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Synthesis, biological evaluation and molecular modeling study of pyrazole derivatives as selective COX-2 inhibitors and anti-inflammatory agents

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ABSTRACT

A novel series of pyrazole derivatives were synthesized and evaluated in vivo for their anti-inflammatory activity in carrageenan-induced rat paw edema model. Among all compounds, **5a**, and **5b** showed comparable anti-inflammatory activity to Nimesulide, the standard drug taken for the studies. In silico (docking) studies were carried out to investigate the theoretical binding mode of the compounds to target the cyclooxygenase (COX-2) using Autodock 4.2.

1. Introduction

Inflammation is a multifactorial process which reflects the response of the organism to various stimuli and is related to many disorders such as arthritis, asthma, and psoriasis, etc [1]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutics. Through their anti-inflammatory, anti-pyretic and analgesic activities, they represent a choice of treatment in various inflammatory diseases such as arthritis, rheumatisms as well as to relieve the pains of everyday life [2]. The pharmacological effect of this class of drugs are due to inhibition of Cyclooxegenase enzyme [3]. Cyclooxygenase is the key enzyme in the biosynthesis of prostanoids, biologically active substances that are involved in several physiological processes and also in pathological conditions, such as inflammation [4]. There exist mainly two form of enzyme COX-1, COX-2, and recently, COX-3 as well as two smaller COX-I derived proteins (partial COX-1 or PCOX-I proteins) are also discovered [5]. Cox-1 is constitutively expressed in the Gastrointestinal tract, kidneys, and platelets and is involved in the regulation of physiological functions in maintaining vascular homeostasis, normal renal function, and gastric mucosal integrity, and the autocrine response to circulating hormones [6,7]. In contrast, the COX-2 isozyme is induced by stimuli such as mitogenes, oncogenes, growth factors, hormones and disorders of water-electrolyte homeostasis linking its involvement to pathological processes such as inflammation and various cancer types [8–10]. Long term use of classical NSAIDs cause side-effects such as gastrointestinal ulcer, renal dysfunction, and hepatotoxicity (Nimesulide and more recently, lumiracoxib). Side-effects are due to high COX-1 versus COX-2 selectivity [11–14]. Therefore, during the last two decades medicinal chemist, develops a new class of drug by taking the advantage of fact that COX-2 active site is 20% larger than COX-1 active site. These molecules, termed as coxibs (i.e., Celecoxib, Rofecoxib, Valdecoxib, and Etoricoxib) showed reduced gastrointestinal side effects as compared to traditional NSAIDs [15,16]. Unfortunately, some selective COX-2 inhibitory drugs that include Rofecoxib, Valdecoxib and Celecoxib alter the natural balance in the COX biochemical pathway, this natural imbalance in the COX pathway are believed to be responsible for increased incidences of high blood pressure and myocardial infarction in long term use and high dosage, that ultimately prompted the withdrawal of these drugs from the market [17,18].

This has led intense efforts in search for potent and selective COX-2 inhibitors which could provide anti-inflammatory drugs with fewer side-effects. Therefore, medicinal chemists are working on various scaffolds for development of NSAIDs with improved safety profile [19–22]. Many pyrazole derivatives are

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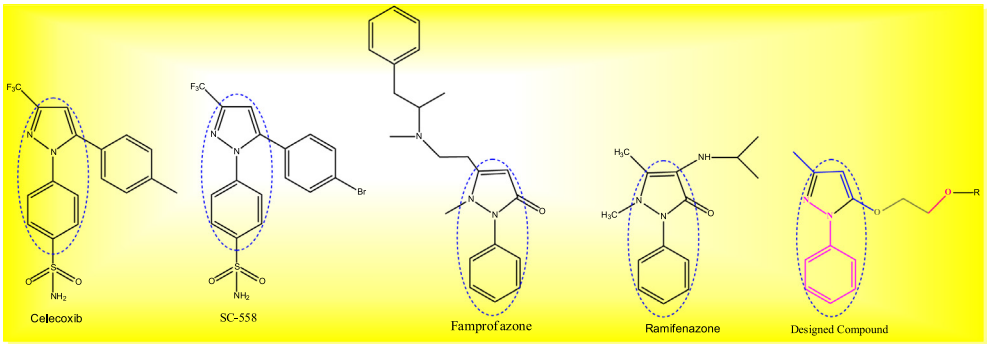


Fig. 1. Chemical structures of anti-inflammatory agents (Pyrazole derivative).

acknowledged to possess a wide range of bioactivities. The pyrazole motif makes up the core structure of numerous biologically active compounds. Some representatives of this heterocycle exhibit anti-viral/anti-tumor [23–25], antibacterial [26–29], antiinflammatory [30–33], analgesic [34], fungistatic [35], and anti-hyperglycemic activity [36,37].

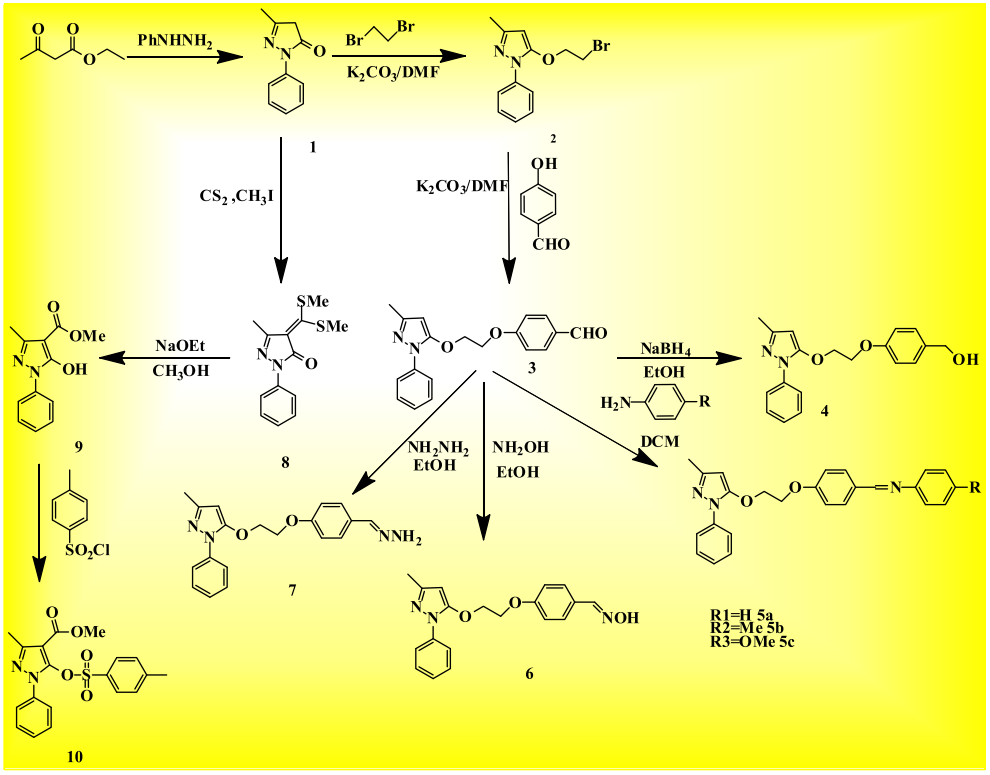
The well known anti-inflammatory drug, Celecoxib, is pyrazole derivative, some other examples of pyrazole derivative as NSAIDs are Mefobutazone, Ramifenazone, Famprofazone. (Fig. 1) Uses of few pyrazole derivatives are limited, as it is associated with serious side-effects such as bone marrow depression, water and salt retention and carcinogenesis [38]. This gave a great impetus to speculate for a new pyrazole derivative as anti-inflamatory agent with more potent activity and less toxicity.

Encouraged with the above literature and as a continuation of our research interest in the synthesis and biological activities of novel pyrazole derivatives [39–42]. The present study aimed at synthesis of the novel pyrazole derivative as they are the pharmacophore of various anti-inflammatory drugs [30,38]. The validity of this design was assessed through preliminary in vitro, in vivo anti-inflammatory screening, and docking simulation in our attempt to accentuate anti-inflammatory activity of compounds.

2. Results and discussion

2.1. Chemistry

The compounds were synthesized as shown in Scheme 1 via a general nucleophilic substitution, condensation and reduction reactions. The starting compound **1** was prepared according to previously reported procedure [39]. Compound **3** was prepared in two step procedure starting from **1**. In the first step, the compound **1** was treated with excess of 1, 2 dibromoethane (5 equivalent) in presence of K_2CO_3 in DMF to afforded the compound **2** in good yield. In subsequent step compound **2** was treated with p-hydroxybenzaldehyde (1 equivalent) in presence of K_2CO_3 in DMF, leading to formation of compound **3** in moderate yield. The intermediate **3**



Scheme 1.

smoothly reacted with various substituted aniline, hydroxylamine hydrogensulfate and hydrazine hydrate to provide corresponding Schiff base **5a**, **5b**, **5c** oxime **6** and hydrazone **7** in good yield. In case of Schiff base there should be two products syn and anti, but this isomerism is present only in case of **5a**. Two isomer were separated by crystallization and differentiated on basis of their melting point and λ_{max} absorption [43]. Melting point of syn and anti isomer are 88, 96 °C respectively, whereas λ_{max} of syn and anti are 320, 331 respectively. Syn isomer is the major product whereas anti isomer is the minor product. But in case of **5b**, **5c** there exist only syn isomer, this may be due to bulkier size of substituent. Whereas aromatic hydrazone and oxime exist only in syn isomer because aromatic aldehydes always give syn isomer in these type of reactions. Compound **8** was prepared by the reaction of phenylpyrazolone **1** with carbon disulfide followed by methylation. Further base catalyzed alcholysis of **8** followed by acidification gave pyrazole-ester **9** in good yield [44–46]. The reaction of **9** with the para methylbenzene-1-sulfonyl chloride resulted the formation of compound **10** in moderate yield. The newly synthesized compounds 1-10 were established on the basis of spectroscopic data. ^{1}H NMR spectra of compounds were studied in $CDCl_3$ showed characteristic peak at δ 5.5 ppm correspond to CH proton of pyrazole, peak at δ 2.2 ppm correspond to methyl proton of pyrazole ring. The characteristic peaks observed at δ 4.3–4.4 ppm correspond to OCH_2 of linker. ^{13}C NMR spectrum of compounds show signals at 14.5 ppm assigned for the methyl carbon of pyrazole ring, while signal at 66.1 and 70.0 correspond to linker carbon. The mass spectra of all compounds showed molecular ion peak corresponding to their molecular formula.

2.2. Biological studies

2.2.1. In vivo anti-inflammatory studies

All the newly synthesized compounds and Nimesulide, as a reference drug, were subjected to in vivo anti-inflammatory studies using the well-known rat carrageenan-induced foot paw edema model [47]. In carrageenan stimulated peripheral inflammation model, edema formation as inflammatory response is developed in multiphase mechanisms as reported, thus, (i) proinflammatory cytokines such as IL-6, IL-1b, and TNF-a are released by the peripheral stimulus, (ii) COX-2 mRNA and COX-2 protein are inducted and/or upregulated, and (iii) COX-2-dependent induction/upregulation of PGE_2 and PGI_2 causes increases of vasodilation, vascular permeability, extravasation of plasma proteins, and leakage to waterwithin the site to form PGG_2, and then (iii) the hydroperoxide group of PGG_2 is reduced (PGG_2 is subjected to the reduction of two electrons) to form PGH_2 at peroxidase site where haem (ironeporphyrin complex) is bound and histidine groups are linked [48–50]. The results of anti-inflammatory activities against carrageenan-induced rat paw edema model are shown in Tables 1 and 2. Compound **5b, 5c** & **4** show maximum inhibitory effect, while compound **6, 7** & **10** showed intermediate effect. The result of present study had shown that compounds **5a** and **5b** possess maximum inhibitory effect when compared to control group. It has been observed that maximum percentage of paw edema growth in control group after 90 minutes was 38.7% which was found to decrease up to 13.4, 19.4% in the group of rats treated with **5a, 5b** respectively, compared to Nimesulide treated group where it was found 23.4%, also the values were found statistically very significant. Compound **4** and **5c** found to possess good anti-inflammatory property as the percentage paw edema growth was shown to be only 19.9, 23.8% when compared to that of control group. Compound **6** show moderate effect, while compound **3**, **7** & **10** showed almost negligible effects (Fig. 2). Therefore, the results showed that **5a** and **5b** are potent inhibitor of inflammation.

Table 1
Percentage edema growth relative to control at different time intervals (mean ± S.E.M.).

Group	0 min	30 min	90 min
Control	100 ± 0	130.1 ± 6.54 (30.1)	138.7 ± 4.47 (38.7)
Nimesulide	100 ± 0	115.1 ± 2.88 (15.1)	123.4 ± 3.27* (23.4)
3	100 ± 0	115.1 ± 2.88 (15.1)	125.8 ± 8.42* (25.8)
4	100 ± 0	111.5 ± 2.89 (11.5)	119.9 ± 3.30*(19.9)
5a	100 ± 0	110.1 ± 5.14 (10.1)	113.4 ± 7.77* (13.4)
5b	100 ± 0	114.1 ± 2.88 (14.1)	119.4 ± 3.17* (19.4)
5c	100 ± 0	109.5 ± 2.68 (9.5)	123.8 ± 7.12* (23.8)
6	100 ± 0	114.8 ± 4.03 (14.8)	124.4 ± 6.78* (24.4)
7	100 ± 0	115.8 ± 5.19 (15.8)	128.4 ± 4.78* (28.4)
10	100 ± 0	122.1 ± 5.11 (22.1)	132.6 ± 3.13* (32.6)

Table 2
Paw edema at different time interval (ml/Rat) (mean ± S.E.M.).

Group	0 min	30 min	90 min
Control	0.99 ± 0.067	1.27 ± 0.043**	1.36 ± 0.070
Nimsulide	1.02 ± 0.054	1.16 ± 0.065	1.26 ± 0.038
3	0.78 ± 0.033	0.89 ± 0.041	0.98 ± 0.044
4	1.16 ± 0.054	1.29 ± 0.061	1.39 ± 0.068
5a	0.98 ± 0.031	1.06 ± 0.038	1.11 ± 0.045
5b	1.22 ± 0.061	1.35 ± 0.056	1.46 ± 0.068
5c	1.06 ± 0.041	1.20 ± 0.048	1.31 ± 0.057
6	1.08 ± 0.038	1.25 ± 0.054	1.38 ± 0.058
7	1.06 ± 0.044	1.21 ± 0.058	1.31 ± 0.061
10	0.98 ± 0.053	1.19 ± 0.058	1.29 ± 0.061

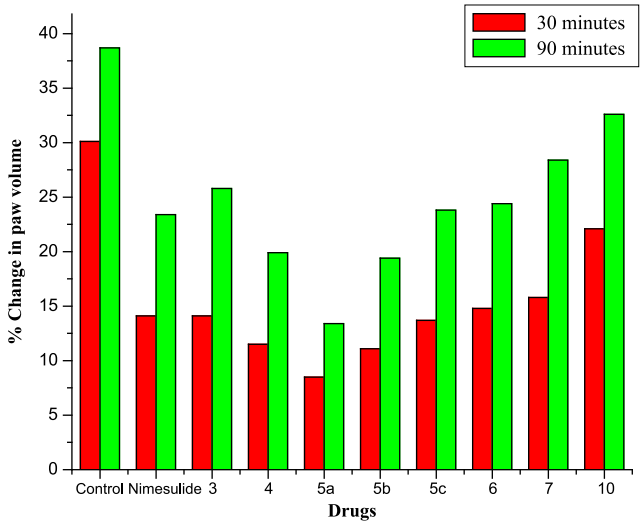


Fig. 2. Effect of different drugs on carrageenan induced rat paw edema.

2.2.2. COX assays

All the synthesized compounds were evaluated for their anti-inflammatory activity by biochemical COX (COX-1 & COX-2) inhibitory assay. The ratio of IC_{50} of COX-2 to IC_{50} of COX-1 (COX-2/COX-1) would suggest the selectivity of the compound (Table 3).

2.3. Molecular docking

Molecular docking is a frequently used tool in computer-aided structure-based rational drug design. It evaluates how small molecule (substrate, inhibitor, drug or drug candidate) and the target macromolecule (receptor, enzyme or nucleic acid) fit together. This can be useful for developing better drug candidates and also for the understanding the nature of the binding. Insights into the

Table 3
Cox-2/Cox-1 ratio of pyrazole derivatives.

Compound	Cox-1 IC_{50} (μM)[a]	Cox-2 IC_{50} (μM)[a]	SI[b]
Celecoxib	15	0.04	0.0028
3	24.0	18.5	0.78
4	28.3	15.0	0.53
5a	32.9	16.8	0.5100
5b	32.5	14.3	0.4400
5c	28.5	15.4	0.5400
6	28.8	15.3	0.5300
7	3.39	17.4	5.2100
10	20.1	13.1	1.53

differences between the binding sites of COX-1and COX-2 obtained from X-ray crystal structure data provided useful guidelines that facilitated the design of the selective COX-2 inhibitors. For example, the COX-2 binding site possesses an additional secondary pocket, that is, absent in COX-1, which is highly relevant to the design of selective COX-2 inhibitors. This COX-2 2^0 pocket arises due to a conformational change at Tyr355, that is, attributed to the presence of Ile523 in COX-1 relative to Val523 having a smaller side chain in COX-2 [51,52]. It has also been reported that replacement of His513 in COX-1 by Arg513 in COX-2 plays a key role with respect to the H-bond network in the COX-2 binding site. Access of ligands to the secondary pocket of COX-2 is controlled by histidine (His90), glutamine (Gln192), and tyrosine (Tyr355) [53]. Interaction of Arg513 with the bound drug is a requirement for time dependent inhibition of COX-2 [54].

Docking (in silico) studies were in good agreement with pharmacological results. Compounds **4**, **5a**, **5c** and **6** were the most promising compounds by computational studies, which is almost consistent with biological evaluation assays. Due to the increased flexibility introduced by the linker group the pyrazole derivatives show a potential higher affinity to COX-2. The results of the docking studies, docking scores, and involved amino acids interacted ligand moieties and hydrogen bond length for each compound and the reference native ligand are listed in Table 2, Figs. 3 and 4.

(i) Compound **4** fits in the active site of COX-2 and form one hydrogen bonds between H of the CH_2OH group and Gln 356 with distance 2.16 Å which is consistent with the moderate anti-inflammatory activity.
(ii) Docking study for the compound **5b** indicates that the phenyl ring of pyrazole, exhibit pi-pi interaction with amino acids Phe367, Phe191, Trp373which is consistent with the good anti-inflammatory activity.

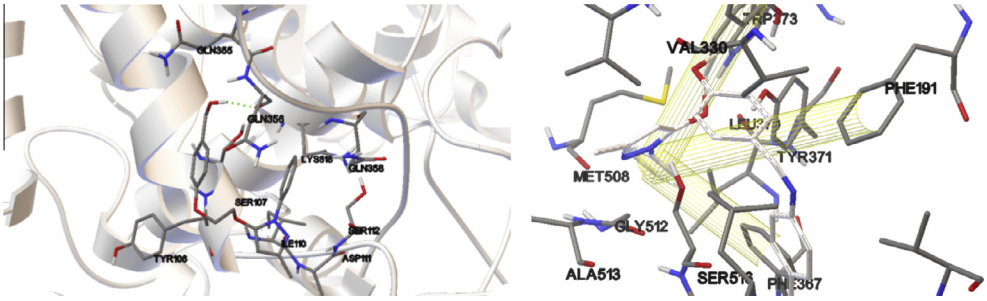


Fig. 3. Interaction diagrams for compounds 4 and 5b active site of COX-2 Green lines indicate H-bonds formed between the compound and the enzyme active site residues yellow cylinder represent π–π interaction.

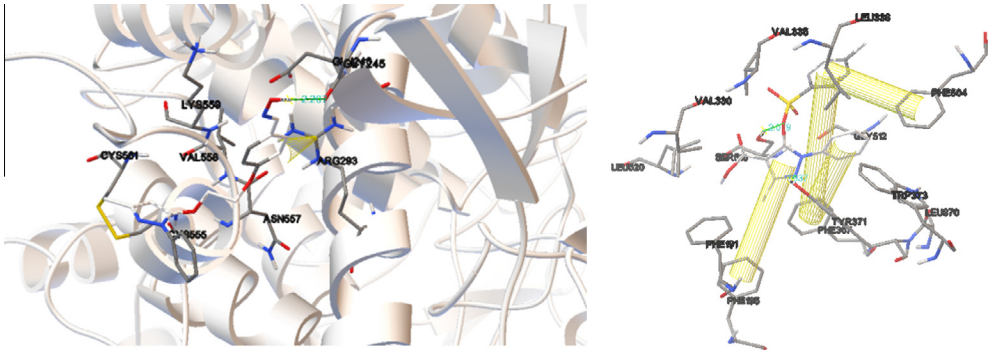


Fig. 4. Interaction diagrams for compounds 6 and 10 in active site of COX-2 Green lines indicate H-bonds, yellow cylinder represent π–π interaction, and yellow cone represent cation–π interaction.

Table 4
Dock scores and summary of molecular interactions of compounds after docking into COX-2 active site.

S. No.	Dock score	Type of interactions	Amino acid interactions	Interacting groups and distance
4	−6.66	H bond	Gln 356, Ser 107, Lys 158, Ile 110, Tyr 108	—CH_2OH (2.62 Å)
5a	−6.66	–	Gly121, Tyr120, His119	
5b	−8.93	π–π	Phe367, Phe191, Trp373	
6	−5.33	H bond, cation–π	Arg 293, Gly245, Cys555, Asn557	—NH (2.28 Å)
7	−5.66	–	Cys 44 Cys 42	
10	−6.67	2H bond, π–π	Val 335, Tyr371, Gly542, Phe367, Leu338, Phe 504	N & O of pyrazole ring (2.07 Å, 1.93 Å)

(iii) The binding mode of **6** into the active site of COX-2 enzyme revealed one hydrogen bonds with Gly 245 and show strong cation–pi interaction with Arg 293. Hydrogen bond and cation–pi interaction are responsible for moderate activity of compound.
(iv) Finally, Compound **10** formed two hydrogen bonds, one hydrogen bond between Tyr 371 with N of pyrazole and another one between Val 335 with O of pyrazole with a distance of 1.93 and 2.07 Å, respectively and also show pi–pi interaction with amino acids Phe 195, Phe 504 (see Table 4).

3. Conclusion

A series of novel pyrazole analogues were synthesized and their anti-inflammatory activity was determined using carrageenan mouse paw edema bioassay. In synthesized compounds, **5b** exhibited good anti-inflammatory activity, and optimal COX-2 inhibitory potency (IC_{50} = 0.44) μM). Molecular modeling showed that pyrazole analogues interact with COX-2 active site by forming classical hydrogen bonding, π–π interaction and cation–π interaction these interactions increase the residence time of ligand in the active site consequently augmenting anti-inflammatory activity of compounds.

4. Experimental

4.1. General

Melting points were determined in open capillaries on a Buchi Melting-point apparatus and are uncorrected. All the reactions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F254 (mesh); spots were visualized with UV light. Merck silica gel (60–120 mesh) was used for column chromatography. The IR spectra were recorded on VARIAN 3300 FTIR spectrophotometers as KBr pellets and the wave numbers were given in cm^{-1}. ^1H NMR (300 MHz), and ^{13}C NMR (75 MHz) spectra were recorded on a JEOL AL 300 MHz NMR spectrometer in $CDCl_3$ solution using TMS as an internal standard. All chemical shifts were reported in δ (ppm) using TMS as an internal standard. Mass spectra of the compounds were taken with JEOL SX 102/Da-600 mass spectrometer. Elemental analysis was performed on Exter Analytical Inc. 'Model CE-400 CHN Analyzer. The well known compounds were prepared following the procedures reported in the literature.

4.1.1. Synthesis of 3-methyl-1-phenyl-pyrazol-5-one (1)

In a 250-ml round-bottom flask, ethyl acetoacetate (48 ml, 0.385 mol) was taken and phenyl hydrazine (41.54 ml, 0.385 mol) was added slowly with stirring. The reaction mixture was refluxed on a heating mantle for 2 h. The completion of reaction was checked via TLC. The reaction mixture was cooled and diethyl ether was slowly added with stirring. After 10 min, light yellow colored precipitate appeared which was filtered and washed with ether. Pure white colored 3-methyl-1-phenyl-pyrazol-5-one (1) was then recrystallized with hot aqueous ethanol.

Mp = 127 °C. Yield = 60.20 g (90%). ^1H NMR (300 MHz, $CDCl_3$, δ ppm); d 2.20 (s, 3H, CH_3), d 3.44 (s, 2H, CH_2), d 7.18–7.87 (m, 5H, Ar—H).

4.1.2. Synthesis of 5-(2-bromo-ethoxy)-3-methyl-1-phenyl-1H pyrazole (2)

A mixture of compound 1(2.60 g, 11.2 mmol), anhydrous K_2CO_3 (2.31 g, 16.8 mmol) in 10 ml DMF was stirred for half an hour. 1,2-dibromoethane (4.8 mL, 56 mmol) was added and stirred at room temperature for 24 h. After completion of reaction (evident by TLC), solvent was evaporated under reduced pressure and the residue was dissolved in chloroform. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography with chloroform/ethyl acetate as eluent to afford **2** as yellow powder.

Yellow powder (3.00 g, 79%); R_f = 0.38 (Hexane/EtOAc, 9:1); mp. 48–50 °C; ^1H NMR (300 MHz, $CDCl_3$, δ ppm); 2.27 (3H, s, $C\underline{H}_3$), 3.13 (2H, t, $C\underline{H}_2$, J = 6.0 Hz,), 4.37 (2H, t, $OC\underline{H}_2$, J = 6.3 Hz,), 5.50 (1H, CH pyrazole) 7.23–7.70 (5H, m, Ar $\underline{H}$). MS (m/z): 281 (M++1). Anal. Calcd for $C_{12}H_{13}BrN_2O$ (280.02): C, 51.26; H, 4.66; N, 9.96. Found: C, 51.62; H, 4.52; N, 9.61.

4.1.3. Synthesis of 4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)-ethoxy]-benzaldehyde (3)

A mixture of p-hydroxybenzaldehyde, (2.34 g, 19.25 mmol), and anhydrous K_2CO_3 (4.04 g, 28.8 mmol) in 10 ml DMF was stirred for half an hour. Compound **2** (5.41 g, 19.25 mmol) was added and stirred at room temperature for 24 h. After completion of reaction (evident by TLC), the solvent was concentrated, and the residue was dissolved in chloroform. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography with hexane/ethyl acetate as eluent to afford **3**.

Yield, (3.00 g, 49%); R_f = 0.70 (Hexane/EtOAc, 9:1); mp. 88–90 °C; ^1H NMR (300 MHz, $CDCl_3$, δ ppm); 2.28 (3H, s, $C\underline{H}_3$), 4.41-4.45 (4H, m $C\underline{H}_2C\underline{H}_2$), 5.57 (1H, s, C$\underline{H}$ pyrazole), 7.00-7.85 (9H, m, Ar—$\underline{H}$) 9.90 (1H, s, C$\underline{H}$O). ^{13}C NMR ($CDCl_3$, δ ppm); 14.5($\underline{C}H_3$), 66.1($O\underline{C}H_2$) 70.0($O\underline{C}H_2$), 86.8(C=C of pyrazole), 114.8(Ar$\underline{C}$), 121.7(Ar$\underline{C}$), 125.8(Ar$\underline{C}$), 128.7(Ar$\underline{C}$), 130.3(Ar$\underline{C}$), 131.9, (Ar$\underline{C}$), 138.5(Ar$\underline{C}$),, 148.6(Ar$\underline{C}$), 154.0(pyrazole $\underline{C}$), 163.2(Ar$\underline{C}$), 190.5($\underline{C}$HO). MS (m/z): 323 (M++1). Anal. Calcd for $C_{19}H_{18}N_2O_3$ (322.36): C, 70.80; H, 5.59; N, 8.70%. Found: C, 70.62; H, 5.52; N, 8.61.

4.1.4. Synthesis of {4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)-ethoxy]-phenyl}-methanol (4)

A mixture of compound **3** (0.20 g, 0.62 mmol), and $NaBH_4$ (0.012 g, 0.31 mmol) in 10 ml methanol was refluxed for 15 min. After completion of reaction (evident by TLC), the reaction mixture was concentrated to leave residue which was crystallized from ethanol to afford compound **4**.

Yield, (0.10 g, 40%); R_f = 0.50 (Hexane/EtOAc, 9:1); mp. 120 °C; ^1H NMR (300 MHz, $CDCl_3$, δ ppm); 2.28 (3H, s, $C\underline{H}_3$), 4.40–4.43 (4H, m CH_2 CH_2), 4.63 (2H, d, CH_2, J = 5.4 Hz), 5.56(1H, s, CH pyrazole), 6.90–7.61(9H, m, Ar—H). ^{13}C NMR ($CDCl_3$, δ ppm); 14.4 ($\underline{C}H_3$) 62.5($O\underline{C}H_2$), 65.9($O\underline{C}H_2$), 70.7($O\underline{C}H_2OH$), 87.3(C=C of pyrazole), 115.3(Ar$\underline{C}$), 120.9(Ar$\underline{C}$), 125.6(Ar$\underline{C}$), 128.0(Ar$\underline{C}$), 128.9(Ar$\underline{C}$), 135.0(Ar$\underline{C}$), 138.5(Ar$\underline{C}$), 148.0(Ar$\underline{C}$), 155.1(Ar$\underline{C}$), 157.1(Ar$\underline{C}$). MS (m/z): 325 (M++1). Anal. Calcd for $C_{19}H_{20}N_2O_3$ (324.37) C, 70.37; H, 6.17; N, 8.64. Found: C, 69.98; H, 6.02; N, 8.51.

4.1.5. General method for synthesis of {4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)]-benzylidine}-aryl-amine

A mixture of compound **3** (0.5 g, 1.55 mmol), and appropriate aryl amine (0.14 g, 1.55 mmol) was refluxed in 10 ml of dichloromethane (DCM) with stirring. After completion of reaction (evident by TLC), the reaction mixture was concentrated to leave oily residue which was crystallized from ethanol to afford compound **5a**–**5c**.

4.1.5.1. {4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)]-benzylidine}-phenyl-amine (5a). Yield, (0.18 g, 36%); R_f = 0.52 (Hexane/EtOAc, 9:1); mp. 88 °C; (Major product) ^1H NMR (300 MHz, $CDCl_3$, δ ppm); 2.29 (3H, s, $C\underline{H}_3$); 4.38-4.45 (4H, m, $C\underline{H}_2C\underline{H}_2$), 5.57 (1H, s, C$\underline{H}$ pyrazole), 6.97-8.39 (14H, m, Ar—$\underline{H}$). ^{13}C NMR ($CDCl_3$, δ ppm); 14.5($\underline{C}H_3$), 66.0($O\underline{C}H_2$), 70.3($O\underline{C}H_2$), 86.8(C=C of pyrazole), 114.8(Ar$\underline{C}$), 120.8(Ar$\underline{C}$), 120.8(Ar$\underline{C}$), 125.6(Ar$\underline{C}$), 125.9(Ar$\underline{C}$), 128.7(Ar$\underline{C}$),

129.1(ArC), 129.8(ArC), 130.5(ArC), 138.6(ArC), 148.7(ArC), 152.2(ArC), 155.2(ArC), 159.4(ArC), 160.9(ArC). MS (*m*/*z*): 398 (M++1). Anal. Calcd for $C_{25}H_{23}N_3O_2$(397.18) C, 75.57; H, 5.79; N, 10.58. Found: C, 73.62; H, 5.49; N, 10.48.

4.1.5.2. {4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)-ethoxy]-benzylidine}-p-tolylamine (5b). Yield, (0.29 g, 55%); R_f = 0.54 (Hexane/EtOAc, 9:1);mp 146 °C; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm);; 2.29 (3H, s, CH$_3$); 2.37 (3H, s, CH$_3$), 4.38–4.45 (4H, m, CH_2CH_2), 5.57 (1H, s, CH pyrazole), 6.97–8.39 (13H, m, Ar—H). ^{13}C NMR ($CDCl_3$, δ ppm); 14.5(CH$_3$), 20.9, 66.0, 70.3, 86.8(C=C of pyrazole), 114.8(ArC), 120.7(ArC), 121.8(ArC), 125.9(ArC), 128.7(ArC), 129.7(ArC), 130.0(ArC), 130.4(ArC), 135.4(ArC), 138.6(ArC), 148.7(ArC), 149.6, 154.4, 158.6, 160.8. MS (*m*/*z*): 412 (M++1). Anal. Calcd for $C_{26}H_{25}N_3O_2$ (411.19) C, 75.91; H, 6.08; N, 10.21. Found: C, 75.52; H, 5.90; N, 10.01.

4.1.5.3. (4-Methoxy-phenyl)-{4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)-ethoxy] benzylidine}-amine (5c). Yield, (0.25 g, 67%); R_f = 0.57 (Hexane/EtOAc, 9:1);mp 138 °C; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm); 2.29 (3H, s, CH$_3$), 3.83 (3H, s, OCH$_3$), 4.39–4.43 (4H, m CH_2CH_2), 5.57 (1H, s, CH pyrazole), 6.91–8.41 (13H, m, Ar–H). ^{13}C NMR ($CDCl_3$, δ ppm); 14.5(CH$_3$), 55.4, 66.0, 70.3, 86.8(C=C of pyrazole), 114.7(ArC), 114.8(ArC), 121.8–122.0(ArC), 125.9(ArC), 128.7(ArC), 130.1–130.2(ArC), 132.0(ArC), 138.6(ArC), 145.1(ArC), 148.7(ArC), 155.2, 157.6, 158.0, 160.7, 177.8. MS (*m*/*z*): 428 (M++1).). Anal. Calcd for $C_{26}H_{25}N_3O_3$ (427.19) C, 73.07; H, 5.85; N, 9.84. Found: C, 72.92; H, 5.63; N, 9.71.

4.1.6. Synthesis of 4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)-ethoxy]-benzaldehyde-oxime (6)

To solution of compound **3** (0.20 g, 0.62 mmol) in ethanol (7 mL), hydroxylamine hydrogensulfate (0.143 g, 0.87 mmol), was added and the mixture was stirred for half an hours. Sodium hydroxide (0.004 g) in 5 mL water was added in portion over 5 min. The reaction mixture was refluxed for 4 h, after completion of reaction (evident by TLC), the reaction mixture was cooled and poured into HCl–water (5:1). Oxime crystal formed was filtered and washed with water.

Yield, (0.13 g, 65%); mp 183 °C; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm);; 2.16 (3H, s, CH$_3$), 4.48-4.46 (4H, m, CH$_2$CH$_2$), 5.78(1H, s, CH, *J* = 9 Hz), 6.99–7.63 (9H, m, Ar—H), 8.47(1H, s, CH). ^{13}C NMR ($CDCl_3$, δ ppm); 15.3(CH$_3$), 66.0(OCH$_2$), 70.6, 87.3(C=C of pyrazole), 114.9, 120.9, 125.6, 126.0, 127.9, 128.9, 138.4, 147.5, 147.9, 154.0, 159.0. MS (*m*/*z*): 338 (M++1). Anal. Calcd for $C_{19}H_{19}N_3O_3$ (337.14) C, 67.67; H, 5.64; N, 12.46. Found: C, 67.45; H, 5.40; N, 12.36.

4.1.7. Synthesis of {4-[2-(5-Methyl-2-phenyl-2H-pyrazol-3-yloxy)-ethoxy]-benzylidine}-hydrazine (7)

A mixture of hydrazine hydrate (0.062 ml, 1.24 mmol), 1 ml acetic acid in 0.5 ml water was stirred for 10 min. Compound **3** (0.02 g, 0.62 mmol) was added to reaction mixture and refluxed for 4 h. After completion of reaction (evident by TLC), the reaction mixture was concentrated and crystallized from ethanol to afford compound **7**.

Yield, (0.13 g, 65%); mp 220 °C.^{1}H NMR (300 MHz, $CDCl_3$, δ ppm); 1.2 (2H, s, NH_2), 2.29 (3H, s, CH$_3$), 4.40–4.46 (4H, m, CH_2CH_2), 5.57(1H, s, CH pyrazole), 6.96–7.85 (9H, m, Ar—H), 8.61(1H, s, CH). ^{13}C NMR ($CDCl_3$, δ ppm); 14.5(CH$_3$), 66.6(OCH$_2$), 70.8, 87.7(C=C of pyrazole), 115.4(ArC), 121.4(ArC), 126.1(ArC), 129.2(ArC), 130.1(ArC), 132.2(ArC), 138.5(ArC), 148.5(ArC), 154.4, 163.4, 191.9. MS (*m*/*z*); 337 (M++1). Anal. Calcd for $C_{19}H_{20}N_4O_2$ (336.16) C, 67.86; H, 5.95; N, 16.67. Found: C, 67.43; H, 5.60; N, 16.31.

4.1.8. Synthesis of 4-(bis-methylsulfanyl-methylene)-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one (9)

In a 500-ml round-bottom flask, fitted with guard tube, anhydrous K_2CO_3 (59.48 g, 0.431 mol) was taken, and 20 ml DMF and 40 ml dry benzene were added and stirred in an ice bath for 1 h. In another 500-ml round-bottom flask, fitted with guard tube (1) (30.0 g, 0.172 mol) was taken and 20 ml DMF was added. It was stirred for 15 min. Then, carbon disulfide (10.40 ml, 0.172 mol) was added slowly with stirring. It was also stirred for 1 h in an ice bath. Then, the content of first round bottom flask was added to second round bottom flask with stirring and the reaction mixture was stirred for 6 h. With the use of a dropping funnel, methyl iodide (21.46 ml, 0.345 mol) was added very slowly along with 10 ml dry benzene. The addition of methyl iodide was accompanied with the use of ice bath. After complete addition, the reaction was stirred for 6 h. The completion of reaction was checked by TLC. Reaction was worked up. Solvents were removed under pressure through rotary evaporator and the reaction mixture was extracted with CHCl3/H2O (200/200 9 2 ml). The $CHCl_3$ layer was dried with anhydrous Na_2SO_4 and filtered. Chloroform was removed and the product was purified via SiO_2-column chromatography. Eluent used was 20% ethyl acetate in hexane.

Mp = 46–52 °C. Yield = 40.5 g (85%). ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm); 2.52 (s, 3H, CH_3), 2.69 (s, 3H, SCH_3), 2.76 (s, 3H, SCH_3), 7.12–7.99 (m, 5H, Ar—H).

4.1.9. Synthesis of 3-methyl-1-phenyl-5-(toluene-4-sulfonyloxy)-1H-pyrazole-4 carboxylic acid methyl ester (10)

A mixture of compound **9** (1.0 g, 3.9 mmol), anhydrous K_2CO_3 (0.85 g, 5.86 mmol) in 10 ml DMF was stirred for half an hour. After half hour 4-methylbenzene-1-sulfonyl chloride (0.82 g, 3.9 mmol) was added and stirred at room temperature for 24 h. After completion of reaction (evident by TLC), the reaction mixture was concentrated and the residue was dissolved in chloroform. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography with hexane/ethylacetate as eluent to afford **10**.

Yield, (0.62 g, 49.5%); mp. 88–90 °C; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm); 2.36 (3H, s, CH$_3$), 2.50 (3H, s, CH$_3$), 3.75 (3H, s, OCH$_3$), 7.04–7.07(2H, d, Ar—H, *J* = 6 Hz), 7.24–7.26 (5H, m, Ar—H), 7.44–7.77(1H, s, Ar—H). ^{13}C NMR ($CDCl_3$, δ ppm); 12.1, 14.6, 21.2, 50.9, 91.1, 122.1, 124.0, 125.8, 128.7(ArC), 129.2(ArC), 129.8(ArC), 139.1(ArC), 145.3, 156.6. MS (*m*/*z*): 387 (M++1). Anal. Calcd for $C_{19}H_{18}N_2O_5S$ (387.09) C, 59.06; H, 5.08; N, 7.91. Found: C, 60.13; H, 4.99; N, 7.39.

4.2. Pharmacology

4.2.1. In vitro assays for cyclooxygenase 1 and 2

COX-1 has been isolated from Rat seminal vesicles. Recombinant human COX-2 has been expressed in insect cell expression system. These enzymes have been purified by employing conventional chromatographic techniques. Enzymatic activities of COX-1 and COX-2 were measured according to the method of Copeland et al. [55], with slight modifications using a chromogenic assay based on the oxidation of N,N,N,N,-tetramethyl-4-phenylene diamine (TMPD) during the reduction of PGG_2 to PGH_2 [56,57]. Briefly, the assay mixture contained TriseHCl buffer (100 mM, Ph 8.0), hematin (15 mM), EDTA (3 mM), enzyme (100 mg COX-1 or COX-2) and the test compound. The mixture was pre-incubated at 25 °C for 1 min and then the reaction was initiated by the addition of arachidonic acid and TMPD, in total volume of 1 ml. The enzyme activity was determined by estimating the velocity of TMPD oxidation for the first 25 s of the reaction by following the increase in absorbance at 603 nm. A low rate of non enzymatic oxidation

observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition.

4.2.2. In vivo anti-inflammatory activity

All experiments have been conducted on adult Wistar strain albino rats of either sex, weighing between 150 and 200 g. The animals were obtained from the Central Animal House of the Institute of Medical Sciences, B.H.U. They were kept in colony cages under identical housing conditions at an ambient temperature of 25 ± 2 °C and 45–55% relative humidity with a 12 h light–dark cycle in the departmental animal room and fed on standard diet. Animals were acclimatized for a week before use.

4.2.3. Experimental model of Inflammation

Carrageenin Induced Paw Edema in Rats: Target compounds were suspended in aqueous solution of carboxymethyl cellulose (CMC, 1% w/v) and administered orally at a dose level of 10 mg/kg of NSAID drugs Nimesulide and their equivalent doses of the target derivatives (**3–7, 10**). After 15 min control animals were similarly treated with aqueous solution of carboxymethyl cellulose (CMC, 1% w/v). One percent carrageenin suspension was prepared as a homogeneous solution in distilled water. A volume of 0.1 ml of carrageenin solution was injected through a 26 gauge needle into the plantar surface of the left hind paw below the plantar aponeurosis. The volume of paw was measured before and at different intervals for 2 h after injection of carrageenin. The difference in paw volume before and after administration of the phlogistic agent was taken as the measure of pedal edema.

4.2.4. Measurement of paw volume

The volume of hind paw of the rats up to the ankle joint was measured plathysmographically by the mercury displacement method. The ankle joint of the rats was marked with a skin marking pencil and the paw was dipped in the mercury, so that the mark on the paw coincides with a prefixed line kept constant on the syringe. The level of the mercury was every time brought to the level of this line by adjusting the height of the displaced mercury. The difference in the paw volume before and after injection of the phlogistic agents was taken as a measure of pedal edema. The change in paw volume was expressed in "ml" of mercury displaced.

4.2.5. Statistical analysis

The difference in the means between each experimental group was first analyzed by Analysis of Variance (ANOVA). When the overall ANOVA was significant ($p < 0.05$), the data was further subjected to statistical analysis by the student's *t*-test.

4.3. Molecular docking

Molecular docking studies of pyrazole derivatives was carried out in the active sites of COX-2 in order to get the nature of interactions between the molecule and the active site amino acids using the docking program Autodock 4.2 [58]. The PDB structure 3LN1 (resolution 2.2 Å) was used as a receptor for docking the molecules. Firstly, all bound water, ligands, and cofactors were removed from the proteins. The macromolecule was checked for polar hydrogen; torsion bonds of the inhibitors were selected and defined. Gasteiger charges were computed and the AutoDock atom types were defined using AutoDock 4.2, graphical user interface of AutoDock supplied by MGL Tools [59]. The Lamarckian Genetic Algorithm (LGA), which is considered one of the best docking methods available in AutoDock [60,61], was employed. This algorithm yields superior docking performance compared to simulated annealing or the simple genetic algorithm and the other search algorithms available in AutoDock 4.2. Secondly, the three dimensional grid boxes were created by AutoGrid algorithm to evaluate the binding energies on the macromolecule coordinates. Ligand PDB were prepared using ChemBio3D. The grid maps representing the intact ligand in the actual docking target site were calculated with AutoGrid (part of the AutoDock package). Eventually cubic grids encompassed the binding site where the intact ligand was embedded. Finally, AutoDock was used to calculate the binding free energy of a given inhibitor conformation in the macromolecular structure while the probable structure inaccuracies were ignored in the calculations. The search was extended over the whole receptor protein used as blind docking. Nimesulide (Native Ligand) in the crystal structure was docked as reference.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2014.05.004.

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