Original Article

Copper Nitrate Catalyzed Synthesis Of Novel Thiazolyl Hydrazones: Anti-Inflammatory Effect Via COX-2/IL-6 Dual Antagonistic Action

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Abstract

Inspired by the well documented inflammation protecting ability of thiazoles, hydrazones and Schiff bases, a novel series of thiazolyl hydrazones were designed and synthesized via copper nitrate catalyzed efficient synthetic strategy. The structures of the newly synthesized compounds were elucidated based on IR, 1H NMR, 13C NMR and mass spectroscopic data. The molecular docking was conducted to elucidate the binding interactions of designed compounds with inflammation- associated targets, COX-2 (PDB ID: 3Q7D) and IL-6 (PDB ID: 1ALU). Most of compounds exhibited a stable binding complex with COX-2 and IL-6 receptors with predicted binding affinities -9.59 and -9.32, respectively. Further, the in-vivo study was investigated using the carrageenan induced paw oedema model where compounds **3b** and **3f** reflected a remarkable anti-inflammatory activity against diclofenac as standard drug. Interestingly, the mRNA expression analysis through qRT-PCR study further strengthened the previous findings showing the blockade of COX-2 and IL-6 mediated inflammation. Altogether, these remarkable findings open up possibilities of developing the synthesized molecules as novel candidate for plausible alternatives of NSAIDS.

Keywords: Disubstituted thiazolyl Schiff bases, Anti-inflammatory, NSAIDs, COX-2 and IL-6, Diclofenac

1. Introduction

Inflammation is a basic mechanism in which the body responds to infection, irritation or other injury of the body cells and tissues, and the symptoms being redness, warmth, swelling and pain.[1] Inflammations are mainly as acute and chronic inflammations[2]. The inflammation process is catalyzed by easily accessible molecules in the body such as histamine, prostaglandins, leukotrienes, oxygen- and nitrogen-derived free radicals, serotonin, bradykinin, and interleukins.[3] Cyclooxygenases (COXs) are enzymes responsible for the synthesis of prostanoids that have inflammatory and thrombotic effects.[4] Most of the present anti-inflammatory drugs inhibit the production of cyclooxygenase (COX) enzymes, COX-1 and COX-2 which synthesize prostaglandins and thromboxane, inflammatory mediators[5] Inflammatory diseases are currently treated with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs).[6,7] Nonsteroidal anti-inflammatory drugs (NSAIDS) such as indomethacin and Diclofenac are broadly used therapeutic drugs, primarily for inflammation, pain, and arthritis. The beneficial anti-inflammatory and analgesic effects of NSAIDs is due to their inhibition of cycloxygenase (COX) thereby inhibiting prostaglandin synthesis.

Thiazole ring is an integral part of penicillin antibiotics with a fragment (S-C=N),[8-11] plays an important role in the drug development. Thiazoles are reported to be a building block of various recent therapeutic agents such as anti-microbial,[12,13] anti-tubercular, anti-convulsant,[14] anti-cancer,[15-17] anti-HIV, anti-viral[18], cardiotonic and anti-inflammatory[19,20], analgesic-antipyretic[21], anti-hypertensive[22], and anti-schizophrenia[23], activities.

Hydrazones and their derivatives are known to exhibit interesting diverse biological activities including anti-inflammatory activity.[24,25] Hydrazones are a class of organic compounds that lies under Schiff base family. Schiff bases (>C=N-) are chemical structures that have a significant pharmacological potential. Schiff bases contain an azomethine group obtained through the condensation of primary amines with carbonyl compounds.[26] Hydrazones constitute the basic structure $R_1R_2C=N-NR_3R_4$, generally synthesized by reaction of hydrazine derivatives with carbonyl compounds in presence of strong acid in organic solvents,[27] and so forth. Here in present context, we developed an efficient copper nitrate catalyzed synthetic route of novel hydrazones.

In view of the above facts, the present research deals with the design of some novel thiazolyl hydrazones (THs) by molecular docking study on inflammatory-associated targets, COX-2 (PDB ID: 3Q7D) and IL-6 (PDB ID: 1ALU). A copper nitrate catalyzed efficient synthetic strategy was employed to synthesize the desired compounds. Further, the compounds were subjected to in-vivo anti-inflammatory activity using the carrageenan induced paw oedema model. As a part of study, qRT-PCR analysis was conducted to observe the levels of COX-2 and IL-6 mRNA (gene) expression that further strengthened our previous findings demonstrating the blockade of COX-2 and IL-6 inflammatory markers.

2. Experimental

2.1. General

The chemicals and reagents were acquired from Merk and S.D. Fine Chemicals, TCI, Kemphasol, and Spectrochem, Mumbai. Reaction progression was examined by thin layer chromatography (TLC) by using precoated silica gel G plates, iodine vapours and UV cabinet as visualizing agents. Melting points were determined on an electrothermal digital melting point apparatus. After physical evaluations, compounds were analyzed for elemental and spectral analysis. Elemental study was done on a EuroVector E 3000 Elemental Analyzer. IR spectroscopy was performed on Bruker Alpha-II FTIR Spectrophotometer which is expressed in cm^{-1}. Mass spectra were investigated by using Waters Aliance e2695/HLC-TQM Mass Spectrometer,1HNMR and 13CNMR spectra were observed and recorded on Bruker Advance 400/AivIII HD-300 (FT NMR) Chemical shifts were examined in parts per million (δ ppm for 1HNMR and 13CNMR) at the "Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute (CDRI) Lucknow''.

General procedure for synthesis of aryl thiosemicarbazone (2a-h)

A solution of thiosemicarbazide (5.5mmol) in ethanol or acetonitrile (20 mL) was added to a solution of substituted acetophenones (5.5 mmol) in ethanol or acetonitrile (20 mL). Now, $Cu(NO_3)_2{\cdot}6H_2O$ (1 mol %) was added and the reaction mixture was stirred at room temperature for desired time. The progress of reaction was monitored by thin-layer chromatographic plates (TLC) using the solvent system ethyl acetoacetate: n-hexane (4: 6). After completion of the reaction, 50 mL ice cold water was added to produce the solid product which then filtered dried and recrystallized with ethanol.

General procedure for synthesis of novel thiazolyl hydrazones (3a-f)

In second step, the obtained solution of aryl thiosemicarbazone (5.5 mmol) and the respective α-haloketone (5.5 mmol) in 20 ml of 2-propanol was placed in 100 mL round bottom flask. The reaction mixture was stirred at room temperature and monitored by TLC. After completion of the reaction, the mixture was allowed to stand at room temperature overnight. The resulting solid was filtered and washed with distilled water to obtain product and recrystallized with

ethanol. All the products thus obtained physically appeared as pure needle-shaped crystals, giving a single spot on the TLC plate.

N-[1-(5-Bromo-thiophen-2-yl)-ethylidene]-N'-[4-(4-methoxy-phenyl)-3H-thiazol-2-ylidene]-hydrazine (3a)

Reddish brown; Yield: 45%; mp (ºC)110-112º; FTIR(cm-1): 3411.38 (NH, str.) 2919.20(CH, str.),1553.01 (C=N, str.), 1016.39(-OCH_3, bend), 624.49 (C-Br, bend.); ^{1}H NMR, (DMSO-d6, 300MHz) δ_{ppm}: 3.17(s, 3H, CH_3), 3.78 (s, 3H, OCH_3), 6.88 (s, 1H Ar), 6.94 (d, 2H, Ar) 7.11 (s, 1H, thiazole), 7.59 (s, 1H, Ar) 7.76 (d, 2H, Ar), 8.36 (s, 1H, NH); ^{13}C NMR (DMSO-d6,75.5MHz) δ_{ppm}: 55.2(-OCH_3), 13.1(CH_3), 102.0 (CH, thiazole), 114.0 (CH, thiophene), 121.1(Ar), 122.1(Ar), 124.9(Ar), 125.6(Ar), 126.8(Ar), 126.9 (Ar), 127.2 (Ar), 131.0(Ar), 142.8 (Ar), 145.2(C-N), 169.1 (C=N, thiazole), 158.9 (S-C=N, thiazole); ESI-MS: 410 $[M+2H]^+$; Elemental analysis for $C_{16}H_{14}BrN_3OS_2$: Calculated: C, 47.06 ; H, 3.46; N, 10.29. Found: C, 46.79; H, 3.12; N, 10.01.

N-[1-(5-Bromo-thiophen-2-yl)-ethylidene]-N'-[4-(4-methyl-phenyl)-3H-thiazol-2-ylidene]-hydrazine(3b)

Reddish brown; Yield: 54%; mp (ºC)148-150º; FTIR(cm-1): 3421.45 (NH, str) 2922(CH, str),1696.94 (C=N, str), 1455 (C=C, bend), 624.77(C-Br, bend) ; ^{1}H NMR, (DMSO-d6, 300MHz) δ_{ppm}: 2.40 (s, 3H, CH_3), 4.57(s, 3H, CH_3), 7.03 (d, 2H, Ar), 7.20 (d 2H, Ar), 7.21 (s, 1H, thiazole), 7.67(s, 1H , Ar), 7.78 (s, 1H, Ar), 8.30 (s, 1H, NH); ^{13}C NMR (DMSO-d6,75.5MHz) δ_{ppm}: 55.2(-OCH_3), 38.6(CH_3), 113.8 (CH, thiazole), 114.2 (CH, thiophene), 128.0(CH, Ar), 128.3(Ar), 128.7(Ar), 128.7(Ar), 129.9(Ar), 129.9 (Ar), 130.5 (Ar), 134.2(Ar), 138.6 (Ar), 160.0 (C-N), 164.6 (C=N thiazole), 171.1 (S-C=N, thiazole); ESI-MS: 394 $[M+2H]^+$; Elemental analysis for $C_{16}H_{14}BrN_3S_2$: Calculated: C, 48.98; H, 10.71; N, 16.34; Found: C, 48.45 ; H,10.01; N, 16.10

N-3-(1-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-hydrazono ethyl)-phenol (3c)

Reddish brown; Yield: 61%; mp (ºC)158-160º; FTIR(cm-1):3648.40 (N-H, str.), 2360.03 (C-H, str.), 1541.29 (C=C, str), 1698.19 (C=N, str.); ^{1}H NMR, (DMSO-d6, 300MHz) δ_{ppm}: 3.81 (s, 3H, -OCH_3), 2.45 (s, 3H, CH_3), 3.91 (s, 1H, NH), 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, OH); ^{13}C NMR (DMSO-d6,75.5MHz) δ_{ppm}: 55.1(-OCH_3), 17.2(CH_3), 101.6 (CH, thiazole), 114.0 (CH,), 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: 340 $[M+2H]^+$; Elemental analysis for$C_{18}H_{17}N_3O_2S$: Calculated: C, 63.70; H, 5.05; N, 12.38; Found: C, 62.74; H, 5.13; N, 12.21.

N-3-(1-[4-(4-methyl-phenyl)-thiazol-2-yl]-hydrazono ethyl)-phenol (3d)

Yellowish brown; Yield 58%; mp (ºC):198-200º; FTIR(cm-1):3565.68 (N-H, str.), 2310.68 (C-H, str.), 1543.77 (C=C, str), 1698.12 (C=N, str.), 3747.40 (-OH); ^{1}H NMR, (DMSO-d6, 300MHz) δ_{ppm}: 2.32 (s, 3H , CH_3), 2.72 (s, 3H, CH_3), 2.45 (s, 1H, NH), 6.92 (d, 1H Ar), 7.14-7.33 (m, 5H, Ar), 7.50 (d, 1H, Ar)7.84(d, 2H, Ar) 8.23 (s, 1H, OH); ^{13}C NMR (DMSO-d6,75.5MHz) δ_{ppm}: 14.5 (CH_3), 20.8(CH_3), 101.6 (CH, thiazole), 116.8 (CH,), 118.9 (Ar), 120.6 (Ar), 125.5 (Ar),128.2(Ar), 128.6 (Ar), 128.8 (Ar), 129.2 (Ar), 130.4 (Ar), 130.8 (Ar), 137.3(Ar), 139.5(Ar), 153.3(C-N), 157.4 (C=N, thiazole), 167.8(S-C=N, thiazole); ESI-MS: 324 $[M+H]^+$; Elemental analysis for $C_{18}H_{17}N_3OS$: Calculated: C, 66.85; H, 5.30; N, 12.99; Found: C, 66.15
H, 5.12; N, 12.43.

N-[1-(2-chloro-phenyl)-ethylidene]-N'-[4-(4-methoxy-phenyl)-thiazol-2-yl]-hydrazine(3e)

Yellowish brown; Yield:78%; mp (ºC)136-140º; FTIR(cm-1):3648.54 (N-H, str), 2360.22 (C-H, str), 1557.81 (C=C, str), 1698.29 (C=N, str), 668.75 (C-Cl); ^{1}H NMR, (DMSO-d6, 300MHz) δ_{ppm}: 2.95(s, 3H, -OCH_3), 3.78(s, 3H,CH_3), 6.98(d, 2H, Ar), 7.115 (s, 1H, thiazole), 7.40 (s, 1H, NH)7.38-7.46(m, 3H, Ar), 7.77(d,2H, Ar); ^{13}C NMR (DMSO-d6,75.5MHz) δ_{ppm}: 18.7 (CH_3), 55.1(CH_3), 105 (CH, thiazole), 113.9 (Ar), 114.0 (Ar), 126.7 (Ar),125.1(Ar), 126.8

(Ar), 128.8 (Ar), 129.8 (Ar), 129.9(Ar), 131.2 (Ar), 136.9(Ar), 137.1(Ar), 139.5(Ar), 150.1(C=N), 153.2 (C-N, thiazole), 167.8(S-C=N, thiazole); ESI-MS: 358 [M+H]+; Elemental analysis for $C_{18}H1_{16}ClN_3OS$: Calculated: C, 60.41; H, 4.51; N, 11.74; Found: C,59.69; H, 4.41; N,11.17.

[1-(2-chloro-phenyl)-ethylidene]-N'-[4-(4-methyl-phenyl)-thiazol-2-yl]-hydrazine (3f)

Cream; Yield 72%; mp (ºC); 164-166º; IR (KBr cm-1): 3747.58 (N-H, str), 2310.91 (C-H, str), 1542.11 (C=C, str), 1698.28 (C=N, str), 654.53 (C-Cl); ^{1}H NMR, (DMSO-d6, 300MHz) δ_{ppm}: 2.30(s, 3H,CH_3), 2.31(s, 3H, CH_3), 7.21(d, 2H, Ar)7.22(s, 1H, thiazole), 7.74 (d,2H, Ar), 7.52 (s, 1H, NH), 7.39-7.45 (m, 3H, Ar), 7.50(d, 1H, Ar); ^{13}C NMR (DMSO-d6,75.5MHz) δ_{ppm}: 18.47 (CH_3), 20.8(CH_3), 103.2 (CH, thiazole) 125.5 (CH, thiazole), 127.3 (Ar), 128.9 (Ar), 129.2 (Ar),129.8(Ar), 130.0 (Ar), 130.4 (Ar), 131.1 (Ar), 131.9(Ar), 136.9 (Ar), 138.7(Ar), 147.5(Ar), 148.0(C=N), 150.1 (C-N thiazole), 169..4(S-C=N, thiazole); ESI-MS: 342 $[M+H]^+$; Elemental analysis for $C_{18}H1_{16}ClN_3S$: Calculated: C, 63.24; H, 4.72; N,12.29; Found: C, 61.94; H, 4.21; N, 12.12.

2.2. In silico screening

Molecular docking studies shows binding of the compounds with amino acid residue of cyclo-oxygenase (a promising anti-inflammatory) through hydrogen bonding interaction. The Open Babel, Avogadro, and ChemDraw softwares were used for drawing of 3D structure of ligands and their geometry was optimized six-times with Gauss view 5.0. The crystal structure of target proteins COX-2 (PDB ID: 3Q7D) and IL-6 (PDB ID: 1ALU) was retrieved from RCSB Protein Data Bank.[28] The new synthesized compounds were docked to active sites of the assigned targets using with the help of CASTp database. Furthermore, in silico molecular docking studies of titled compounds were performed using Autodock 4.1 along with its LGA algorithm for automated flexible ligand docking and binding energy evaluated in form of negative kilocalorie per mole. Probable hydrogen bonds and π bonds were evaluated.[29]

2.3. In-vivo anti-inflammatory activity

Carrageenan-induced edema is a nonspecific inflammation but is highly sensitive to NSAIDs. Diclofenac, a potent NSAID was used as a reference standard. COX-2 mediated increase in prostaglandin E2 (PGE2) production contributes to the severity of the inflammatory and pain responses in this model. The novel synthesized compounds were screened for in-vivo anti-inflammatory activity, by using carrageenan induced rat paw edema model. Male Wistar rat of either sex, weight (150-275gm) divided into nine groups. Each group includes 5 animals. Group 1 referred as control group administered with only 0.5% carboxymethyl cellulose (CMC) solution, Group 2 referred as inducer (carrageenan along with 0.5% CMC as vehicle), group 3 referred as standard (Diclofenac along with 0.5% CMC as vehicle prior to administration of carrageenan). Groups 4-9 were assigned as test groups with test drugs along with 0.5% CMC as vehicle prior to administration of carrageenan.

The standard drug, Diclofenac sodium (10mg/kg body weight) and all the test compounds were administered orally (10 mg and 20mg/kg body weight) in a suspension of 0.5% CMC as vehicle. After 1h foot paw edema was induced by injecting inducer (0.1mL of freshly prepared 1% carrageenan in physiological solution) subcutaneously into the planter portion of the left hind paw of each rat. Initial foot paw edema was measured immediately by using a mercury plethysmometer. The volume was measured at 1, 2, 3, 4 and 5 hrs after the administration of carrageenan. The increase in volume of paw was adopted as measure of edema.[30] The level of anti-inflammatory activity (% inhibition of inflammation) was calculated using the formula given below:

$$\frac{V_c - V_t}{V_c} \times 100$$

Where V_c is the increase in paw volume of control (in absence of test compounds), and V_t is the increase in paw volume after administration of the test compound.

2.4. qRT-PCR study

To explore the expression of mRNA for the target genes, 10 mg of tissue samples of each group was taken, and total mRNA was isolated using TriZol reagent and RNeasy mini kit was applied to purify the mRNA. cDNA was prepared according to the manufacturer's protocol for GeneSure first strand cDNA synthesis kit (Genetix Biotech Asia Pvt. Ltd., New Delhi, India). Finally, qRT-PCR was performed in Agilent Stratagene Mx3000P series (Applied Biosystems, Foster City, USA) using Sybr@ green PCR master mix. The mRNA was normalized with housekeeping control GAPDH. ΔCt values were normalized with untreated control samples for all compounds (ΔCt = Ct gene of interest – Ct housekeeping gene). Relative changes in the expression level of one specific gene were calculated in terms of 2-ΔΔCt (ΔΔCt = ΔCt test − ΔCt control).[31] The primer sequences were as follows: IL-6, 5′-TCAATGAGGAGACTTGCCTG-3′(forward), 5′-GATGAGTTGTCATGTCCTGC-3′ (reverse)[32] COX-2, 5′ ATCAGAACCGCATTGCCTCT-3′ (forward), 5′-GCCAGCAATCTGTCTGGTGA-3′ (reverse)[33]and β-actin, 5′-AAGTCCCTCACCCTCCCAAAAG-3′ (forward) and 5′- AAGCAATGCTGTCACCTTCCC-3′ (reverse)[34].

2.5. Statistical data analysis

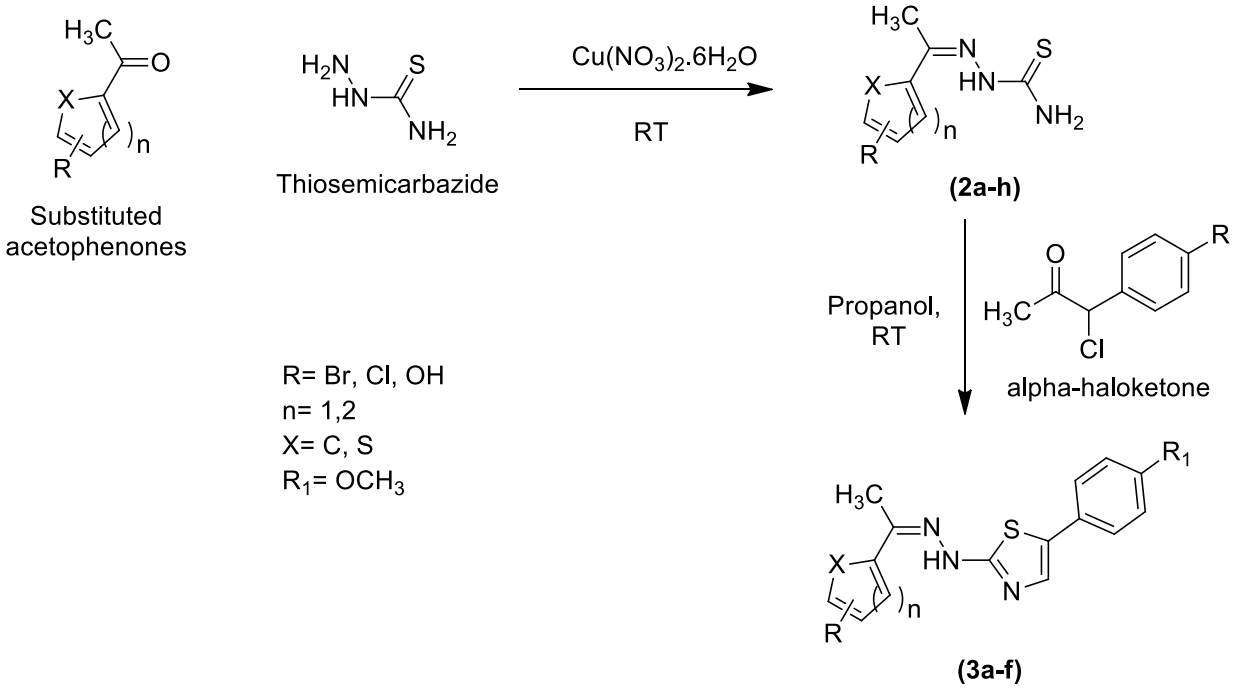
Statistical data analysis was carried out using the software GraphPad Prism 5.0 (San Diago, CA, USA). The results were expressed as mean±standard deviation (SD) (n=5). The statistical data was analyzed by one-way ANOVA (analysis of variance) followed by Bonferroni's multiple comparison test. Statistically significant differences were observed between carrageenin induced (**Inducer**) group and test groups (**3a-3f**) (*p<0.001).

3. Results and discussion

3.1. Chemistry

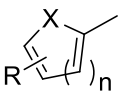
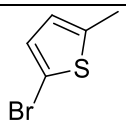
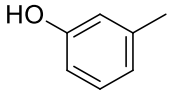
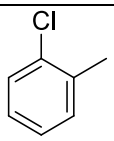
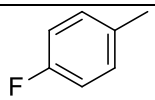
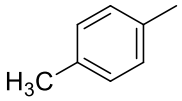
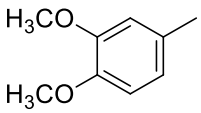
By adopting the reported procedures of Schiff base formation, a thiazole substituted ethanone and thiosemicarbazone underwent an acid-catalyzed reaction to constitute a rapid and facile synthesis of novel thiazole substituted Schiff bases (I to III). The first step in the mechanism is believed to be the condensation between thiazole substituted ethanone and thiosemicarbazone with removal of water molecule. The intermediate so-generated acts as an electrophile for the nucleophilic addition of the α-haloketone in presence of propanol. The resulting adduct undergoes cyclization into new thiazole ring. The possible route of the reaction is delineated in figure 1.

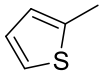
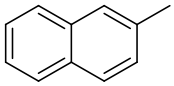
The substituted acetophenones were reacted with a primary amine thiosemicarbazide in the presence of $Cu(NO_3)_2.6H_2O$ as a catalyst. For the first time, 1 mol of thiosemicarbazide was reacted with substituted acetophenones in the presence of $Cu(NO_3)_2$, $6H_2O$ at room temperature (Scheme 1). The reaction of substituted acetophenones with thiosemicarbazide gave corresponding hydrazones (**2a–c**) in good to excellent yields (Table 1). On the other hand, the yield of reaction in the absence of catalyst for the same time was very low. The time of reaction is 10–12 min, which was quite shorter compared to the previously reported procedures of hydrazone Schiff base synthesis. The yield of reactions with the present protocol for the preparation of hydrazone Schiff bases (**2a-c**) was quite fair and the reaction time was found to be very short. The NMR spectra data of all synthesized Schiff bases are consistent with their structures. The aromatic protons resonate as a multiple signal at 6.39–9.45 ppm range depends on the different aromatic groups. The recorded mass spectrum revealed the correct molecular ion (M+1) peak, as evidenced by the molecular formula. The absence of the NH_2 asymmetric and symmetric stretching vibrations at 3281 cm^{-1} and 3186 cm^{-1}, and the presence of the peaks at 1553 cm^{-1} and 3411 cm^{-1} correspond to -C=N (str) and –NH (str) respectively in the IR spectrum of the final compound provided strong evidence for the formation of the Schiff bases. The ^{1}H-NMR and ^{13}C-NMR spectrum of the newly synthesized compound was consistent with the proposed structure. The methyl and the methoxy groups were appeared at 3.17 ppm (-CH_3) and 3.78 ppm (-OCH_3) as singlets whereas the -NH- group of hydrazones was appeared as singlet at 8.36 ppm. The mass spectra of the compound showed m/z 410.0 $[M+2H]^+$ and agrees with the desired molecular formulae. Similarly, the structural elucidation of the remaining pyrazolyl hydrazone Schiff bases (**3a-f**) was characterized as described above.



Scheme 1: General synthesis of compounds (**3a-f**), Reagent and condition: **(A)** $Cu(NO_3)_2.6H_2O$, room temperature, **(B)** propanol, room temperature.

Table 1. Hydrazone Schiff bases (**2a-h**) derived from $Cu(NO_3)_2.6H_2O$ catalyzed reaction of substituted acetophenones with thiosemicarbazide.

Entry	**Ar** = 	**Catalyst**	**Time (min)**	**Products (2a-h)**	**Yield (%)**
1		$Cu(NO_3)_2.6H_2O$	15	**2a**	88
2		$Cu(NO_3)_2.6H_2O$	10	**2b**	91
3		$Cu(NO_3)_2.6H_2O$	12	**2c**	93
4		$Cu(NO_3)_2.6H_2O$	10	**2d**	85
5		$Cu(NO_3)_2.6H_2O$	15	**2e**	87
6		$Cu(NO_3)_2.6H_2O$	10	**2f**	98

7		$Cu(NO_3)_2.6H_2O$	20	**2g**	91
8		$Cu(NO_3)_2.6H_2O$	18	**2h**	85

3.2. In silico study

The aim of the present study was to evaluate the anti-inflammatory activity of a new thiazolyl hydrazone Schiff-bases as COX-2 and IL-6 inhibitors. Thus, molecular docking studies was conducted to assess the binding of the compounds with amino acid residue of target proteins COX-2 (PDB ID: 3Q7D) and IL-6 (PDB ID: 1ALU) through hydrogen bonding and π-π interaction. The new synthesized compounds were docked to active sites of COX-2 and IL-6 using Autodock 4. The 3D structure of ligands was drawn on Chemdraw/Openbabel. The crystal structure of target proteins (3Q7D and 1ALU) were retrieved from RCSB Protein Data Bank[28]. Binding of the ligand molecule into the binding site of COX-2 and IL-6 was found noncovalent and exhibited the geometry of the binding sites at which the ligands produce optimal binding energy and H-bond interactions with amino acid residues. This study clearly demonstrated that maximum binding affinity of compounds **3d** and **3f** was found on COX-2 (-9.59 kcal/mol) and IL-6 (-9.32 kcal/mol) respectively (Table 2 and Figure 1).

Table 2: Binding affinities of the compound with target proteins COX-2 (PDB ID: 3Q7D) and IL-6 (PDB ID: 1ALU). Studies were performed using Autodock 4.1.

Comp Codes	Protein COX-2 (PDB ID: 3Q7D)			Protein IL-6 (PDB ID: 1ALU)		
	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	-8.19	Trp139, Asn537, Pro538, Asn375, Gln374, Pro127, Phe142, His226, Arg376	1	-7.84	Pro538, Asn375, Gln374, Pro127, Phe142, Arg376, Glu322	2
3b	-8.88	Pro538. Phe142, Arg376, Phe142, Pro538, Gly533	2	-8.82	Thr549, Pro538, Phe142, Arg376, Phe142, Gly533, Cys41, His226	2
3c	-8.35	Cys41, Cys47, Glu46, Tyr130, Gln327, Asn34, Glu322, Trp323	1	-7.98	His226, Cys41, Cys47, Glu46, Tyr130, Gln327, Asn34, Glu322, Trp323	1
3d	**-9.59**	**Thr549, Lys137, Cys47, Pro153, Tyr130, Cys41**	**3**	-9.15	Pro538, Lys137, Cys47, Pro153, Tyr130, Cys41	3
3e	-8.58	Asp125, Tyr373, Gln372, Trp139	2	-8.4	Thr549, Asp125, Tyr373, Gln372, Trp139, Asn537	1

3f	-9.45	Cys41, Arg44, Cys47, Pro153 Lys468, Arg469	3	**-9.32**	**Cys41, Arg44, Cys47, Pro153 Lys468, Arg469**	**3**

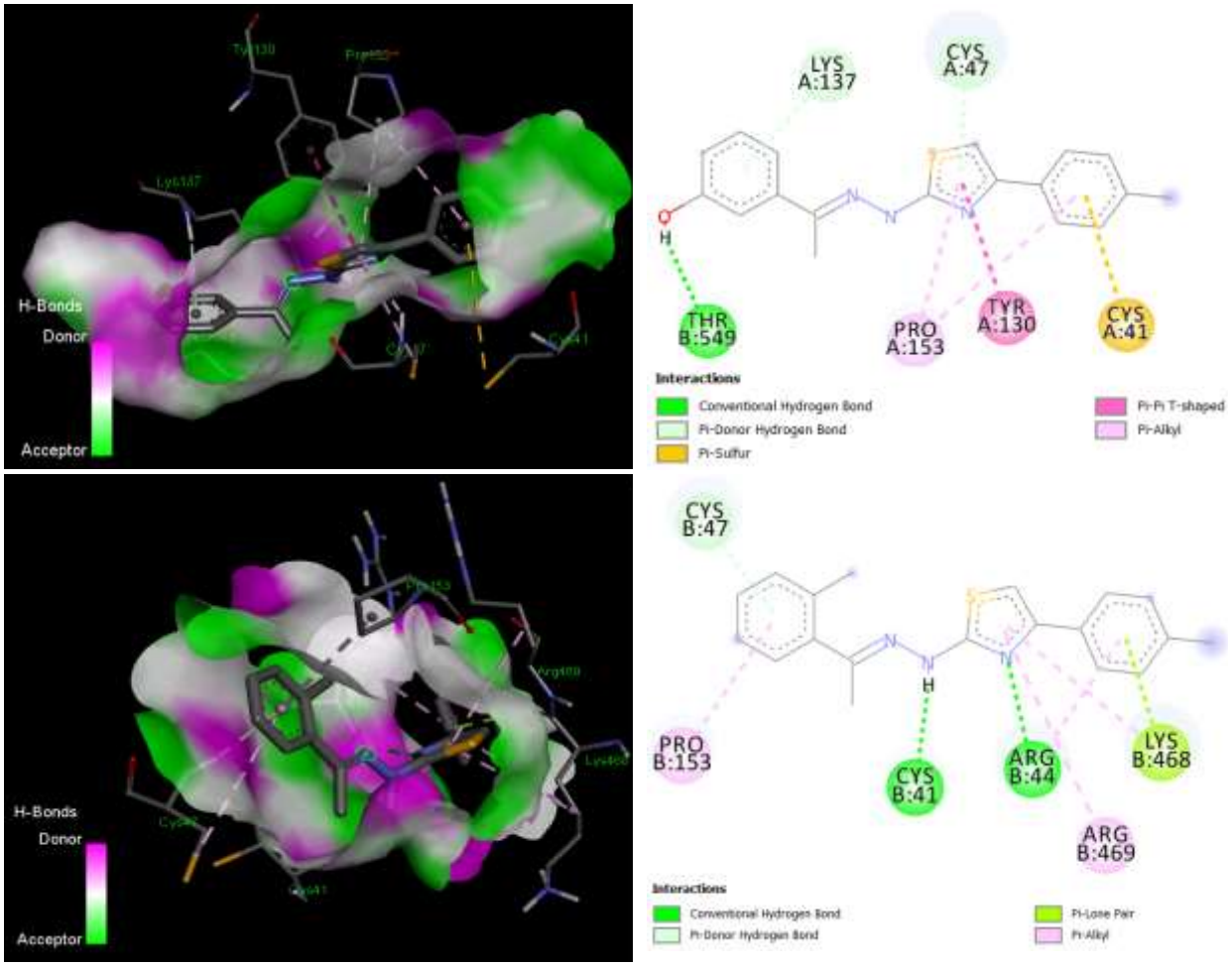


Figure 1: (A) 3D and 2D docking images of compound **3d** with target proteins **COX-2 (PDB ID: 3Q7D)** (binding energy **-9.59kcal/mol**), **(B)** 3D and 2D docking images of compound **3f** with target proteins **IL-6 (PDB ID: 1ALU)** (binding energy **-9.32 kcal/mol**).

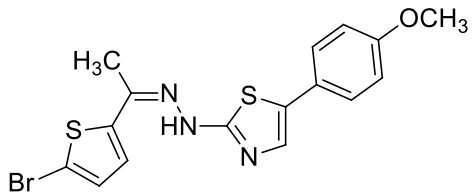
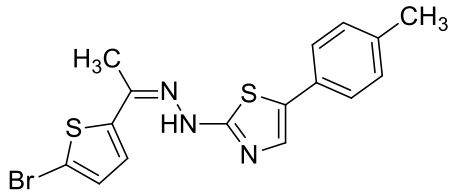
3.3. In-vivo anti-inflammatory activity

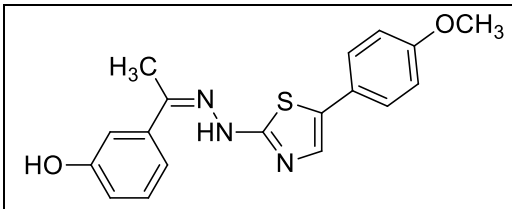
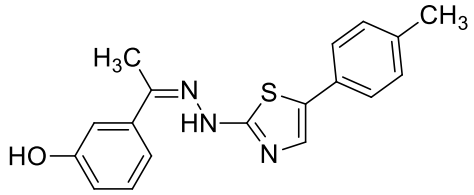
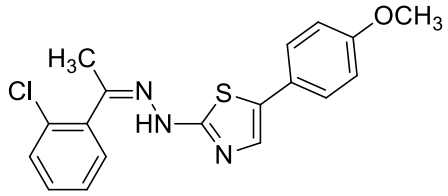
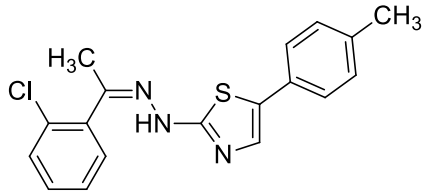
The in-vivo anti-inflammatory activity of the synthesized thiazolyl hydrazone Schiff bases were investigated by using carrageenan induced rat paw oedema model at dose of 10 and 20mg/kg body weight against the standard drug Diclofenac sodium. The results are tabulated in table 3 and 4 where it is observed that compounds **3d** and **3f** (bearing 4-methyl benzene) showed maximum anti-inflammatory activity and compounds **3c** and **3e** (bearing 4-methoxy benzene) showed good anti-inflammatory activity. The compounds **3a** and **3b** (bearing thiophene substituent) displayed moderate anti-inflammatory activity. In general, within the series of the synthesized thiazolyl hydrazones, compounds bearing 4-methyl benzene exhibited pronounced anti-inflammatory activity when compared to the compounds bearing 4-methoxy benzene. Similarly, compounds bearing six-membered ring substitution exhibited more anti-inflammatory activity when compared to the compounds bearing five-membered ring substitution.

Table 3: Mean paw volume of synthesized compounds (**3a-f**). Results are expressed as mean±SD (n=5). Statistically significant differences were observed between carrageenan induced (Inducer) group and test groups (**3a-3f**). *p<0.001.

Group	Dose (mg/kg body wt.)	Mean paw volume (Mean ± SEM)						
		Initial paw volume (mL)	0h	1h	2h	3h**	4h**	5h**
(Control)	0.5% CMC	0.09±0.005	0.084±0.005	0.082±0.003	0.09±0.004	0.084±0.005	0.08±0.003	0.088±0.004
(Inducer)	0.1% Carrageenan	0.088±0.004	0.232±0.017	0.3±0.007	0.386±0.007	0.492±0.005	0.302±0.004	0.488±0.004
(Diclofenac)	**10**	**0.082±0.004**	**0.288±0.006**	**0.292±0.004**	**0.184±0.008**	**0.192±0.004**	**0.188±0.006**	**0.180±0.005**
(3a)	20	0.10±0.004	0.282±0.004	0.27±0.003	0.258±0.004	0.242±0.002	0.22±0.003	0.216±0.003
(3b)	20	0.084±0.004	0.280±0.003	0.274±0.002	0.248±0.004	0.22±0.004	0.212±0.004	0.210±0.003
(3c)	20	0.087±0.005	0.281±0.002	0.284±0.004	0.266±0.005	0.244±0.004	0.201±0.004	0.190±0.005
(3d)	**20**	**0.082±0.004**	**0.284±0.002**	**0.260±0.003**	**0.234±0.002**	**0.216±0.004**	**0.198±0.004**	**0.184±0.005**
(3e)	20	0.081±0.005	0.298±0.004	0.272±0.006	0.246±0.002	0.238±0.006	0.202±0.004	0.191±0.003
(3f)	**20**	**0.081±0.004**	**0.278±0.004**	**0.282±0.002**	**0.250±0.003**	**0.224±0.002**	**0.197±0.004**	**0.184±0.005**

Table 4: % inhibition of paw edema of synthesized compounds (**3a-3f**)

Group	Dose (mg/Kg)	% Inhibition of Paw Volume				
		1h	2h	3h	4h	5h
Control	0.5% CMC	-	-	-	-	-
Diclofenac	**10**	**27.5**	**57**	**58.69**	**65.41**	**75.38**
 **(3a)**	20	32.5	40.9	47.82	54.16	59.61
 **(3b)**	20	32.5	43.1	52.1	56.25	63.46

 **(3c)**	20	30	40.9	47.82	56.25	63.47
 **(3d)**	**20**	**30**	**38.6**	**57.82**	**64.16**	**74.47**
 **(3e)**	20	32.5	43.18	47.82	56.25	63.45
 **(3f)**	**20**	**30**	**43.18**	**53.1**	**66.25**	**73.38**

3.4. mRNA expression of the effectors cytokine IL-6 and COX-2

To confirm gene expression levels of IL-6 and COX-2, we performed qRT-PCR analysis, which showed the overexpression of these genes in carrageenin induced toxic group, compared to the normal control. However, the administration of standard drug diclofenac, test drugs **3d** and **3f** normalized these overexpressed levels to a considerable extent. The potency of **3d** and **3f** at 20 mg/kg dose was comparable to market available anti-inflammatory drug, diclofenac, without any significant difference (Figure 2). Furthermore, it is well documented that IL-6 and COX-2 is released strongly in response to various inflammatory stimuli and the over-expression of IL-6 and COX-2 gene has been clearly linked to elevated inflammation and in cancer prognosis as well. In qRT-PCR analysis, the rapid reduction in over-expressed mRNA level of IL-6 and COX-2 with the treatment of our synthesized compounds **3d** and **3f** provided a trend like those measured by molecular docking study and thereby demonstrating the mechanism of action of synthesized compounds.

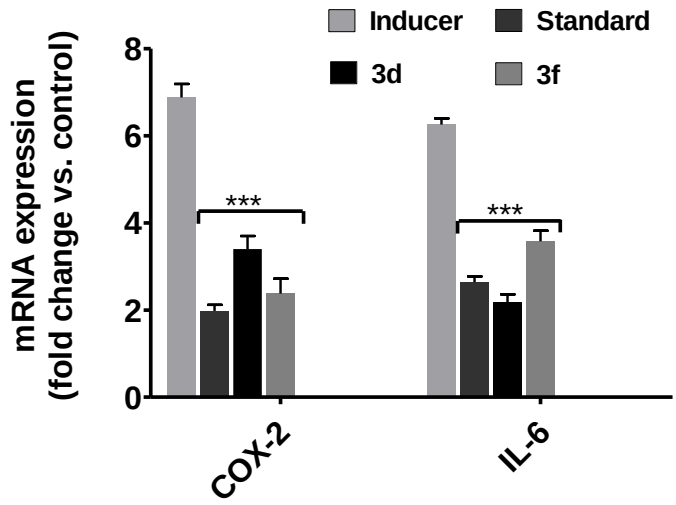


Figure 2. mRNA expression levels of COX-2 and IL-6. qRT-PCR analysis confirms IFBOs potential to regulate the expression of IL-6. Results are expressed as mean±SD (n=5). Statistically significant differences were observed between carrageenin induced (inducer) group and most potent test groups (**3d** and **3f**). *$p<0.001$.

4. Conclusion

We herein employed an efficient and fast synthetic strategy for the synthesis of new thiazolyl hydrazone Schiff-bases. First step involved $Cu(NO_3)_2.6H_2O$ catalysed synthesis of hydrazone Schiff bases which further reacted with substituted α-haloketones to produce thiazolyl hydrazone Schiff-bases. The in-vivo anti-inflammatory activity showed that all compounds **3a-f** exhibited good anti-inflammatory activity at 20mg/kg against reference drug Diclofenac 10 mg/kg after fifth hour of dose administration. Compounds **3d** and **3f** (bearing 4-methyl benzene) showed maximum anti-inflammatory activity. In general, within the series of the synthesized thiazolyl hydrazones, compounds bearing 4-methyl benzene exhibited pronounced anti-inflammatory activity when compared to the compounds bearing 4-methoxy benzene. Similarly, compounds bearing six-membered ring substitution exhibited more anti-inflammatory activity when compared to the compounds bearing five-membered ring substitution. Furthermore, it is well documented that IL-6 and COX-2 is released strongly in response to various inflammatory stimuli and their over-expression has been clearly linked to elevated inflammation and in cancer prognosis as well. The molecular docking study clearly demonstrated that maximum binding affinity of compounds **3d** and **3f** was found on COX-2 (-9.59 kcal/mol) and IL-6 (-9.32 kcal/mol) respectively. Further, in qRT-PCR analysis, the rapid reduction in over-expressed mRNA level of IL-6 and COX-2 with the treatment of our synthesized compounds **3d** and **3f** further strengthened our findings of molecular docking study, demonstrating the mechanism of action of the synthesized compounds. Taken together, all the results indicate that the synthesized compounds show exceptional potential to obliterate inflammation by COX-2 and IL-6 dual antagonistic action and could serve as potential lead molecules for the development of anti-inflammatory drugs.

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6. Conflict of interest

Authors declare no conflict of interest.

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