivatives in designing potent and selective COX-2 inhibitors with diminished gastric injuries.

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References

[1] A.M. Alanazi, A.S. El-Azab, I.A. Al-Suwaidan, K.E. ElTahir, Y.A. Asiri, N.I. Abdel-Aziz, A.A. Abdel-Aziz, Structure-based design of phthalimide derivatives as potential cyclooxygenase-2 (COX-2) inhibitors: anti-inflammatory and analgesic activities, Eur J Med Chem, 92 (2015) 115-123.

[2] X. Lu, H. Zhang, X. Li, G. Chen, Q.S. Li, Y. Luo, B.F. Ruan, X.W. Chen, H.L. Zhu, Design, synthesis and biological evaluation of pyridine acyl sulfonamide derivatives as novel COX-2 inhibitors, Bioorg Med Chem, 19 (2011) 6827-6832.
[3] E.A. Meade, W.L. Smith, D.L. DeWitt, Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs, J Biol Chem, 268 (1993) 6610-6614.
[4] W.L. Xie, J.G. Chipman, D.L. Robertson, R.L. Erikson, D.L. Simmons, Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing, Proc Natl Acad Sci U S A, 88 (1991) 2692-2696.
[5] A.A. Abdel-Aziz, K.E. ElTahir, Y.A. Asiri, Synthesis, anti-inflammatory activity and COX-1/COX-2 inhibition of novel substituted cyclic imides. Part 1: Molecular docking study, Eur J Med Chem, 46 (2011) 1648-1655.
[6] J.R. Vane, Y.S. Bakhle, R.M. Botting, Cyclooxygenases 1 and 2, Annu Rev Pharmacol Toxicol, 38 (1998) 97-120.
[7] J.R. Vane, R.M. Botting, Anti-inflammatory drugs and their mechanism of action, Inflamm Res, 47 Suppl 2 (1998) S78-87.
[8] A.A. Moneer, K.O. Mohammed, H.B. El-Nassan, Synthesis of Novel Substituted Thiourea and Benzimidazole Derivatives Containing a Pyrazolone Ring as Anti-Inflammatory Agents, Chem Biol Drug Des, 87 (2016) 784-793.
[9] K.L. Cook, J.L. Schwartz-Roberts, W.T. Baumann, R. Clarke, Linking autophagy with inflammation through IRF1 signaling in ER+ breast cancer, Molecular & cellular oncology, 3 (2016) e1023928.
[10] M.H. Barcellos-Hoff, How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues, Radiation research, 150 (1998) S109-120.
[11] G. Desmarais, G. Charest, D. Fortin, R. Bujold, D. Mathieu, B. Paquette, Cyclooxygenase-2 inhibitor prevents radiation-enhanced infiltration of F98 glioma cells in brain of Fischer rat, International journal of radiation biology, 91 (2015) 624-633.
[12] M.T. Khayyal, M.A. El-Ghazaly, R.M. El-Hazek, A.S. Nada, The effects of celecoxib, a COX-2 selective inhibitor, on acute inflammation induced in irradiated rats, Inflammopharmacology, 17 (2009) 255-266.
[13] M.A. El-Ghazaly, M.T. Khayyal, The use of aqueous propolis extract against radiation-induced damage, Drugs Exp Clin Res, 21 (1995) 229-236.
[14] M. el-Ghazaly, S. Kenawy, M.T. Khayyal, H. Roushdy, S. Saleh, Effect of exposure to radiation on the inflammatory process and its influence by diclofenac, Br J Pharmacol, 85 (1985) 45-50.
[15] T.J. FitzGerald, J. Aronowitz, M. Giulia Cicchetti, G. Fisher, S. Kadish, Y.C. Lo, C. Mayo, S. McCauley, J. Meyer, R. Pieters, A. Sherman, The effect of radiation therapy on normal tissue function, Hematol Oncol Clin North Am, 20 (2006) 141-163.
[16] A. Zarghi, S. Arfaei, Selective COX-2 Inhibitors: A Review of Their Structure-Activity Relationships, Iran J Pharm Res, 10 (2011) 655-683.
[17] M.D. Tortorella, Y. Zhang, J. Talley, Desirable Properties for 3rd Generation Cyclooxygenase-2 Inhibitors, Mini Rev Med Chem, (2016).
[18] A. Zarghi, S. Kakhgi, A. Hadipoor, B. Daraee, O.G. Dadrass, M. Hedayati, Design and synthesis of 1,3-diarylurea derivatives as selective cyclooxygenase (COX-2) inhibitors, Bioorg Med Chem Lett, 18 (2008) 1336-1339.
[19] A. Zarghi, S. Arfaee, P.N. Rao, E.E. Knaus, Design, synthesis, and biological evaluation of 1,3-diarylprop-2-en-1-ones : a novel class of cyclooxygenase-2 inhibitors, Bioorg Med Chem, 14 (2006) 2600-2605.
[20] S.J. Lin, W.J. Tsai, W.F. Chiou, T.H. Yang, L.M. Yang, Selective COX-2 inhibitors. Part 2: synthesis and biological evaluation of 4-benzylideneamino- and 4-phenyliminomethyl-benzenesulfonamides, Bioorg Med Chem, 16 (2008) 2697-2706.

[21] H.F. Al-Shareef, H.A. Elhady, A.H. Aboellil, E.M. Hussein, Ammonium chloride catalyzed synthesis of novel Schiff bases from spiro[indoline-3,4'-pyran]-3'-carbonitriles and evaluation of their antimicrobial and anti-breast cancer activities, SpringerPlus, 5 (2016) 887.
[22] N.N.E. El-Sayed, M.A. Abdelaziz, W.W. Wardakhan, R.M. Mohareb, The Knoevenagel reaction of cyanoacetylhydrazine with pregnenolone: Synthesis of thiophene, thieno[2,3-d]pyrimidine, 1,2,4-triazole, pyran and pyridine derivatives with anti-inflammatory and anti-ulcer activities, Steroids, 107 (2016) 98-111.
[23] M.M. Ghorab, F.A. Ragab, H.I. Heiba, R.M. El-Hazek, Anticancer and radio-sensitizing evaluation of some new thiazolopyrane and thiazolopyranopyrimidine derivatives bearing a sulfonamide moiety, European journal of medicinal chemistry, 46 (2011) 5120-5126.
[24] R.M. Mohareb, M.Y. Zaki, N.S. Abbas, Synthesis, anti-inflammatory and anti-ulcer evaluations of thiazole, thiophene, pyridine and pyran derivatives derived from androstenedione, Steroids, 98 (2015) 80-91.
[25] Y.H. Hyup, J.K. Kwan, S.H. Kang, M.S. Noh, J.Y. Ha, J.K. Kyu, K.M. Min, C.H. Hoon, S. Chung, 2,3-Diarylbenzopyran derivatives as a novel class of selective cyclooxygenase-2 inhibitors, Bioorg Med Chem Lett, 13 (2003) 413-417.
[26] F. Caturla, J.M. Jiménez, N. Godessart, M. Amat, A. Cárdenas, L. Soca, J. Beleta, H. Ryder, M.I. Crespo, Synthesis and biological evaluation of 2-phenylpyran-4-ones: a new class of orally active cyclooxygenase-2 inhibitors, J Med Chem, 47 (2004) 3874-3886.
[27] H.M. Aly, N.M. Saleh, H.A. Elhady, Design and synthesis of some new thiophene, thienopyrimidine and thienothiadiazine derivatives of antipyrine as potential antimicrobial agents, European journal of medicinal chemistry, 46 (2011) 4566-4572.
[28] C.-H. Park, C. Lee, J.S. Yang, B.-Y. Joe, K. Chun, H. Kim, H.Y. Kim, J.S. Kang, J.I. Lee, M.-H. Kim, G. Han, Discovery of thienopyrimidine-based FLT3 inhibitors from the structural modification of known IKKβ inhibitors, Bioorganic & Medicinal Chemistry Letters, 24 (2014) 2655-2660.
[29] A.E. Rashad, A.H. Shamroukh, R.E. Abdel-Megeid, A. Mostafa, R. El-Shesheny, A. Kandeil, M.A. Ali, K. Banert, Synthesis and screening of some novel fused thiophene and thienopyrimidine derivatives for anti-avian influenza virus (H5N1) activity, European journal of medicinal chemistry, 45 (2010) 5251-5257.
[30] B. Sirisha, B. Narsaiah, T. Yakaiah, G. Gayatri, G.N. Sastry, M.R. Prasad, A.R. Rao, Synthesis and theoretical studies on energetics of novel N- and O- perfluoroalkyl triazole tagged thienopyrimidines – Their potential as adenosine receptor ligands, European journal of medicinal chemistry, 45 (2010) 1739-1745.
[31] J.K. Gierse, S.D. Hauser, D.P. Creely, C. Koboldt, S.H. Rangwala, P.C. Isakson, K. Seibert, Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase, Biochem J, 305 (Pt 2) (1995) 479-484.
[32] M. Barone, A.C. Graziano, A. Marrazzo, P. Gemmellaro, A. Santagati, V. Cardile, Synthesis and biological evaluation of new benzo-thieno[3,2-d]pyrimidin-4-one sulphonamide thio-derivatives as potential selective cyclooxygenase-2 inhibitors, Mol Divers, 17 (2013) 445-458.
[33] Y. Hu, C.Y. Li, X.M. Wang, Y.H. Yang, H.L. Zhu, 1,3,4-Thiadiazole: synthesis, reactions, and applications in medicinal, agricultural, and materials chemistry, Chemical reviews, 114 (2014) 5572-5610.
[34] J. Matysiak, Biological and pharmacological activities of 1,3,4-thiadiazole based compounds, Mini Rev Med Chem, 15 (2015) 762-775.
[35] G. Simsek, S. Gurocak, N. Karadag, A.B. Karabulut, E. Demirtas, E. Karatas, E. Pepele, Protective effects of resveratrol on salivary gland damage induced by total body irradiation in rats, The Laryngoscope, 122 (2012) 2743-2748.
[36] C.A. Winter, E.A. Risley, G.W. Nuss, Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs, Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.), 111 (1962) 544-547.

[37] L.O. Randall, J.J. Selitto, A method for measurement of analgesic activity on inflamed tissue, Archives internationales de pharmacodynamie et de therapie, 111 (1957) 409-419.
[38] R. Esser, C. Berry, Z. Du, J. Dawson, A. Fox, R.A. Fujimoto, W. Haston, E.F. Kimble, J. Koehler, J. Peppard, E. Quadros, J. Quintavalla, K. Toscano, L. Urban, J. van Duzer, X. Zhang, S. Zhou, P.J. Marshall, Preclinical pharmacology of lumiracoxib: a novel selective inhibitor of cyclooxygenase-2, British journal of pharmacology, 144 (2005) 538-550.
[39] S.K. Kulkarni, Handbook of experimental Pharmacology, Vallabh Prakashan, New Delhi, 2002.
[40] Q.E. Smith, Pharmacological screening test progress in medicinal chemistry, Butterworths, London, 1960.
[41] A. Tanna, R. Nair, S. Chanda, Assessment of anti-inflammatory and hepatoprotective potency of Polyalthia longifolia var. pendula leaf in Wistar albino rats, Journal of natural medicines, 63 (2009) 80-85.
[42] S. Reitman, S. Frankel, A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases, American journal of clinical pathology, 28 (1957) 56-63.
[43] J. Schirmeister, H. Willmann, H. Kiefer, W. Hallauer, [FOR AND AGAINST THE USEFULNESS OF ENDOGENOUS CREATININE CLEARANCE IN FUNCTIONAL KIDNEY DIAGNOSIS], Deutsche medizinische Wochenschrift (1946), 89 (1964) 1640-1647.
[44] S. Talath, A.K. Gadad, Synthesis, antibacterial and antitubercular activities of some 7-[4-(5-amino-[1,3,4]thiadiazole-2-sulfonyl)-piperazin-1-yl] fluoroquinolonic derivatives, European journal of medicinal chemistry, 41 (2006) 918-924.
[45] N.S. Cho, G.N. Kim, C. Parkanyi, ChemInform Abstract: Synthesis of 5-Aroylamino-3H-1,3,4-thiadiazole-2-thiones and Their Tautomerism, ChemInform, 24 (1993) no-no.
[46] R.W. Sabnis, The Gewald Synthesis, Sulfur Reports, 16 (1993) 1-17.
[47] F. Bigi, M.L. Conforti, R. Maggi, A. Piccinno, G. Sartori, Clean synthesis in water: uncatalysed preparation of ylidene malononitriles, Green Chem, 2 (2000) 101-103.
[48] F.M.A.A. El-Taweel, Studies with quinolines: New synthetic routes to 4H,5H,6H,9H-benzo[ij]pyrano[2,3-b]quinolizine-8-one, 4H-pyrano[2,3-b]pyridine, 2H-pyran-2-one and pyranopyridoquinoline derivatives, Journal of Heterocyclic Chemistry, 42 (2005) 943-946.
[49] M.M. Ghorab, F.A. Ragab, H.I. Heiba, M.G. El-Gazzar, M.G. El-Gazzar, Synthesis, in vitro anticancer screening and radiosensitizing evaluation of some new 4-[3-(substituted)thioureido]-N-(quinoxalin-2-yl)-benzenesulfonamide derivatives, Acta pharmaceutica (Zagreb, Croatia), 61 (2011) 415-425.
[50] J.H. Hong, C.S. Chiang, I.L. Campbell, J.R. Sun, H.R. Withers, W.H. McBride, Induction of acute phase gene expression by brain irradiation, International journal of radiation oncology, biology, physics, 33 (1995) 619-626.
[51] C. Linard, A. Ropenga, M.C. Vozenin-Brotons, A. Chapel, D. Mathe, Abdominal irradiation increases inflammatory cytokine expression and activates NF-kappaB in rat ileal muscularis layer, American journal of physiology. Gastrointestinal and liver physiology, 285 (2003) G556-565.
[52] P.J. Trocha, G.N. Catravas, Prostaglandin levels and lysosomal enzyme activities in irradiated rats, International journal of radiation biology and related studies in physics, chemistry, and medicine, 38 (1980) 503-511.
[53] V. Eisen, D.I. Walker, Effect of ionizing radiation on prostaglandin-like activity in tissues, Br J Pharmacol, 57 (1976) 527-532.
[54] E. Kereskenyiova, B. Smajda, Endogenous opioids and analgesic effects of ionizing radiation in rats, Acta Vet Brno, 73 (2004) 195-199.
[55] A.A. Mohamed Eissa, G.A. Soliman, M.H. Khataibeh, Design, synthesis and anti-inflammatory activity of structurally simple anthranilic acid congeners devoid of ulcerogenic side effects, Chemical & pharmaceutical bulletin, 60 (2012) 1290-1300.

[56] M.A. El-Sayed, N.I. Abdel-Aziz, A.A. Abdel-Aziz, A.S. El-Azab, Y.A. Asiri, K.E. Eltahir, Design, synthesis, and biological evaluation of substituted hydrazone and pyrazole derivatives as selective COX-2 inhibitors: Molecular docking study, Bioorg Med Chem, 19 (2011) 3416-3424.

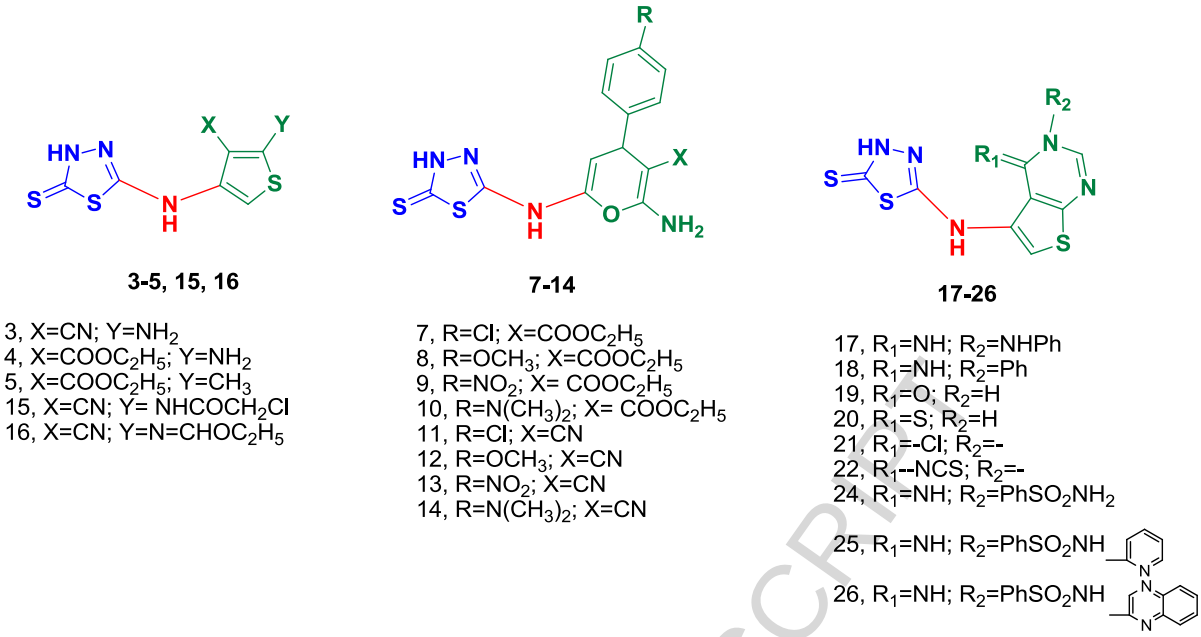
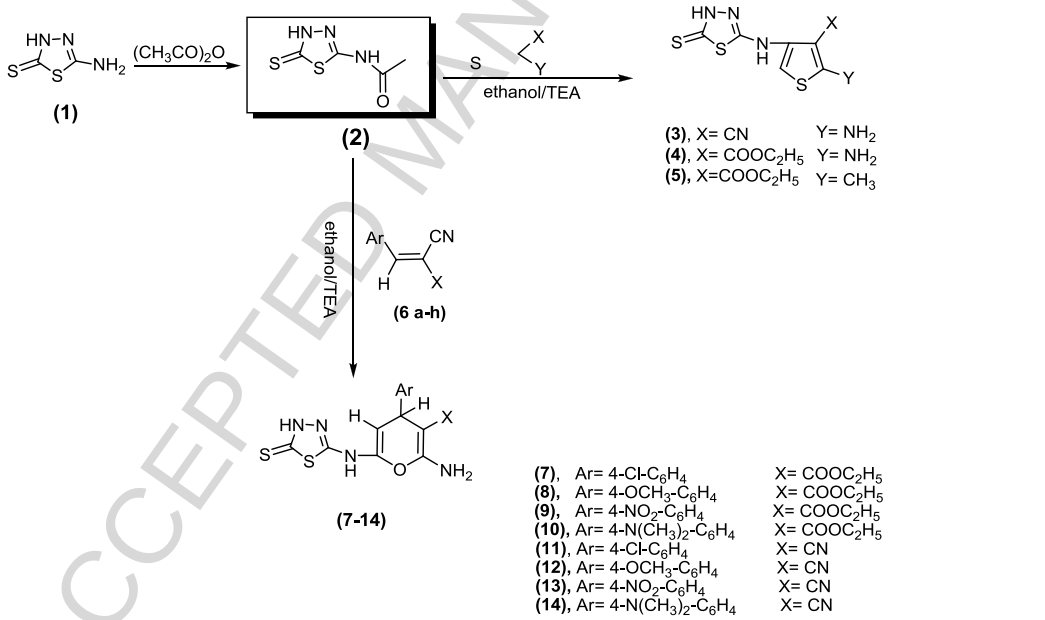
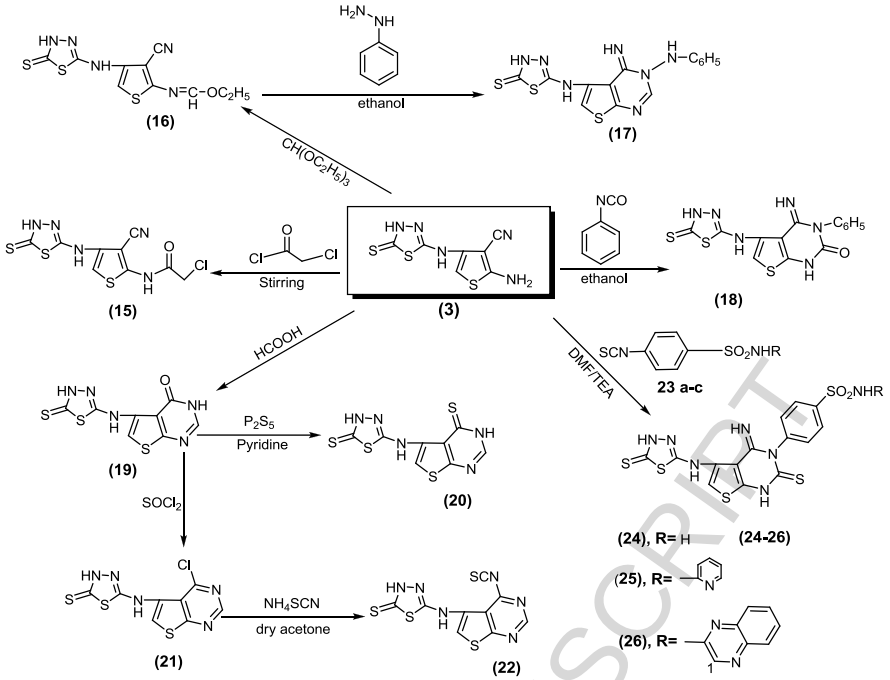


Figure 1. The designed synthesized compounds.



Scheme 1. Synthetic pathways for compounds **3-5**, **7-14**.



Scheme 2. Synthetic pathways for compounds **15-18**, **19-22** and **24-26**.

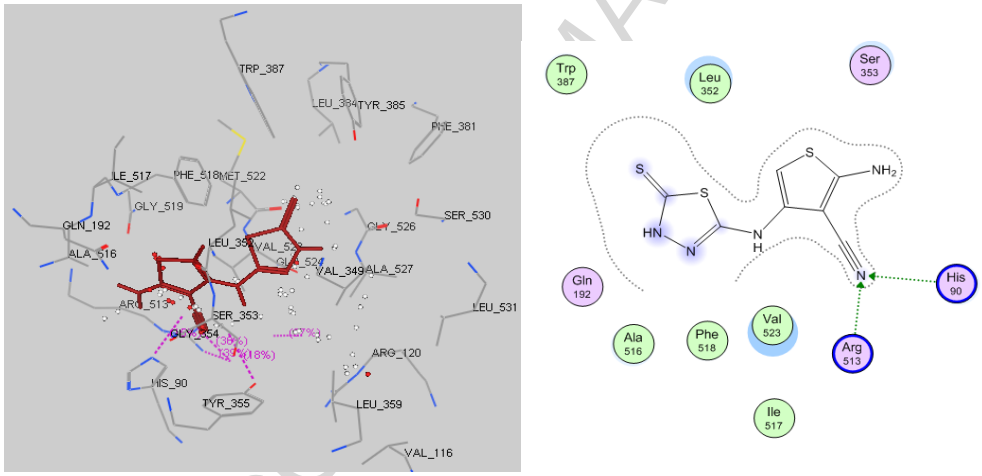


Figure 2. 3D binding interactions of **3** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **7** within the active site amino acids of COX-2 (right panel).

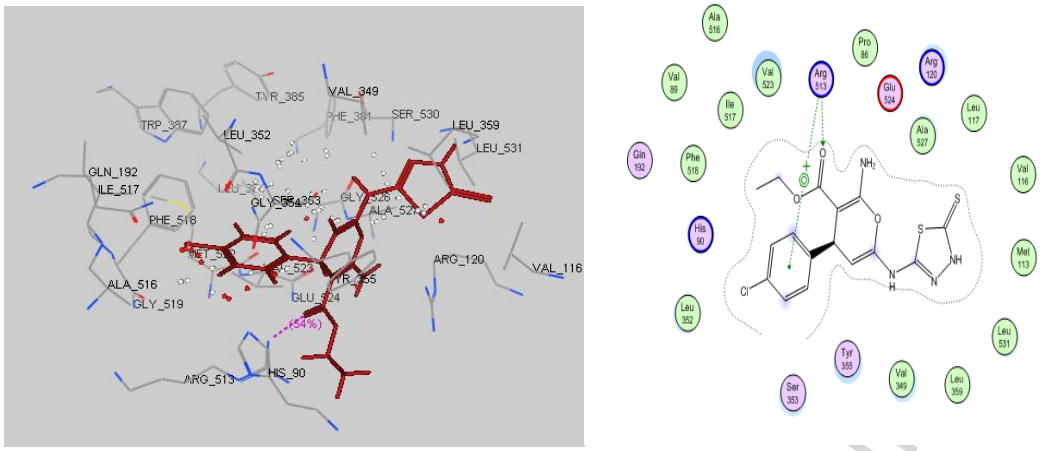


Figure 3. 3D binding interactions of **7** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **7** within the active site amino acids of COX-2 (right panel).

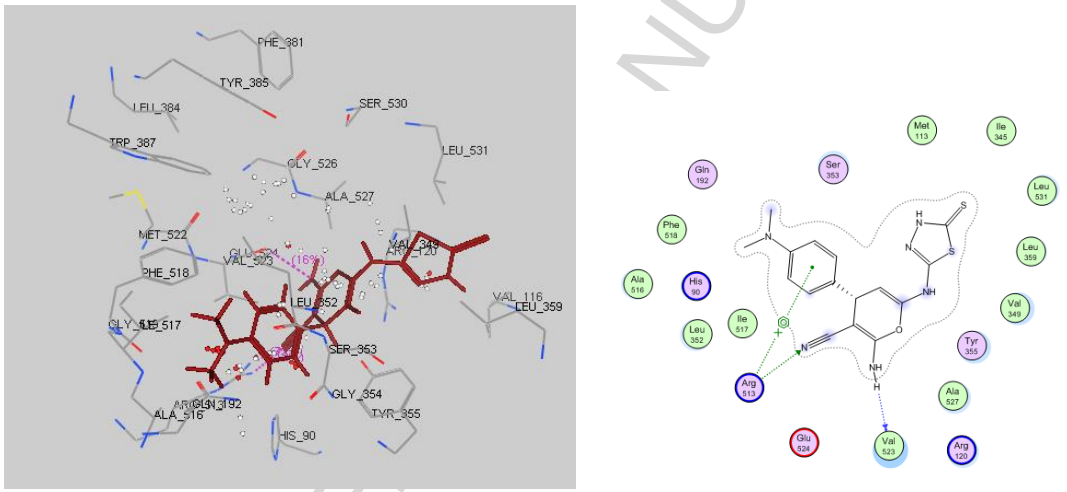


Figure 4. 3D binding interactions of **14** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **14** within the active site amino acids of COX-2 (right panel).

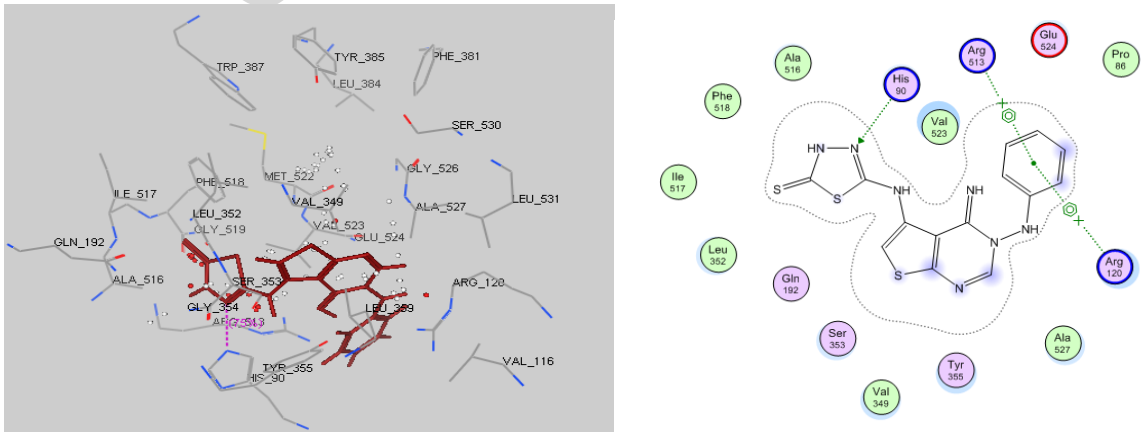


Figure 5. 3D binding interactions of **17** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **14** within the active site amino acids of COX-2 (right panel).

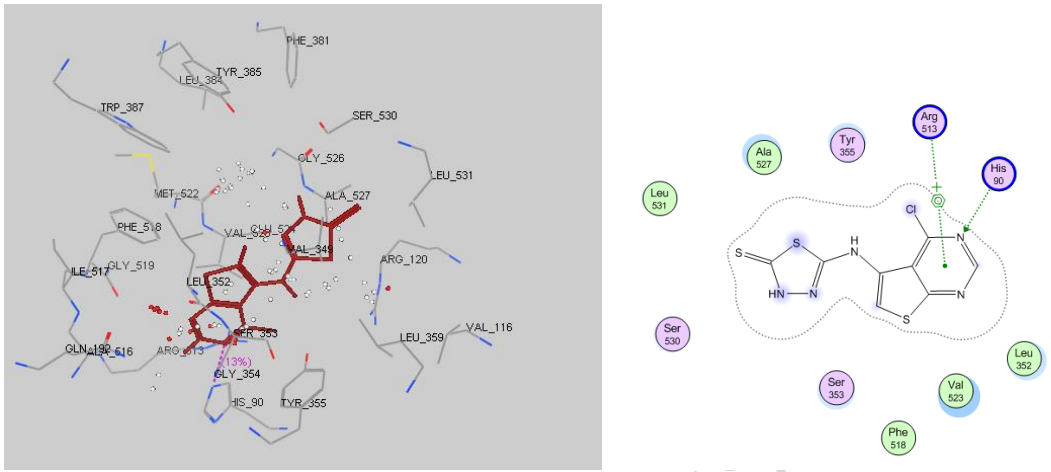


Figure 6. 3D binding interactions of **21** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **21** within the active site amino acids of COX-2 (right panel).

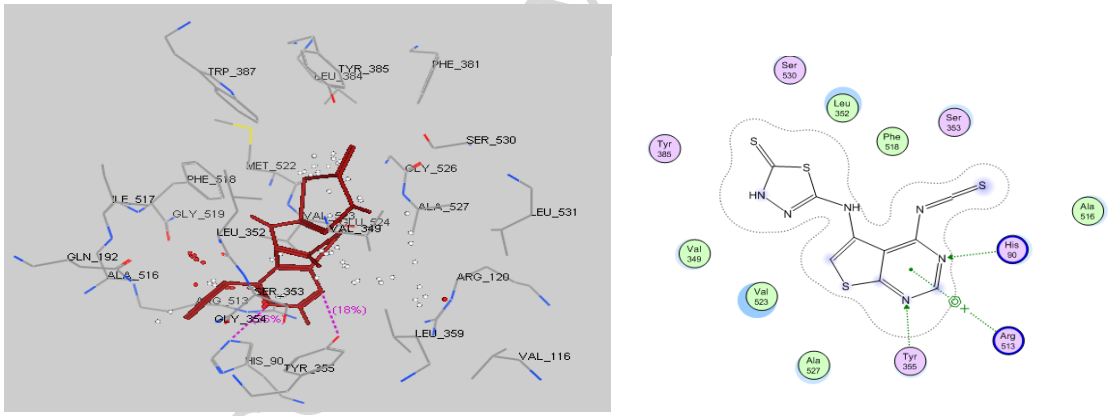


Figure 7. 3D binding interactions of **22** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **22** within the active site amino acids of COX-2 (right panel).

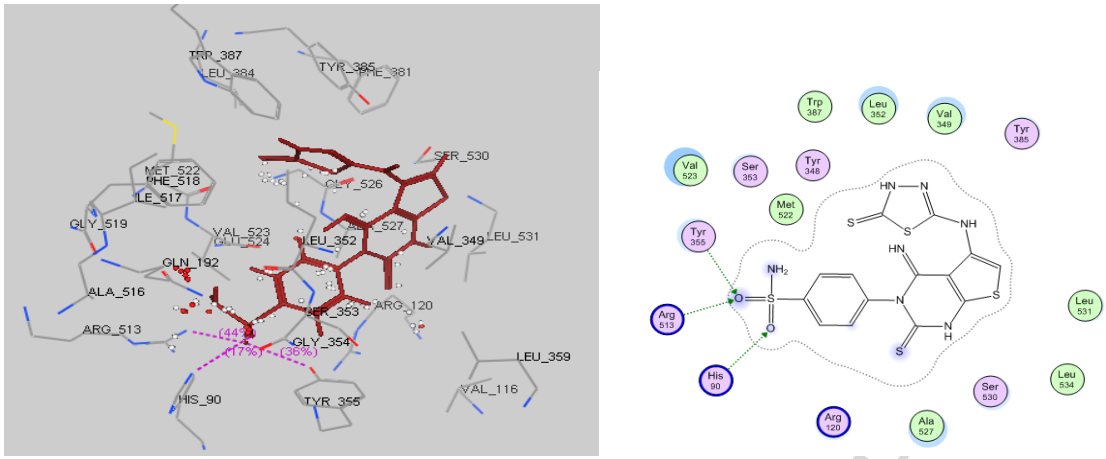


Figure 8. 3D binding interactions of **24** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **24** within the active site amino acids of COX-2 (right panel).

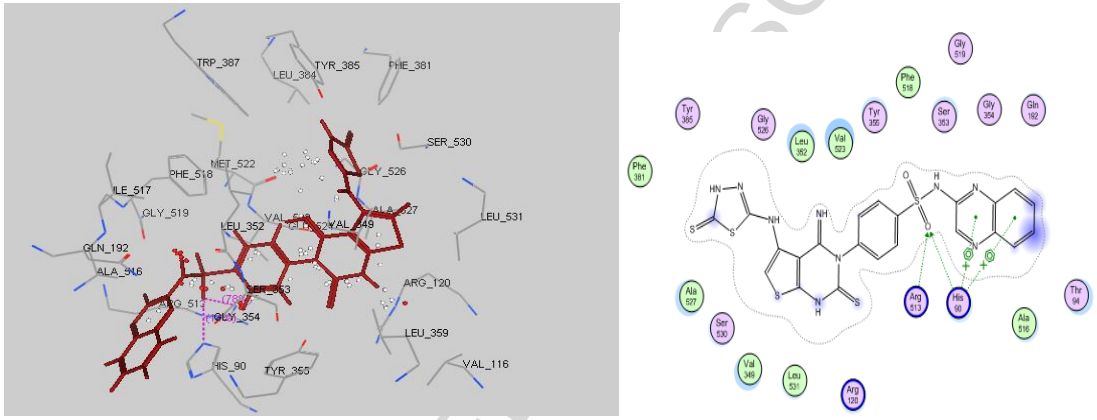


Figure 9. 3D binding interactions of **26** within the active site amino acids of COX-2, hydrogen bonds are shown as magenta lines (left panel); 2D binding interactions of **26** within the active site amino acids of COX-2 (right panel).

Table 1. *In-vitro* COX-1/COX-2 enzyme inhibition assay.

Compound no.	IC_{50} (μM)[a]		SI[b]
	COX-1	COX-2	
1	>50	>50	>1.00
2	23.5	0.366	64.2
3	>50	0.15	>333.3
4	>50	>50	>1.00
5	>50	>50	>1.00
7	>50	0.171	>292.3
8	>50	>50	>1.00
9	>50	>50	7.05
10	>50	>50	>1.00
11	40.1	0.257	156.03
12	>50	>50	>1.00
13	>50	>50	>1.00
14	>50	0.168	>297.6
15	>50	>50	>1.00
16	>50	0.193	>384.61
17	>50	0.151	>331.1
18	>50	>50	>1.00
19	25.3	0.209	121.05
20	>50	>50	>1.00
21	>50	0.162	>308.6
22	>50	0.216	>231.48
24	>50	0.156	>320.51
25	>50	>50	>1.00

26	>50	0.09	>555.55
Diclofenac	**0.25**	**0.26**	**0.96**
Celecoxib	**>50**	**0.18**	**>277.77**

[a] IC_{50} value is the compound concentration required to produce 50% inhibition of COX-1 or COX-2 for means of two determinations using an ovine COX-1/COX-2 assay kit (catalog no. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) and deviation from the mean is <10% of the mean value.

[b] Selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).

Table 2. Binding scores, amino acid interactions and bond length of the synthesized compounds within the active site of COX-2.

Compound	Binding Scores (Kcal/mol)	Ligand atom	Residue	Bond length (Å)
Celecoxib	-11.03	O of SO_2NH_2 O of SO_2NH_2	His 90 Arg 513	2.90 1.56
Dichlofenac	-10.71	O of COOH O of COOH O of COOH	Ser 530 Ser 530 Tyr 385	3.09 2.12 2.78
1	-6.93	H of NH thiadiazole	Leu 352	2.18
2	-8.04	H of NH thiadiazole O of CO	Leu 352 Arg 513	1.92 2.78
3	-11.49	N of CN N of CN	His 90 Arg 513	2.95 2.10
4	-9.67	H of NH thiadiazole O of CO O of CO	Leu 352 Arg 120 Tyr 355	2.16 2.38 2.78
5	-9.18	O of CO	Arg 120	2.54
7	-10.51	O of CO Arene	Arg 513 Arg 513	2.09 3.34
8	-10.39	H of NH thiadiazole O of OCH_3	Ser 353 Arg 120	1.87 3.05
9	-9.59	H of NH_2 H of NH thiadiazole Arene	Leu 352 Ser 530 Arg 120	1.84 2.16 3.94
10	-10.26	H of NH_2 N of thiadiazole	Tyr 355 Arg 120	2.38 2.92
11	-10.08	H of NH N of thiadiazole N of CN N of CN Arene	Tyr 355 Arg 513 Ile 517 Phe 518 His 90	2.02 1.81 2.81 2.72 4.07
12	-10.22	H of NH O of OCH_3 O of OCH_3	Leu 352 Tyr 385 Ser 530	1.63 2.19 2.3
13	-10.36	H of NH_2 N of CN N of thiadiazole	Ala527 Arg 120 Tyr 385	1.85 2.85 2.26
14	-9.82	H of NH_2 N of CN Arene	Val523 Arg 513 Arg 513	2.52 1.80 2.99
15	-10.09	H of NH O of CO	Leu 352 Arg 120	1.44 2.56

16	-10.28	N of N=CH N of CN N of CN H of NH	Arg 513 Arg 513 Tyr 355 Tyr 355	2.39 2.89 2.79 2.01
17	-10.45	N of thiadiazole Arene Arene	His 90 Arg 513 Arg 120	2.62 4.04 4.47
18	-9.09	H of NH pyrimidine O of CO pyrimidine O of CO pyrimidine	Tyr 355 Arg 120 Tyr 355	2.37 2.70 1.81
19	-9.25	H of NH pyrimidine N of pyrimidine Arene	Leu 352 His 90 Arg 513	1.99 2.52 4.06
20	-9.54	H of NH thiadiazole N of pyrimidine	Leu 352 Tyr 385	2.21 2.2
21	-9.54	N of pyrimidine Arene	His 90 Arg 513	2.33 4.39
22	-10.75	N of pyrimidine N of pyrimidine Arene	His 90 Tyr 355 Arg 513	2.85 2.72 3.38
24	-12.73	O of SO_2NH_2 O of SO_2NH_2 O of SO_2NH_2	His 90 Tyr 355 Arg 513	1.95 2.88 1.65
25	-9.29	H of NH thiadiazole N of NH imino	Pro191 Gln192	1.91 2.07
26	-12.64	O of SO_2NH O of SO_2NH Arene Arene	His 90 Arg 513 His 90 His 90	1.78 1.40 1.33 2.85

Table 3. *In-vivo* anti-inflammatory and analgesic activities.

Treatment	Anti-inflammatory activity[a] % of edema	Analgesic activity[a] (nociceptive threshold) (g)
normal	-	100±1.91
Control inflammation	89.50± 1.47	37.60 ± 0.79^{n}
Control radiation	$109.70\pm 0.8^{\#}$	30.24 ± 1.42^{n}
Celecoxib (100 mg/kg.b.wt)	$18.61\pm1.4^{\#*}$	$90.23\pm4.31^{\#*}$
2	$60.51\pm1.18^{*c}$	$85.72\pm5.32^{\#*}$
3	$42.58\pm2.04^{\#*c}$	$46.61\pm2.15^{n*c}$
7	$24.49\pm1.45^{\#*}$	$50.12\pm2.14^{n*c}$
11	$50.17\pm1.358^{*\#c}$	$62.12\pm3.21^{n\#*c}$
14	$40.08\pm3.2^{*\#c}$	$73.41\pm40.2^{n\#*c}$
16	$41.30\pm1.978^{\#*c}$	$62.81\pm4.06^{n\#*c}$
17	$24.70\pm3.02^{\#*}$	42.31 ± 2.14^{nc}
19	$41.\ 85\pm1.35^{\#*c}$	$61.50\pm2.21^{n\#*c}$
21	$60.47\pm3.01^{\#*c}$	$50.01\pm2.17^{n\#*c}$
22	$54.44\pm3.02^{\#*c}$	$54.01\pm4.14^{n\#*c}$
24	$62.50\pm3.57^{\#*c}$	$61.21\pm3.05^{n\#*c}$
26	$25.40\pm1.97^{\#*}$	$50.81\pm3.08^{n*c}$

Note: Number of animals used: six. [a] Compounds tested at a dose of 100 mg.kg.bwt.
Statistical analysis was carried out by one-way ANOVA test.
Each value is the mean of three values ± standard Error
*. Significant from control irradiation at $p \leq 0.05$.
[#]. Significant from control inflammation at $p \leq 0.05$.
[c]. Significant from celecoxib
[n] . Significant from normal

Table 4. Ulcerogenic liability in rats.

Compound no.	Animals no.	Average no of ulcers (UN)	Average severity (US)	%of animals with ulcers per group (UP)	Ulcer index (UI)
Normal	6	0.0± 0.0	0.0± 0.0	0	0.0± 0.0
Diclofenac	6	15.0± 0.9	7.0± 0.6	100%	2.37± 0.09[n]
Celecoxib	6	0.0± 0.0	0.0± 0.0	0%	0.0±0.0[d]
2	6	1.0± 0.09	3.5± 0.07	50%	0.50± 0.033[ndc]
3	6	0.0± 0.0	0.0± 0.0	0%	0.0±0.0[d]
7	6	0.0± 0.0	0.5± 0.02	0%	0.05± 0.007[d]
11	6	3.0± 0.21	1.5± 0.10	66.6%	0.52± 0.042[ndc]
14	6	0.0± 0.0	0.67± 0.04	0%	0.07± 0.005[d]
16	6	0.0± 0.0	0.0± 0.0	0%	0.0± 0.0[d]
17	6	0.0± 0.0	0.5± 0.05	0%	0.05± 0.006[d]
19	6	3.0± 0.25	1.0± 0.01	50%	0.45 ± 0.04[ndc]
21	6	0.0± 0.0	0.83± 0.08	0%	0.083 ± 0.003[d]
22	6	0.0± 0.0	0.0± 0.0	0%	0.0± 0.0[d]
24	6	0.0± 0.0	1.0± 0.02	0%	0.10± 0.004[d]
26	6	0.0± 0.0	0.0± 0.0	0%	0.0± 0.0[d]

n. significant from normal
d. significant from diclofenac
c. significant from celecoxib

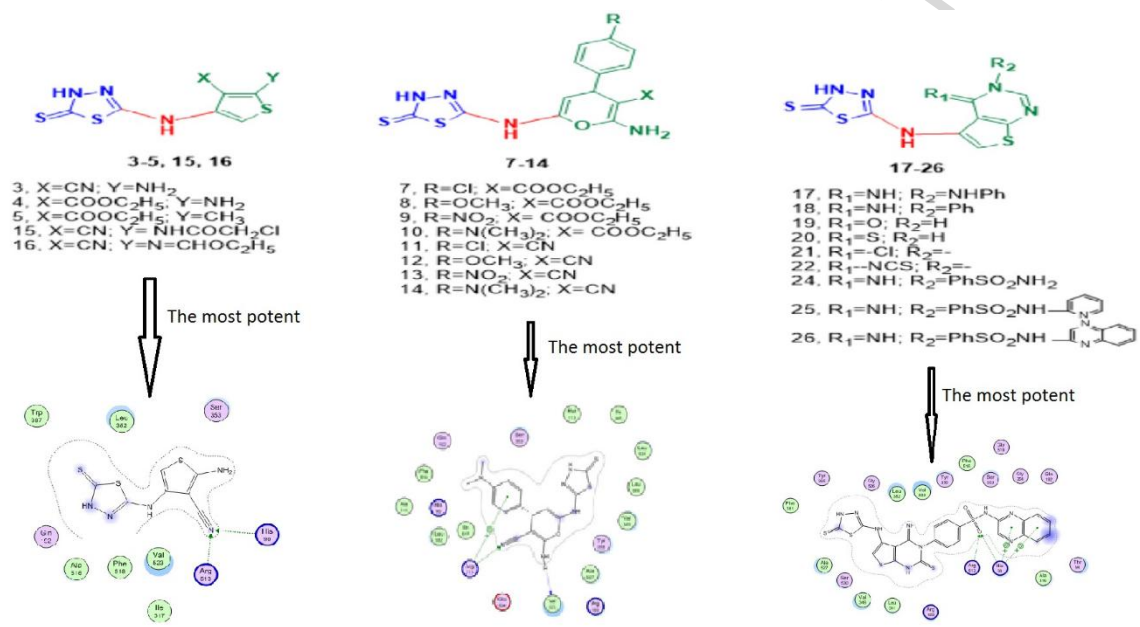
Table 5. *In-vivo* acute toxicity, kidney and liver Function Parameters.

Compound no.	SGPT (U/ml)	SGOT (U/ml)	SCr (mg/dl)	Acute toxicity ALD_{50}(mg/Kg bwt)
Normal	8.85± 0.155	19.04± 1.07	0.38± 0.006	-
Celecoxib	8.925± 0.246	18.03± 0.36	0.43± 0.02	-
Diclofenac	15.69± 0.3134*[c]	21.83± 0.58[c]	0.515± 0.01*[c]	-
17	11.1± 0.86	18.1± 0.42	0.43± 0.006	**>1000**
21	11.63± 0.22	18.23± 0.63	0.41± 0.01	>1000
26	11.53± 0.92*[c]	17.48± 0.3	0.39± 0.009	>1000

* significant from normal
[c] significant from celecoxib

Graphical Abstract

Novel series of pyran, thiophene and thienopyrimidine derivatives based on 2-acetamide-thiadiazole scaffold were designed and synthesized for evaluation as selective COX-2 inhibitors *in-vitro* and investigated *in-vivo* as anti-inflammatory and analgesic agents against carrageenan-induced rat paw oedema model in irradiated rats. Toxicological studies were evaluated for the most potent compounds. Docking study was performed for the synthesized compounds within the active site of COX-2.



Compound **3**; SI >333.3 µM

Compound **14**; SI >297.6 µM

Compound **26**; SI >555.5 µM

Highlights

- Novel thiadiazole derivatives were designed and synthesized.
- COX1/COX2 enzyme inhibition was performed.
- Anti-inflammatory and analgesic activities was evaluated.
- Toxicological Studies were performed.
- Docking study was performed.