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Cu(II) and Fe(III) Catalyzed Synthesis of Novel Thiophene Hybridized Thiadiazolyl Schiff Bases (TTS) as COX-2 Selective Inhibitor

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A novel series of thiophene hybridized thiadiazolyl Schiff bases (TTS) were designed and synthesized using copper nitrate (Cu^{II}) catalyzed synthesis of Schiff bases and their subsequent ferric chloride (Fe^{III}) catalyzed cyclization into thiadiazoles. The design of molecules was inspired by the well-documented anti-inflammatory properties of thiadiazoles, Schiff bases and thiophene. Based on information from mass spectroscopy, IR, ^{1}H and ^{13}C NMR measurements, the structures of the recently synthesized compounds were determined. The purpose of molecular docking was to better understand the interactions between drug candidates and COX-1 (PDB ID: 3KK6) and COX-2 (PDB ID: 3Q7D) inflammation-related targets. Compounds **3a-f** showed a more stable binding complex with COX-2 (-8.09 to -9.13 kcal/mol) when compared to COX-1 (-4.86 to -5.94 kcal/mol). Additionally, the carrageenan-induced rat paw oedema model was used for the *in vivo* analysis and compound **3a** demonstrated excellent anti-inflammatory activity when compared to the reference drug, diclofenac. Interestingly, the mRNA expression analysis using qRT-PCR demonstrates a specific suppression of COX-2 as compared to COX-1, further supporting the earlier findings. Altogether, the findings could provide an opportunity for these compounds to be developed as novel lead molecules for rational alternatives of NSAIDS.

Keywords: Thiophene-thiadiazolyl Schiff base, Catalysis Anti-inflammatory activity, NSAIDs, COX-1, COX-2.

INTRODUCTION

Inflammation is a complex defense mechanism against any adverse stimuli that is characterized by the buildup of fluids and leukocytes causing edema and pain [1]. There are different physiological and immunological factors involved in both acute and chronic inflammation that often mediate this inflammatory response [2]. Non-steroidal anti-inflammatory medicines, in particular rheumatoid arthritis, are frequently used to treat pain and inflammation associated with these conditions. However, long-term use of these drugs has been linked to kidney damage, bleeding and GIT ulceration [3]. Therefore, even if there are many anti-inflammatory medications on the market, it is necessary to discover new medications with improved safety profiles.

Cyclooxygenases (COXs) are enzymes producing prostanoids, which can cause inflammation and thrombosis [4]. The majority of anti-inflammatory medications block COX-1 and COX-2 enzymes that are responsible for producing the inflammatory mediators, prostaglandins and thromboxane [5]. There are several steroidal and non-steroidal anti-inflammatory drugs currently used to treat diseases associated with inflammation [6,7]. Nonsteroidal anti-inflammatory medications (NSAIDS), mostly used for arthritis, pain and inflammation, include indomethacin and diclofenac. The therapeutic effect of NSAIDs is mainly due to the inhibition of cyclooxygenase (COX) enzyme, leading to prevention of prostaglandins synthesis.

A significant group of heterocyclic compounds known as 1,3,4-thiadiazoles has a wide range of biological actions including anticancer [8], antiviral [9], antibacterial [10], antioxidant [11], antitubercular [12], anticonvulsant [13] and anti-inflammatory [14,15] properties. Cruz *et al.* [16] reviewed the importance of thiophene-based compounds as privileged structures for the design and discovery of novel anti-inflammatory agents. The

most well-known examples of commercially available medications containing thiophene ring as a pharmacophoric group with anti-inflammatory properties include tinoridine, tiaprofenic acid, tenidap and zileuton. The first three are NSAIDs that are used to treat inflammation and discomfort. Zileuron is a 15-LOX inhibitor, whereas tinoridine and tiaprofenic acid act by inhibiting COX enzymes [17,18].

Schiff bases and their derivatives have a variety of biological properties, including the ability to reduce inflammation [19-24]. They have an azomethine (>C=N-) group, produced when primary amines condense with carbonyl compounds [25]. Hydrazones, another member of the Schiff base family, are often produced by reacting carbonyl compounds with hydrazine derivatives in the presence of strong acid [26].

Inspired by the molecular hybridization concept, the current study deals with the design of some novel thiophene-thiadiazolyl Schiff bases (TTS) by molecular docking studies on the inflammatory associated targets, COX-1 (PDB ID: 3KK6) and COX-2 (PDB ID: 3Q7D). To synthesize the desired compounds, hydrazones were first synthesized using a copper nitrate (Cu^{II}) catalyst and their subsequent cyclization into thiadiazoles by ferric chloride (Fe^{III}) catalyst. The carrageenan-induced paw oedema model was used to test the *in vivo* anti-inflammatory efficacy of synthetic compounds. In addition, qRT-PCR analysis was also carried out to investigate the gene (mRNA) expression levels of COX-1 and COX-2 to confirm the earlier findings showing the preferential suppression of COX-2 over COX-1.

EXPERIMENTAL

The chemicals and reagents were procured from Spectrochem, Mumbai, TCI, Kemphasol and Merck and S.D. Fine Chemicals, India. Thin layer chromatography (TLC) was used to examine the progress of the reaction where iodine vapours and a UV cabinet were used as visualizing agents. Melting points were estimated using an electrothermal digital melting point equipment and are not corrected. Compounds were examined physically before being subjected to elemental and spectrum analysis. A Euro-Vector E 3000 Elemental Analyzer was used for elemental investigation. IR spectroscopy was carried out on Bruker Alpha-II FTIR Spectrophotometer which is expressed in cm^{-1}. Mass spectra were recorded by using Waters Aliance e2695/HLC-TQM mass spectrometer, whereas ^1H NMR and ^{13}C NMR spectra were observed on Bruker Advance 400/AivIII HD-300 (FT NMR). Chemical shifts were examined in parts per million (δ ppm for ^1H NMR and ^{13}C NMR). All the spectral studies were performed at Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute (CDRI) Lucknow, India.

Synthesis of thiophene thiosemicarbazone (1): Thiophene-2-caboxaldehyde (0.1 mol) in ethanol (10 mL) was mixed with thiosemicarbazide (0.1 mol) in ethanol followed by addition of $Cu(NO_3)_2{\cdot}6H_2O$ (1 mol%) as catalyst and then the reaction mixture was agitated for the required time at room temperature. Thin-layer chromatography (TLC) with the solvent system ethyl acetoacetate:*n*-hexane (4:6) was used to observe the progress of the chemical reaction. Once the reaction was completed, 50 mL of ice-cold water was added to obtain a solid product. The product was then dried, suction-filtered and recrystallized with 75% ethanol to obtain compound **1**.

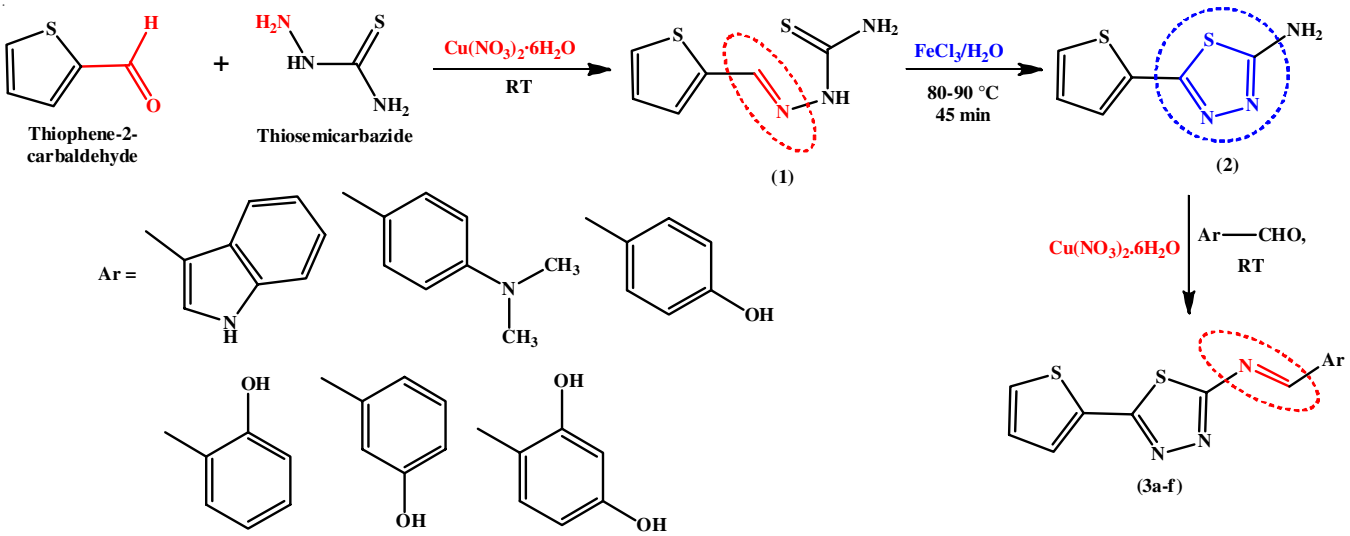
Synthesis of thiophene thiadiazoles (2): Compound **1** (0.1 mol) was dissolved in 67 mL of warm water. In a separate beaker, $FeCl_3$ (0.3 mol) was dissolved in 54.7 mL of water and added slowly to the compound **1** suspension while being constantly stirred. The mixture was heated for 45 min at 80-90 ºC and filtered. The filtrate was mixed with citric acid (0.2 mol) and sodium citrate (0.10 mol) while being continuously stirred followed by the addition of 10% aq. NH_3 solution to neutralize the resultant mixture. The product was filtered by suction filteration, drying and recrystallizing it in 75% alcohol to obtain compound **2**.

Synthesis of thiophene-thiadiazolyl Schiff bases (3a-f): Compound **2** (0.1 mol) was mixed to aromatic aldehyde derivatives (0.1 mol) in 10 mL ethanol followed by the addition of $Cu(NO_3)_2{\cdot}6H_2O$ (1 mol%) was then added and the reaction mixture was agitated for the required amount of time at room temperature. The TLC was used to monitor the reaction mixture using a suitable solvent system. Once the reaction was finished, the mixture was allowed to stand at room temperature overnight (**Scheme-I**). To obtain product, the resultant solid was filtered, washed with distilled water and recrystallized with ethanol. All the resulting products appeared as pure needle-shaped crystals.

1-(1*H*-Indol-3-yl)-N-(5-(thiophen-2-yl)-1,3,4-thiadiazol-2-yl)methanimine (3a): Reddish brown; yield:: 93%; m.p.: 195-197 ºC; FTIR (KBr, cm^{-1}): 3098 [N-H *str.* (indole NH)], 1628 (C=N, *str.*), 1574 (C=C *str.*), 1240 (C-N, *str.*), 786 (C-S *str.*); ^1H NMR (DMSO-d_6, 300 MHz) δ ppm: 7.18-7.28 (m, 3H, Ar), 7.30 (m, 2H, Ar), 7.49 (m, 2H, Ar), 7.69 (d, 1H, Ar), 8.10 (d, 1H, Ar), 9.93 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ ppm: 112.4, 112.5, 120.8, 122.0, 122.1, 122.1, 124.1, 125.2, 127.5, 127.6, 137.0, 138.4, 163.04 (C=N), 168.3 (S-C=N); ESI-MS: 311 $[M+H]^+$; Elemental analysis for $C_{15}H_{10}N_4S_2$: calcd. (found) %: C, 58.04 (59.24); H, 3.25 (2.98); N, 18.05 (18.32); S, 20.66 (20.76).

N,N-Dimethyl-4-(((5-(thiophen-2-yl)-1,3,4-thiadiazol-2-yl)imino)methyl)aniline (3b): Reddish brown; yield:: 91%; m.p.: 186-188 ºC; FTIR (KBr, cm^{-1}): 1686 (C=N, *str.*), 1598 (C=C, *str.*), 1250 (C-N *str.*), 694 (C-S *str.*); ^1H NMR (DMSO-d_6, 300 MHz) δ ppm: 3.36 (s, 3H, -CH_3), 5.22 (s, 3H, -CH_3), 7.11 (t, 1H, Ar), 7.21 (d, 2H, Ar), 7.32-7.48 (m, 2H, Ar), 7.62 (d, 2H, Ar), 7.87 (d, 1H, -CH); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ ppm: 40.35 (2 CH_3), 115.2, 115.2, 120.7, 129.7, 115.5, 125.3, 127.5, 128.5, 129.7, 129.8, 136.2, 163.2 (S-C=N), 168.1 (N=C-S); ESI-MS: 315 $[M+H]^+$; Elemental analysis for $C_{15}H_{14}N_4S_2$: calcd. (found) %: C, 57.30 (56.88); H, 4.49 (4.13); N, 17.82 (17.22); S, 20.40 (10.44).

4-(((5-(Thiophen-2-yl)-1,3,4-thiadiazol-2-yl)imino)-methyl)phenol (3c): Reddish brown; yield:: 93%; m.p.: 182-184 ºC; FTIR (KBr, cm^{-1}):3648.40 (N-H, *str.*), 2360.03 (C-H, *str.*), 1541.29 (C=C, *str.*), 1698.19 (C=N, *str.*); ^1H NMR (DMSO-d_6, 300 MHz) δ ppm: 3.81 (s, 3H, -OCH_3), 2.45 (s, 3H, CH_3), 3.91 (s, 1H, OH), 6.92 (d, 2H, Ar), 7.04 (d, 2H, Ar), 7.16 (s, 1H, Ar), 7.26 (t, 1H, Ar), 7.55 (d, 2H, Ar), 7.58 (d, 1H, Ar), 7.81 (d, 1H Ar), 8.36 (s, 1H, NH); ^{13}C NMR (DMSO-d_6,



Scheme-I: General synthesis of compounds (**3a-f**), Reagent and condition: (A) $Cu(NO_3)_2{\cdot}6H_2O$, room temperature, (B) aqueous solution of $FeCl_3$, 80-90 ºC, 45 min

75.5 MHz) δ ppm: 55.1 (-OCH_3), 17.2 (CH_3), 101.6 (CH, thiazole), 114.8 (Ar), 116.7 (Ar), 125.5 (Ar), 118.7 (Ar), 120.2 (Ar), 125.2 (Ar), 125.3 (Ar), 128.2 (Ar), 130.5 (Ar), 134.5 (Ar), 139.5 (Ar), 152.3 (C-N), 158.4 (C=N, thiazole), 172.1 (S-C=N, thiazole); ESI-MS: 288 $[M+H]^+$; Elemental analysis for $C_{13}H_9N_3OS_2$: calcd. (found) % of $C_{18}H_{117}N_3O_2S$: C, 63.70 (62.74); H, 5.05 (5.13); N, 12.38 (12.21).

2-(((5-(Thiophen-2-yl)-1,3,4-thiadiazol-2-yl)imino)-methyl)phenol (3d): Yellowish brown; yield: 85%; m.p.: 204-206 ºC; FTIR (KBr, cm^{-1}): 3101 (-OH *str.*), 1599 (C=N, *str.*), 1510 (C=C, *str.*), 1229 (C-N, *str.*), 745 (C-S *str.*); ^{1}H NMR (DMSO-d_6, 300 MHz) δ ppm: 7.09 (d, 2H, Ar), 7.25 (s, 1H, Ar), 7.35 (m, 2H, Ar), 7.46 (s, 1H, Ar), 7.72 (s, 1H, -OH), 8.9 (s, 1H, -CH); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ ppm: 114.72, 115.2, 121.8, 122.0, 128.6, 128.7, 130.3, 132.1, 135.7, 137.7, 157.9, (C=O) 160.1 (N=C), 169.7 (S-C=N); ESI-MS: 288 $[M+H]^+$; Elemental analysis for $C_{13}H_9N_3OS_2$: calcd. (found) %: C, 54.34 (54.16); H, 3.16 (4.34), N, 14.62 (14.18), S, 22.32 (21.45).

3-(((5-(Thiophen-2-yl)-1,3,4-thiadiazol-2-yl)imino)-methyl)phenol (3e): Cream; yield: 87%; m.p.: 184-186 ºC; FTIR (KBr, cm^{-1}): 3101 (-OH *str.*), 1599 (C=N, *str.*), 1510 (C=C *str.*), 1229 (C-N, *str.*), 745 (C-S *str.*); ^{1}H NMR (DMSO-d_6, 300 MHz) δ ppm: 7.09 (d, 2H, Ar), 7.25 (s, 1H, Ar), 7.35 (m, 2H, Ar), 7.46 (s, 1H, Ar), 7.72 (s, 1H, -OH), 8.9 (s, 1H, -CH); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ ppm: 114.72, 115.2, 121.8, 122.0, 128.6, 128.7, 130.3, 132.1, 135.7, 137.7, 157.9, (C=O) 160.1 (N=C), 169.7 (S-C=N); ESI-MS: 288 $[M+H]^+$; Elemental analysis for $C_{13}H_9N_3OS_2$: calcd. (found) %: C, 54.34 (54.16); H, 3.16 (4.34); N, 14.62 (14.18); S, 22.32 (21.44).

4-(((5-(Thiophen-2-yl)-1,3,4-thiadiazol-2-yl)imino)-methyl)benzene-1,3-diol (3f): Yellowish brown; yield: 88%; m.p.: 188-190 ºC; IR (KBr, cm^{-1}): 3101 (-OH *str.*), 1629 (C=N, *str.*), 1496 (C=C, *str.*), 1227 (C-N, *str.*), 725 (C-S bend.); ^{1}H NMR (DMSO-d_6, 300 MHz) δ ppm: 6.37 (s, 2H, -OH), 6.48 (d, 2H, Ar), 7.23 (t, 1H, Ar), 7.54 (d, 1H, Ar), 7.72 (m, 1H, Ar), 7.83 (d, 1H, Ar), 9.02 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ ppm: 102.2, 108.6, 112.2, 125.8, 125.9, 128.5, 130.2, 132.9, 158.97 (C=O), 159.0 (C=O), 163.2 (C=N), 165.2, (S-C=N) 166.8, (S-C=N); ESI-MS: 304 $[M+H]^+$; Elemental analysis for $C_{13}H_9N_3O_2S_2$: calcd. (found) %: C, 51.47 (51.88); H, 2.99 (2.13); N, 13.85 (13.12); S, 21.14 (20.67).

***In silico* screening:** Molecular docking studies exhibit the binding complementarity of the compounds **3a-f** with the amino acid residue of COX-1 and COX-2 through hydrogen bonding interaction. The 3D structure of the ligands was drawn using the Open Babel, Avogadro and Chem Draw ultra12 tools and their geometry was optimized six times using Gauss view 5.0. The RCSB Protein Data Bank was used to get the crystal structures of the target proteins COX-1 (PDB ID: 3KK6) and COX-2 (PDB ID: 3Q7D) [27]. The CASTp database was used to dock the newly synthesized compounds to the targets' active sites. Additionally, using Autodock 4.1 and its LGA algorithm for automated flexible ligand docking, molecular docking of the proposed compounds was carried out. The binding energy was measured in terms of negative kcal/mol and the number of probable hydrogen bonds and π-bonds interactions were calculated [28].

***In vivo* anti-inflammatory activity:** All the *in vivo* experimental procedures were approved by the Institutional Animal Ethical Committee vide letter number BBDNIIT/IAEC/2022/14. The newly synthesized compounds were tested for *in vivo* anti-inflammatory action using carrageenan induced rat paw edema model. This model is highly used to perform the activity of NSAIDs or COX inhibitors. Male Wistar rat of either sex, weighing 150-200 g were distributed into nine groups. Each group consists of 5 animals. Group 1 was designated as the control group and received only 0.5% carboxymethyl cellulose (CMC) solution, Group 2 was designated as the inducer and received carrageenan along with 0.5% CMC as the vehicle and Group 3 was designated as the standard and received diclofenac along with 0.5% CMC as the vehicle before receiving carrageenan. Groups 4-9 were designated as test groups with test drugs and 0.5% CMC as the vehicle prior to the administration of carrageenan.

All the test compounds and standard drugs were given orally (10 mg and 20 mg/kg body weight) in a suspension of 0.5% CMC as the vehicle. After 1 h, foot paw edema was induced by subcutaneously injecting every rat with an inducer (0.1mL of 1% carrageenan in physiological solution). A mercury plethysmometer was used to take an instantaneous measurement of the initial foot paw edema. The volume was measured at 1, 2, 3, 4 and 5 h after carrageenan administration. The expansion in the volume of paw was chosen as the measurement of edema [29]. The level of anti-inflammatory activity (% inhibition of inflammation) was calculated using the formula given below:

$$\text{Inhibition of inflammation (\%)} = \frac{V_c - V_t}{V_c} \times 100$$

where V_c is the increase in paw volume of control and V_t is the increase in paw volume after administration of the test compound.

qRT-PCR study: A 10 mg of tissue sample of affected area from each group were used to isolate total mRNA employing TriaZol reagent. The RNeasy mini kit was used to further purify the mRNA and the Nano Drop instrument was used to measure concentration at 260/280 nm. The complementary deoxyribose nucleic acid (*c*DNA) was calculated in accordance with the manufacturer's instructions using the GeneSure first strand *c*DNA synthesis kit (Genetix Biotech Asia Pvt. Ltd., India). The SybrVR green PCR master mix was used to perform the qRT-PCR on an Agilent Stratagene Mx3000P series (Applied Biosystems, Foster City, USA) instrument. The *c*DNA was first denatured for 5 min at 94 ºC, followed by 30 s of annealing at 58 ºC. These were ultimately elongated for 35 s at 72 ºC. The normalization of the mRNA was done using β-actin as control. ΔCt values were normalized with untreated control samples for all compounds ($\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{housekeeping gene}}$). Relative changes in the expression level of one specific gene were calculated in terms of $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct_{test} - \Delta Ct_{control}$) [30]. The primer sequences were as follows: COX-1, 5′-CAGATGCGGAGTTTCTGAGTCG-3′(forward), 5′-GGGTAGTGCATCAGCACGG-3′(reverse) [31] COX-2, 5′-ATCAGAACCGCATTGCCTCT-3′ (forward), 5′-GCCAGCAATCTGTCTGGTGA-3′ (reverse) [32] and β-actin, 5′-AAGTCCCTCACCCTCCCAAAAG-3′ (forward) and 5′-AAGCAATGCTGTCACCTTCCC-3′ (reverse) [33].

Statistical data analysis: Statistical data analysis was performed using the software GraphPad Prism 5.0 (San Diago, USA). The results were expressed as mean ± standard deviation (SD) (n = 5). One-way ANOVA (analysis of variance) and Bonferroni multiple comparison test were used to analyze the statistical data. Statistically significant differences were found between inducer group and test groups (**3a-f**) (*$p < 0.001$).

RESULTS AND DISCUSSION

By adopting the reported a new synthetic method of Schiff base (hydrazone) formation [34], a thiophene-2-carbaldehyde and thiosemicarbazide underwent a hydrated copper nitrate-catalyzed reaction to constitute a rapid and facile synthesis of thiophene substituted Schiff base hydrazones (**1**). The first step in the mechanism is believed to be the condensation between thiophene-2-carbaldehyde and thiosemicarbazide with removal of water molecules. The resulting adduct undergoes ferric chloride (Fe^{III})-catalyzed cyclization into a new thiophene substituted aminothiadiazole ring, which further undergoes to copper nitrated (Cu^{II}) catalyzed Schiff base formation, giving rise to final products. For the first time, 1 mol of thiosemicarbazide was reacted with thiophene-2-carbaldehyde in the presence of $Cu(NO_3)_2{\cdot}6H_2O$ at room temperature (**Scheme-I**). The reaction produced corresponding hydrazone (**1**) in excellent yields. On the other hand, the yield of reaction in absence of this catalyst for the same time was very low. The time of reaction is 8-12 min, which was quite shorter compared to the conventional procedures of hydrazone formation in presence of glacial acetic acid and ethyl alcohol (CH_3COOH/C_2H_5OH) (Table-1). Further, ferric chloride catalyzed cyclization of resulting hydrazone (**1**) with subsequent Schiff base formation resulted into quite fair yield in short reaction time.

TABLE-1
COPPER NITRATE CATALYZED EFFICIENT SYNTHESIS OF THIOPHENE HYBRIDIZED THIADIAZOLYL SCHIFF BASES (**3a-f**)

Compd.	Catalyst	Time	Yield (%)
3a	$Cu(NO_3)_2{\cdot}6H_2O$	12 min	93
	CH_3COOH/C_2H_5OH	4 h	47
3b	$Cu(NO_3)_2{\cdot}6H_2O$	10 min	91
	CH_3COOH/C_2H_5OH	4 h	41
3c	$Cu(NO_3)_2{\cdot}6H_2O$	8 min	93
	CH_3COOH/C_2H_5OH	4 h	45
3d	$Cu(NO_3)_2{\cdot}6H_2O$	15 min	85
	CH_3COOH/C_2H_5OH	4 h	41
3e	$Cu(NO_3)_2{\cdot}6H_2O$	9 min	87
	CH_3COOH/C_2H_5OH	4 h	43
3f	$Cu(NO_3)_2{\cdot}6H_2O$	10 min	88
	CH_3COOH/C_2H_5OH	4 h	52

The NMR spectral data of all the newly synthesized thiophene-thiadiazolyl Schiff bases were found consistent with their structures. The aromatic protons resonate as a multiple signal at δ 6.25-9.15 ppm range as per the different aromatic groups with N-H group of hydrazones appeared at δ 9.93 ppm. The recorded mass spectrum revealed the correct molecular ion (M+1) peak, as evidenced by the molecular formula. The presence of the peaks at 1628 cm^{-1} and 3098 cm^{-1} correspond to -C=N (*str.*) and –NH (*str.*), respectively, in IR spectrum provided strong evidence for the formation of the Schiff bases. The ^1H NMR and ^{13}C NMR spectrum of the newly synthesized compound was found to be corresponding to the proposed structure. The mass spectra of the compound showed *m/z* 311.0 $[M+H]^+$ and agreed with the desired molecular formulae. Similarly, the structural elucidation of the remaining thiophene-thiadiazolyl Schiff bases (**3a-f**) was also characterized.

***In silico* study:** The objective of current study was to assess the ability of newly synthesized thiophene-thiadiazolyl Schiff bases to mitigate inflammation as a new class of COX-1 and COX-2 inhibitors. A molecular docking study was carried out to determine if chemicals bind to the amino acid residues of the target proteins COX-1 (PDB ID: 3KK6) and COX-2 (PDB ID: 3Q7D) through hydrogen bonding and π-π-interaction.

The newly prepared compounds were docked to the COX-1 and COX-2 active sites using Autodock 4. Further, the 3D structure of the ligands was generated using Chemdraw/Openbabel software. The crystal structures of proteins targets (3KK6 for COX-1 and 3Q7D for COX-2) were acquired from RCSB Protein Data Bank [27]. Binding of the ligand molecule into the binding site of COX-1 and COX-2 was found noncovalent and exhibited the configuration of the binding sites where the ligands provide the best binding energy and H-bond interactions with amino acid residues. According to the present findings, all the compounds (**3a-f**) exhibited a substantially higher binding affinity for COX-2 (-8.09 to -9.13 kcal/mol) than for COX-1 (-4.86 to -5.94 kcal/mol) (Table-2 and Fig. 1). Compound **3a** displayed highest binding energy of -9.13 kcal/mol on COX-2 protein target out of all the synthesized compounds.

Anti-inflammatory activity: The *in vivo* anti-inflammatory property of the synthesized thiophene-thiadiazolyl Schiff bases (TTS) was performed by using carrageenan induced rat paw edema model at dose of 10 and 20 mg/kg b.w. using the standard drug diclofenac. The obtained results of study are tabulated in Tables 3 and 4, where it is observed that compounds **3a** (bearing indole ring substitution) and **3f** (bearing 2,4-dihydroxybenzene substitution) exhibited highest anti-inflammatory activity. In general, within the series of synthesized derivatives, compounds bearing indole ring substitution exhibited substantially higher anti-inflammatory activity than those of the compounds bearing other substituted phenyl rings.

mRNA expression of the effectors cytokine COX-1 and COX-2: Expression levels and the results demonstrated that mRNA level were over-expressed in the carrageenin-induced toxic group in comparison to the healthy control. However, these overexpressed levels were significantly normalized by the administration of the test drugs **3a** and **3f** as well as the standard drug diclofenac. The efficacy of **3a** and **3f** at a dose of 20 mg/kg was found comparable to that of the commercially available anti-inflammatory drug, diclofenac, without any noticeable differences (Fig. 2). Additionally, it is well known that COX-1/COX-2 are significantly released in response to a variety of inflammatory stimuli and the overexpression of COX-1 and COX-2 genes has been positively related to inflammation as well as cancer prognosis. The quick decrease in COX-1/COX-2 overexpression observed in qRT-PCR analysis after treatment

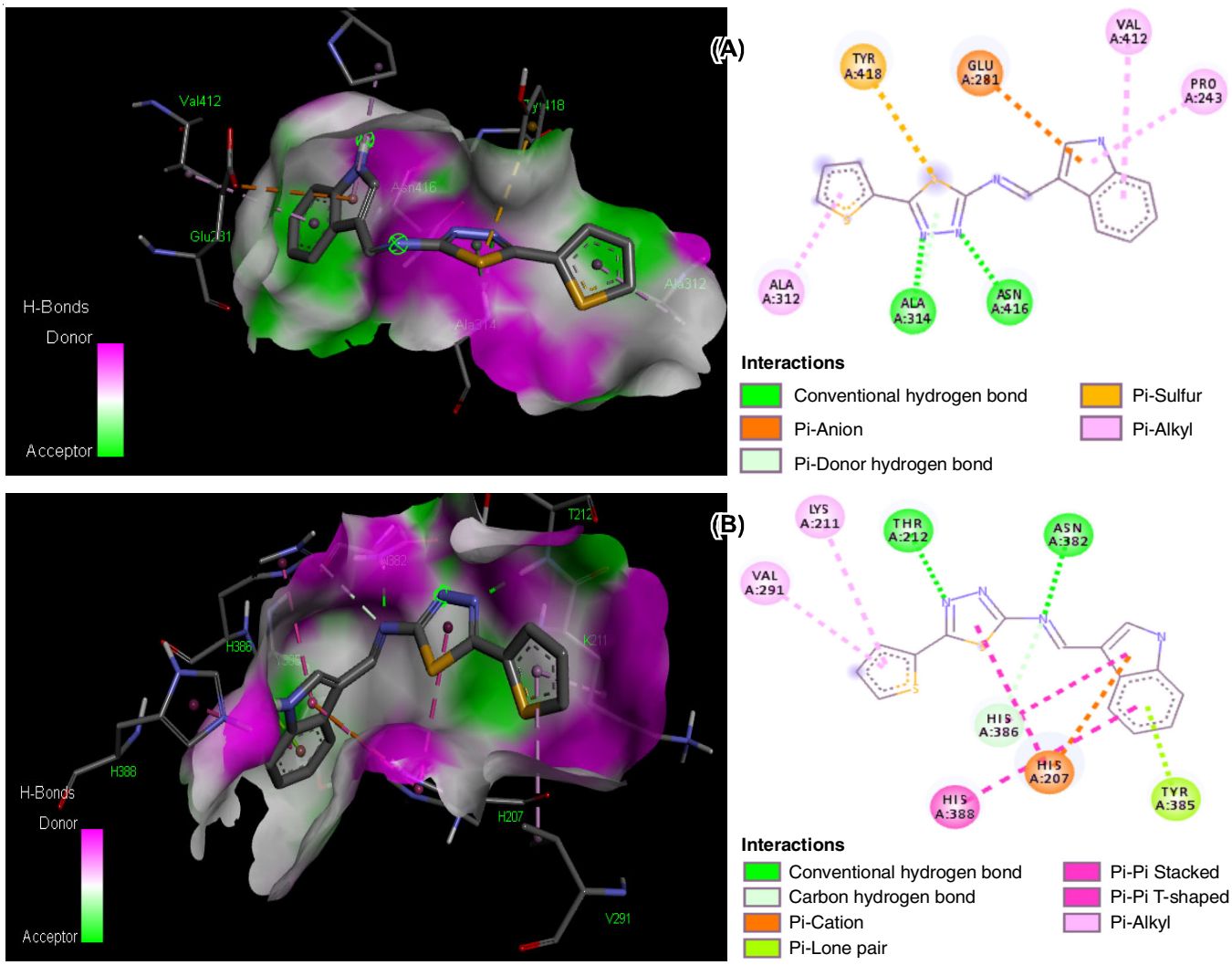


Fig. 1. 3D and 2D docking images of (A) compound **3a** with target proteins COX-1 (PDB ID: 3KK6) and (B) compound **3a** with target proteins COX-2 (PDB ID: 3Q7D)

TABLE-2
BINDING AFFINITIES OF THE COMPOUND WITH TARGET PeROTEINS COX-1 (PDB ID: 3KK6) AND COX-2 (PDB ID: 3Q7D). STUDIES WERE PERFORMED USING AUTODOCK 4.1

Compd.	Protein COX-1 (PDB ID: 3KK6)			Protein COX-2 (PDB ID: 3Q7D)		
	Binding energy (kcal/mol)	Amino acids involved in interactions	No. of H-bond	Binding energy (kcal/mol)	Amino acids involved in interaction	No. of H-bond
3a	-5.94	Ala312, Ala314, Asn416, Tyr418, Glu281, Val412 Pro243	2	-9.13	His207, Lys 211, Thr212, Val291, Asn382, Tyr385, His386, His388	2
3b	-5.54	Phe257, Tyr567, Ile558, Ile555, His553, Pro552, Ile534	0	-8.55	Gln327, Ser548, Lys137, Tyr130, Tyr136, Cys47, Pro153, Cys41, Gln461, Leu152	2
3c	-5.92	Glu301, Val391, Ala300, Gln390, Arg376, Arg298, Asp697, Arg424, Pro392	2	-8.13	Phe147, Asn375, Gly227, Gly536, Gly533, Val228, Asn537	3
3d	-5.8	Glu301, Val391, Lys393, Gln395, Gln390, Gly299, Arg376	2	-8.09	Gln327, Cys47, Tyr136, Pro153, Tyr130, Lys137	2
3e	-4.86	Tyr567, Ile555, Leu253, Phe257	1	-8.57	Leu390, Ala199, Leu391, His207, His388, Trp387, His386, Asn382, Thr212	4
3f	-5.86	Lys393, Ala300, Glu301, Val391, Val389, Arg376, Gln390, Gly299	3	-8.6	Cys41, Cys47, Pro153, Tyr130, Glu46, Lys137, Ser548, Thr549	4

TABLE-3
MEAN PAW VOLUME OF SYNTHESIZED COMPOUNDS (**3a-f**)

Group	Dose (mg/kg body wt.)	Mean paw volume (Mean ± SEM)						
		Initial paw volume (mL)	0 h	1 h	2 h	3 h**	4 h**	5 h**
(Control)	0.5% CMC	0.081 ± 0.005	0.084 ± 0.005	0.082 ± 0.003	0.091 ± 0.004	0.084 ± 0.005	0.085 ± 0.003	0.086 ± 0.004
(Inducer)	0.1% Carrageenan	0.087 ± 0.004	0.216 ± 0.017	0.34 ± 0.011	0.386 ± 0.007	0.491 ± 0.005	0.314 ± 0.004	0.490 ± 0.004
(Diclofenac)	10	0.083 ± 0.004	0.288 ± 0.007	0.291 ± 0.004	0.194 ± 0.008	0.185 ± 0.008	0.170 ± 0.005	0.161 ± 0.007
3a	20	0.086 ± 0.003	0.284 ± 0.002	0.241 ± 0.003	0.224 ± 0.002	0.206 ± 0.004	0.178 ± 0.004	0.164 ± 0.005
3b	20	0.085 ± 0.004	0.281 ± 0.003	0.273 ± 0.002	0.250 ± 0.004	0.220 ± 0.004	0.213 ± 0.004	0.212 ± 0.003
3c	20	0.084 ± 0.005	0.285 ± 0.002	0.280 ± 0.004	0.266 ± 0.005	0.234 ± 0.004	0.211 ± 0.004	0.192 ± 0.005
3d	20	0.098 ± 0.005	0.283 ± 0.004	0.272 ± 0.004	0.258 ± 0.004	0.242 ± 0.003	0.22 ± 0.003	0.216 ± 0.003
3e	20	0.081 ± 0.006	0.298 ± 0.004	0.262 ± 0.005	0.256 ± 0.002	0.238 ± 0.006	0.202 ± 0.005	0.191 ± 0.003
3f	20	0.085 ± 0.004	0.277 ± 0.004	0.272 ± 0.002	0.250 ± 0.003	0.224 ± 0.002	0.197 ± 0.004	0.184 ± 0.005

Results are expressed as mean ± SD (n = 5). Statistically significant differences were observed between carrageenan induced (Inducer) group and test groups (**3a-f**). *$p < 0.001$

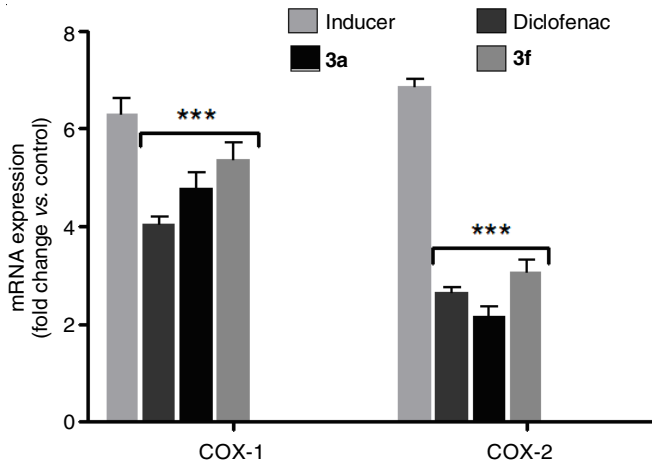


Fig. 2. mRNA expression levels of COX-1 and COX-2. The ability of the synthesized compounds **3a** and **3f** to downregulate the expression of COX-2 more selectively than COX-1 is confirmed by qRT-PCR analysis. Results are expressed as mean ± SD (n = 5). Statistically significant differences were observed between the carrageenin induced (inducer) group and most potent test groups (**3a** and **3f**). *$p < 0.001$

TABLE-4
% INHIBITION OF PAW EDEMA OF SYNTHESIZED COMPOUNDS (**3a-f**)

Group	Dose (mg/Kg)	% Inhibition of paw volume				
		1 h	2 h	3 h	4 h	5 h
Control	0.5% CMC	–	–	–	–	–
Diclofenac	10	26.00	56.00	58.69	66.41	77.38
3d	20	31.00	40.00	56.82	65.16	78.40
3b	20	31.50	43.30	52.60	57.20	62.50
3c	20	30.50	40.90	47.82	56.25	63.42
3a	20	30.00	41.00	48.30	55.20	60.50
3e	20	32.70	43.00	47.52	57.50	64.50
3f	20	30.00	43.18	53.10	66.25	73.38

with our synthesized compounds **3a** and **3f** showed a tendency similar to that observed in a molecular docking study, probably revealing the mechanism of action of synthesized compounds.

Conclusion

In this article, the effective and quick synthetic method to synthesize novel thiophene hybridized thiadiazolyl Schiff bases

(TTS) was described. Thiophene hydrazones were synthesized using $Cu(NO_3)_2 \cdot 6H_2O$, which then underwent cyclization to produce a thiadiazole ring, followed by the formation of desired compounds. The *in vivo* anti-inflammatory activity revealed that all compounds **3a-f** had good anti-inflammatory activity at 20 mg/kg against the reference drug diclofenac at 10 mg/kg after the 5th hour of dose administration. Compound **3f** (2,4-dihydroxybenzene substitution) and compound **3a** (indole substitution) both shown remarkable anti-inflammatory action. Additionally, it is well known that COX-1 and COX-2 are significantly released in response to a variety of inflammatory stimuli and that their over-expression is directly related to both increased inflammation and the prognosis for cancer. The results of the molecular docking investigation clearly showed that compound **3a** had maximal binding affinities of -9.13 kcal/mol on COX-2 and -5.94 kcal/mol on COX-1. Furthermore, in qRT-PCR analysis, the quick decline in overexpressed mRNA levels of IL-6 and COX-2 after the treatment with the synthesized compounds **3a** further supported the findings of the molecular docking study, demonstrating the higher selectivity of synthesized compounds for COX-2 inhibition than for COX-1 inhibition. All the results indicate to the potential of the synthesized compounds to effectively reduce inflammation through COX-2 antagonistic activity and suggest that they may be used as lead molecules for the development of anti-inflammatory drug.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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