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Original article

Design, synthesis and pharmacological screening of novel nitric oxide donors containing 1,5-diarylpyrazolin-3-one as nontoxic NSAIDs

Shashikant V. Bhandari*, Sudarshan C. Dangre, Kailash G. Bothara, Ajit A. Patil, Aniket P. Sarkate, Deepak K. Lokwani, Suraj T. Gore, Bhavana J. Deshmane, Vyankatesh T. Raparti, Chetan V. Khachane

AISSMS College of Pharmacy, Near RTO, Kennedy Road, Pune 411001, Maharashtra, India

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ABSTRACT

Various substituted 1,5-diarylpyrazol-3-one derivatives were synthesized and screened for analgesic, anti-inflammatory activities, ulcerogenic potential and for their ability to release nitric oxide. Most compounds exhibited significant analgesic and anti-inflammatory activities. It was interesting to note that out of ten compounds, **7j** (59.64%) was found to have anti-inflammatory activity greater than the standard drug Indomethacin (57.89%), whereas compound **7b** (57.89%) was found to be equipotent to that of standard, Indomethacin. The pharmacological studies suggested that the presence of 4-nitro and 2-methoxy on phenyl ring at C_5 of pyrazole has a significant anti-inflammatory activity and 4-chloro substitution on same phenyl ring was found to have decreased activity. However only a phenyl substituted derivative was found to have most potent activity. Compound **7j** containing plane phenyl at C_5 of pyrazole was found to have significant analgesic activity (56.86%) in acetic acid induced writhing model. Compounds **7d** and **7i** having 4-chloro substituted phenyl ring showed least analgesic activity (10.78%) and (6.86%) respectively. The compounds also showed significantly reduced GI-ulcerogenicity and gastroprotective results in histopathological studies i.e. they were found to be causing no mucosal injury. All the synthesized compounds were found to exhibit significant nitric oxide releasing activity, in both in vitro and in vivo models. Molecular docking studies served to be an important tool for the study of binding of compounds with that of a COX-2 enzyme. The results of the docking studies were found to endorse the result of experimental work. Thus, the rationale used to design the NCEs was found to produce the promising results as anticipated. Therefore it can be said that the strategy employed can serve as an important tool in future for the design and development of novel therapeutic agents of various categories too.

1. Introduction

The NSAIDs are among the most widely used of all therapeutic agents. They are useful in the treatment of rheumatoid arthritis and some inflammatory diseases. However, long term use of the NSAIDs has been associated with gastrointestinal (GI) toxicities viz. ulceration, bleeding and nephrotoxicity [1]. Therefore investigation of new anti-inflammatory agents is still a major challenge [2–4]. Pyrazolinone is an important pharmacophore which exhibits widespread pharmacological properties such as analgesic, antipyretic, antiphlogistic, antirheumatic, antiarthritic and uricosuric activities [5]. The prostaglandin (PG) synthase (cyclooxygenase), the key enzyme of inflammatory process, and an important target of most of the currently used NSAIDs, exists in two isoforms (COX-1 and COX-2) [6]. While COX-1 plays a cytoprotective role [7], COX-2, induced at the time of injury, causes inflammation, pain, and fever [8]. Thus, conventional non-steroidal anti-inflammatory drugs (NSAIDs), being inhibitors of both, exhibit anti-inflammatory activity along with gastrointestinal (GI) toxicity on extended use [9]. But, the selective COX-2 inhibitors, viz. Nimesulide [10], Celecoxib [11] Rofecoxib [12] Valdecoxib [13], and Etoricoxib [14], treat the chronic rheumatoid and osteoarthritis without causing GI damage. A few COX-2 inhibitors have also been studied for the treatment of cancer [15] and Alzheimer's disease [16]. However a mild cardiac toxicity associated with COX-2 inhibitors (COXIBs) has raised a cautionary flag on this research [17]. So, it is desirable to discover safe, potent, selective and patient-acceptable COX-2 inhibitors to completely abandon the use of steroidal and narcotic drugs.

Recent strategies adopted to minimize the side effects of NSAIDs include the use of the dual LOX/COX inhibitors and the use of hybrid

* Corresponding author. Tel.: +91 9423574082; fax: +91 20 26058208.
E-mail address: drugdesign1@gmail.com (S.V. Bhandari).

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approach i.e. molecule made up of non-selective or selective COX inhibitors together with a vasodilator (nitric oxide releasing) function [18,19]. Recent data revealed serious cardiovascular side effects associated with selective COX-2 inhibitors like COXIBs [17]. The strategy involving the use of hybrid molecules made up of non-selective COX-2 inhibitors together with a nitric oxide donating moiety constitutes one of the most promising approaches, because nitric oxide supports several endogenous GI defence mechanisms, including increase in mucus, bicarbonate secretions, increase in mucosal blood flow and inhibition of the activation of pro-inflammatory cells. Moreover, because of the beneficial cardiovascular effects of NO, such drugs are expected to be devoid of the potential adverse cardiovascular effects associated with the use of selective COX-2 inhibitors. Among those NO-NSAIDs that came into clinical trials are Nitroaspirin, Nitronaproxene, Nitroketoprofen, Nitroibuprofen, etc. [20].

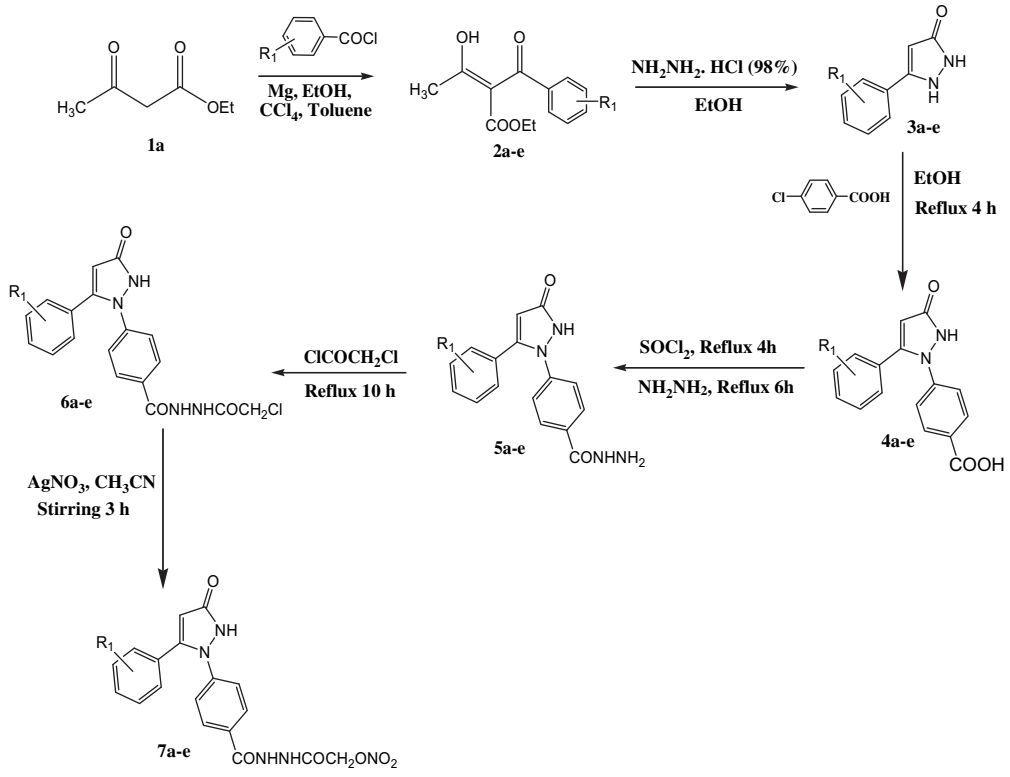
There are several reviews which are mainly focused on the molecular and functional bases of the inhibition of COX enzymes by non-selective and COX-2 selective inhibitors [21,22]. Therefore as an attempt to optimize the pharmacophore requirement for potent, nontoxic NO-NSAIDs, we thought worth optimizing structural requirement for COX-2 binding pocket and to get rid of cardiovascular toxicities associated with COXIBs by incorporating nitric oxide (NO) releasing function (vasodilator) onto COX-2 selective pharmacophore.

In this paper we report structure optimization, design, synthesis and pharmacological screening of some 1,5-diarylpyrazolin-3-one pharmacophore containing compounds. We have carried out docking studies using Glide 4.5 [23], docking programme to identify the binding modes of synthesized derivatives required for the potential analgesic and anti-inflammatory activities. The crystal structure was downloaded from RCSB protein data bank (PDB ID: 1CX2) [24].

2. Results and discussion

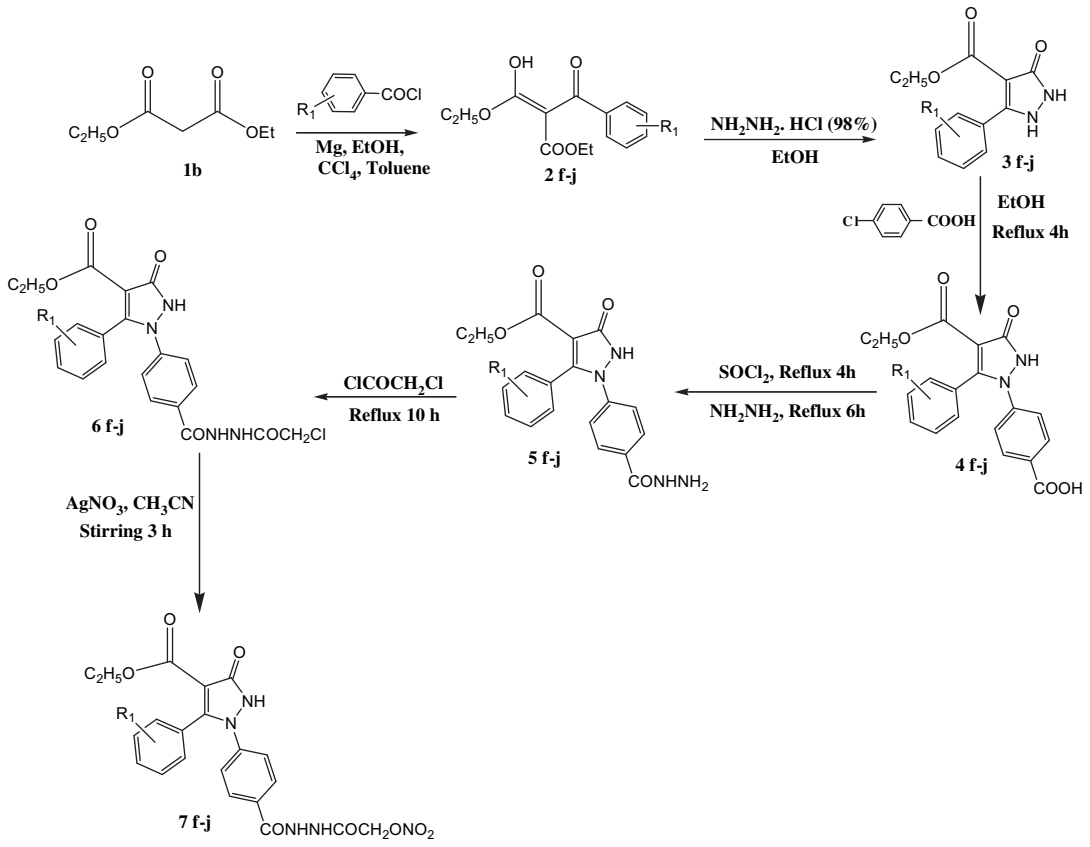
2.1. Chemistry

The synthetic route used to synthesize title compounds is outlined in Scheme 1 and Scheme 2. The α-acylated esters (**2a**–**2j**) could be synthesized, in high yields, by the treatment of ethyl acetoacetate (**1a**) or diethylmalonate (**1b**) with the appropriate acylchloride in the presence of magnesium/ethanol in anhydrous toluene. Further pyrazolinones (**3a**–**3j**) were easily and cleanly synthesized from (**2a**–**2j**) by treatment with hydrazine HCl (98%) in ethanol at room temperature. In next step 4-(3-oxo-5-substituted phenyl-2,3-dihydropyrazol-1-yl)benzoic acids (**4a**–**4j**) were synthesized by refluxing 4-chloro benzoic acid with (**3a**–**3j**) for 4 h. The intermediates 4-(3-oxo-5-substituted phenyl-2,3-dihydropyrazol-1-yl)benzohydrazides (**5a**–**5j**) were further synthesized from (**4a**–**4j**) by refluxing thionyl chloride and hydrazine hydrate for 10 h. Refluxing (**5a**–**5j**) with



Compound code	1a	-R_1
7a	Ethyl acetoacetate	-4-NO_2
7b	Ethyl acetoacetate	-2-OCH_3
7c	Ethyl acetoacetate	-2-OH
7d	Ethyl acetoacetate	-4-Cl
7e	Ethyl acetoacetate	-H

Scheme 1. Synthesis of (**7a**–**7e**) using ethyl acetoacetate.



Compound code	1b	-R_1
7f	Diethyl malonate	-4-NO_2
7g	Diethyl malonate	-2-OCH_3
7h	Diethyl malonate	-2-OH
7i	Diethyl malonate	-4-Cl
7j	Diethyl malonate	-H

Scheme 2. Synthesis of (**7f**–**7j**) using diethylmalonate.

chloroacetylchloride in the presence of dry benzene and ethylamine yielded *N*′-(2-chloroacetyl)4-(3-oxo-5-substituted phenyl-2,3-dihydropyrazol-1-yl)benzohydrazides (**6a**–**6j**). Finally the terminal chloro-function in (**6a**–**6j**) is then converted to target compounds (**7a**–**7j**) with $AgNO_3$ and acetonitrile. The structures of various synthesized compounds were assigned on the basis of different chromatographic and spectral studies. The physical data, FTIR, ^1H NMR and mass spectral data for all synthesized compounds are reported in experimental protocols.

The FTIR spectra of the final compounds exhibited very similar features and showed the expected bands for the characteristic groups which are present in the compounds such as C–H and the C═N stretching vibrations and another specific stretch for two Aromatic rings in the range of 3010–3083. Compounds (**7a**–**7j**) have C═O stretching bands in the range 1678–1783. Similarly the compounds also exhibit the symmetric and asymmetric stretch for NO_2 in the range of 1260–1512. In the proton NMR spectral data, all protons were seen according to the expected chemical shift and integral values. The aromatic protons appeared as multiplet peaks within the range 7.4–8.2 δ ppm, singlet signals derived from secondary amine of pyrazolinone ring appeared at 6.2 δ ppm. Ethyl protons resonated as singlet at 4.6 δ ppm.

The detailed results of various physicochemical studies are presented in experimental protocols.

2.2. Pharmacology

In the pharmacological studies, we have investigated anti-inflammatory and analgesic activities as well as the acute ulcerogenicity of 1,5-diarylpyrazolin-3-ones derivatives (**7a**–**7j**). Indomethacin was used as a reference standard. The experiments were performed on albino rats of Wistar strain of either sex,

weighing 120–140 g. The animals were maintained at 25 ± 2 °C, 50 ± 5% relative humidity and 12 h light/dark cycle. The animals were fasted for 24 h prior to the experiments and water provided ad libitum. The test compounds were suspended in 1% aqueous carboxymethyl cellulose (CMC) solution and administered orally to experimental animals.

2.2.1. Anti-inflammatory activity

Anti-inflammatory activity of the synthesized compounds was evaluated by carrageenan induced rat paw oedema model, at equimolar doses, to that of 6 mg/kg p.o of Indomethacin [25]. Subplantar injection of 0.1 mL, 1% carrageenan produced increase in paw volume (oedema) in all the animals of various groups. The onset of action was evident from 1 h in various test groups. The significant reduction ($P < 0.01$) of rat paw oedema was observed by most of the test compounds at 3 h compared to control group (Table 1).

2.2.2. Analgesic activity

The analgesic activities of the compounds were studied by using acetic acid induced writhing test in mice. The analgesic activity was evaluated at equimolar doses equivalent to 6 mg/kg p.o (Indomethacin) body weight. These compounds exhibited an important analgesic profile measured by the classical acetic acid induced writhing model. From the results of acetic acid induced writhing test, it was noticed that all compounds exhibited significant analgesic activity (Table 2). The analgesic effects of (**7j**) (56.86%) were found to be better than that of Indomethacin (54.90%).

2.2.3. Acute ulcerogenicity studies

Compounds with significant anti-inflammatory profile were subjected to ulcerogenicity potential test at 12 times the therapeutic dose of diclofenac with additional physical (cold) stress for 2 h at −20 °C. A thorough examination of the results of histopathological studies indicated the absence of the disruption of gastric epithelial morphology and absence of ulcers/erosion in test group animals compared to reference standard, Diclofenac acid, and control group animals. The results of the ulcerogenicity studies are presented in Table 3 and results of histopathological studies are depicted in Fig. 1a–f.

Ulcerogenic effect of 1,5-diarylpyrazolin-3-one derivatives (**7a**, **7b**, **7g**, **7j**) in animal efficacy model was evaluated for gastric ulcerogenic potential in rat stress model. When compared with Diclofenac acid, these four compounds did not cause any gastric ulceration and disruption of gastric epithelial cells at the above-mentioned oral doses. Hence gastric tolerance to these compounds was better than that of Diclofenac acid. This led us to conclude that because of the presence of NO releasing moiety vasodilation, mucous production and in turn gastrointestinal protection occur. Thus, such functionalization of free –COOH of Diclofenac to pyrazolinone ring and presence of additional NO releasing function have resulted into more potent and nontoxic New Chemical Entities (NCEs). The results of ulcerogenicity studies are presented in Table 3.

2.2.4. Histopathological studies

The stomach specimen of diclofenac acid treated rats was characterized by complete disruption of protective mucosal layer (Fig. 1 specimen b). Histopathological analyses also showed characteristic features of ulceration in diclofenac acid treated group of animals. The tissue of diclofenac acid treated rats has shown that some epithelial cells in the ulcer margin had proliferated and migrated over and into the ulcer crater, which was strongly infiltrated by inflammatory cells, fibroblasts and endothelial cells indicating complete disruption of gastric epithelial layer. Scanning of stomach specimens using electron microscope revealed that in the rats treated with 1,5-diarylpyrazolinone derivatives (**7a**, **7b**, **7g**, **7j**) there was no injury observed in stomach mucosa. As illustrated in Fig. 1, specimens c–f which are identical to that of the control, specimen a.

2.2.5. Vasorelaxing activity

In isolated Wistar rat aorta rings, compounds **7a–7j** competitively inhibited norepinephrine-induced contraction effects, causing a shift to the right of the norepinephrine concentration response curves. EC_{50} (μg/mL) values were calculated from the cumulative concentration (dose) response curves. In order to prove the involvement of nitric oxide in the relaxation process, the abilities of the test compounds to release NO were ensured by assessing nitric oxide releasing properties of synthesized compounds in phosphate buffer, pH 7.4, in the presence of L-cysteine, relative to nitric oxide released from standard sodium nitrite solution (Table 4).

3. Docking studies

The molecular docking tool, Glide (Schrödinger Inc. USA) software was used for ligand docking studies in the enzyme (COX-2) binding pocket (PDB Code 1CX2) [26].

Glide is one of the most accurate docking programmes available for ligand–protein, protein–protein binding studies. Glide was found to produce least number of inaccurate poses and 85% of Glides binding models had an RMSD of 1.4 Å or less from native co-crystallized structures [27]. Glide is validated software designed for calculating the accurate binding interaction energies of the 3D structures of a known receptor protein with ligand or another protein molecule [28].

Table 1
Results of anti-inflammatory activity of title compounds (**7a–7j**) against carrageenan induced rat paw oedema model in rats.

Mean reduction in paw volume (mL) after treatment with test compounds (mean ± SEM)				Anti-inflammatory activity (% inhibition)		
Compounds	1 h	2 h	3 h	1 h	2 h	3 h
Control	1.868 ± 0.07	1.922 ± 0.07	1.71 ± 0.04	–	–	–
Indomethacin	0.888 ± 0.04	0.808 ± 0.07	0.7224 ± 0.09	52.68**	58.33**	57.89**
7a	1.436 ± 0.03	1.402 ± 0.03	1.3 ± 0.05	23.56**	27.08**	23.97**
7b	0.846 ± 0.06	0.85 ± 0.05	0.728 ± 0.08	55.08**	55.72**	57.89**
7c	1.444 ± 0.04	1.556 ± 0.01	1.43 ± 0.03	22.99**	19.27**	16.37*
7d	1.764 ± 0.06	1.704 ± 0.04	1.616 ± 0.04	5.88ns	11.45ns	5.84ns
7e	1.504 ± 0.08	1.614 ± 0.07	1.368 ± 0.05	19.78**	16.14**	20.46**
7f	1.35 ± 0.04	1.388 ± 0.08	1.332 ± 0.05	27.80**	28.12**	22.22**
7g	1.296 ± 0.01	1.33 ± 0.02	1.288 ± 0.02	31.01**	30.72**	25.14**
7h	1.356 ± 0.04	1.412 ± 0.02	1.354 ± 0.04	27.80**	26.56**	21.05**
7i	1.8126 ± 0.07	1.724 ± 0.04	1.604 ± 0.05	3.20ns	10.41ns	6.43ns
7j	0.86 ± 0.08	0.814 ± 0.08	0.69 ± 0.07	54.01**	57.81**	59.64**

Data analyzed by one-way ANOVA followed by Dunnett's test, ($n = 6$), *$P < 0.05$, **$P < 0.01$ significant from control; ns, not significant.

Table 2
Results of analgesic activity of title compounds (**7a**–**7j**) against acetic acid induced writhing tests in mice.

Compounds	Dose (mg/kg, p.o) (0.00001911 mol/kg)	No. of writhes in 25 min after treatment (mean ± SEM)	% Inhibition
Control	–	20.4 ± 0.74	–
Standard	6.0	9.2 ± 0.37	54.9**
7a	7.41	11.2 ± 0.58	45.09**
7b	7.16	9.80 ± 0.66	51.96**
7c	6.93	17 ± 0.83	16.66*
7d	7.24	18.2 ± 1.02	10.78^{ns}
7e	6.66	9.4 ± 0.6	53.92**
7f	8.62	11.2 ± 0.86	45.9**
7g	8.37	10 ± 0.7	50.98**
7h	8.13	18.2 ± 1.15	10.78^{ns}
7i	8.44	19 ± 0.83	6.86^{ns}
7j	15.73	8.8 ± 0.66	56.86**

Data analyzed by one-way ANOVA followed by Dunnett's test, ($n = 6$), $^{**}P < 0.01$ significant from control.

The Glide docking programme approximated a complete systematic search of the conformational, orientational and positional space of the docked ligand molecules into the receptor (protein) binding pocket [29].

The results of molecular docking studies of 1,5-diarylpyrazolin-3-one containing compounds are presented in (Table 5).

The docking results were evaluated based on following aspects.

3.1. G score (Glide Score)

The Gscore of the standard compounds Celecoxib, SC-558, Indomethacin, Ibuprofen were found to be −11.37, −8.36, −7.37 and −4.38 respectively. While the G score for the designed series of compounds **7a**–**7c** and **7h** were found to be −9.10, −9.37, −9.26 and −8.90 respectively. While the G score for compounds **7d**–**7h** and **7j** were found to be −8.41, −8.38, −8.10, −8.00, −7.80 and −7.77 respectively.

The close analysis of those results suggests that the designed series of compounds have G score comparable with that of a standard compound Celecoxib and is more when compared with the Indomethacin, Ibuprofen and COX-2 selective prototype SC-558.

3.2. H-bond

H-bond is one of the most widely used alternative parameters for the evaluation of docking results. It is an influential parameter in deciding selective binding of compounds. The H-bond for designed compounds **7a**–**7e** was found to be 2, 1, 3, 2 and 2 respectively. While for standard Indomethacin, SC-558, Celecoxib, Ibuprofen it was found to be 1, 2, 2, and 1 respectively.

From the above studies it was found that the functional group –CONHNHCOCH$_2$ONO$_2$ moiety forms good H-bond interaction revealing its importance in the compound.

3.3. Contacts (Van der Waals (vdw) Interaction)

The contacts are represented in the form of

- Good vdw Contact.
- Bad vdw Contact.
- Ugly vdw Contact.

Table 3
Ulcerogenic effects of synthesized compounds in comparison with diclofenac acid.

Compounds	Dose (mg/kg, p.o)	Ratio of ulcerated animals	Ulcer index (mean ± SEM)
Diclofenac	24	6/6	2.3 ± 0.2
7a	33.70	–	–
7b	34.84	–	–
7g	40.71	–	–
7j	38.27	–	–

It is well established and accepted fact that number of good van der Waals interactions decides the binding affinity for any ligand with receptor/enzyme protein. Therefore we have analyzed the binding modes and abilities, taking into consideration the number of vdw contacts.

The designed compounds **7a**–**7e** and **7i** showed good vdw contacts i.e. 470, 455, 437, 426, 411 and 593 respectively, as compared to that of a standard Indomethacin and Celecoxib showing good vdw of 414 and 394 respectively. While the ugly vdw contacts were found to be 3, 5, 1, 3, 6 and 2 for designed compounds **7a**–**7f** respectively, as compared to that of a standard Indomethacin and Celecoxib showing ugly vdw of 2 and 1 respectively.

These contacts reveal the importance of functional group which can form good vdw interaction with the receptor and accordingly the ligand molecules (NCE's) can be designed and synthesized.

Standard drug Indomethacin as well as molecules **7c** and **7e** docked into COX-2 binding pocket is shown in Figs. 2–5.

It was indicated from docking results that all designed and synthesized '1,5-diarylpyrazolin-3-one' derivatives show common binding mode i.e. hydrogen bonding in the binding pockets of COX-2 enzyme (PDB Code 1CX2) when compared with standard Celecoxib and Indomethacin. Thus when compounds were docked into COX-2 enzyme, Celecoxib shows hydrogen binding with Arg120 and His90 whereas Indomethacin was docked by showing binding to Arg120. Also all 1,5-diarylpyrazolin-3-one derivatives show a common hydrogen binding to Arg120 and His90 indicating the binding affinity of compounds to COX-2 enzyme.

4. Conclusions

Various substituted 1,5-diarylpyrazolin-3-one derivatives were synthesized and screened for analgesic, anti-inflammatory and ulcerogenic potential. Most compounds exhibited significant analgesic and anti-inflammatory activities. Compound (**7j**) which is benzene analogue shows strong analgesia in acetic acid induced writhing tests. Among all the synthesized compounds, compounds (**7b**) and (**7j**) exhibited most prominent and consistent anti-inflammatory activity. From the detailed analysis of the results of histopathological studies, we conclude that the synthesized compounds have not only retained the anti-inflammatory profile of Indomethacin but also have helped in enhancing the anti-inflammatory activity and are devoid of the deadlier gastrointestinal toxicities. Also, the results of docking studies were found to be comparable with that of an experimental work. Thus, the strategy employed can serve as an important tool in future for the design, synthesis and development of novel NSAIDs with greater selectivity, potency and minimal side effects.

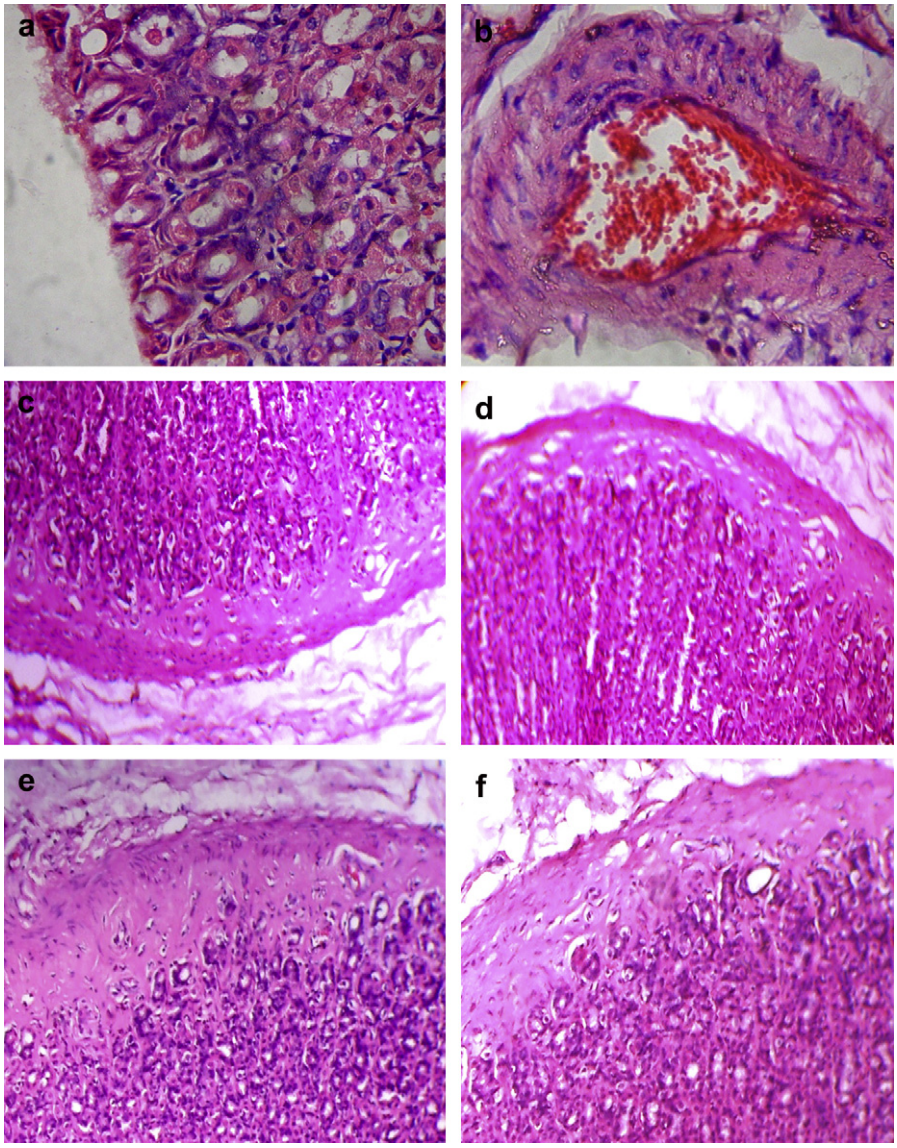


Fig. 1. Haematoxylin and eosin immunohistochemical staining of gastric ulcers after ulcer induction in rats. As illustrated in figure specimen (a) shows intact mucous membrane in control treated rat showing granular tissues composed of macrophages, fibroblasts and endothelial cells forming microvessels. Congestion of mucosal blood vessels in diclofenac treated group, specimen (b). No damage was seen to mucosa of rat treated with test compounds – **7a** specimen (c), **7b** specimen (d), **7g** specimen (e), and **7j** specimen (f), these specimens c–f were identical to that of the control, specimen (a). Original magnification 200×.

Table 4
EC_{50} and nitric oxide releasing properties of the compounds (**7a**–**7j**).

Compound code	EC_{50}	% NO release[a] (%)
7a	49.11	0.39
7b	66.85	0.32
7c	17.82	0.69
7d	21.32	0.66
7e	26.74	0.51
7f	28.18	0.49
7g	63.09	0.35
7h	70.79	0.27
7i	50.18	0.37
7j	35.48	0.46

[a] Percentage of NO released ($n = 2$) relative to a theoretical maximum release of 1 mol NO/mol of test compound; determined by Griess reagent in the presence of 5 mM L-cysteine, at pH 7.4.

Table 5
Results of extra precision docking studies of 1,5-diarylpyrazolin-3-one series of compounds (**7a**–**7j**) along with selective COX-2 inhibitors.

Sr. No.	Title	GScore	Energy	Hbnd	Good vdw	Bad vdw	Ugly vdw
1	Celecoxib	−11.37	−43.7	4	394	10	1
2	**7b**	−9.37	−38.7	1	455	37	5
3	**7c**	−9.26	−34.2	3	437	28	1
4	**7a**	−9.10	−41.7	2	470	29	3
5	**7i**	−8.10	−35.7	2	593	41	2
6	**7d**	−8.41	−32.7	2	426	26	3
7	**7e**	−8.38	−33.7	2	411	23	6
8	SC-558	−8.36	−34.0	2	398	10	4
9	**7f**	−8.10	−34.7	1	490	10	3
10	**7g**	−8.00	−37.8	2	414	23	6
11	**7h**	−7.80	−32.4	1	427	11	5
12	**7j**	−7.77	−31.00	2	438	10	6
13	Indomethacin	−7.37	−33.10	1	414	8	2
14	Ibuprofen	−4.38	−33.15	1	220	8	3

Hbnd = No. of hydrogen bonds. Bad vdw = No. of bad van der Waals contacts. Ugly vdw = No. of ugly van der Waals contacts.

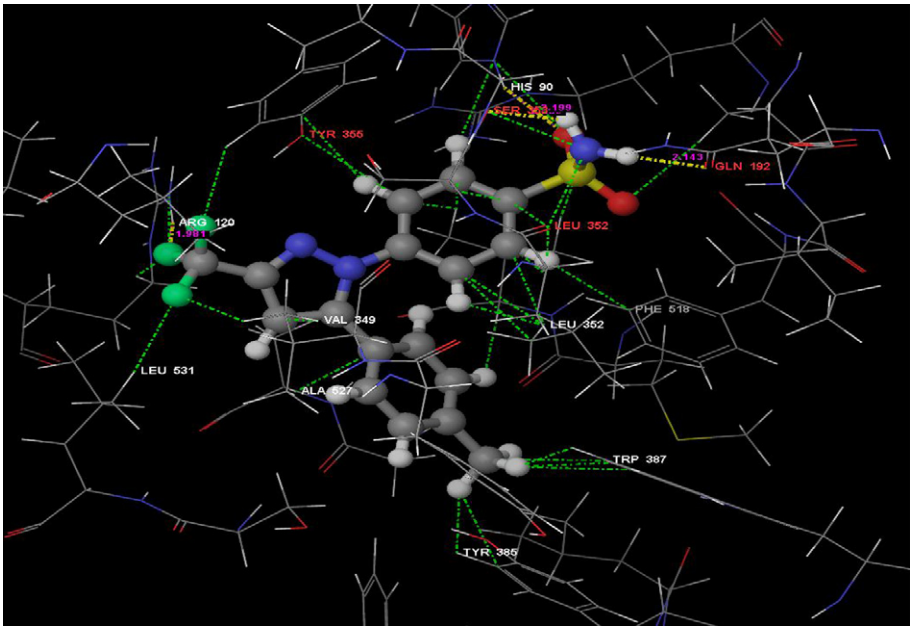


Fig. 2. Molecule celecoxib docked into COX-2 binding pocket.

5. Experimental

5.1. Synthetic studies

All the reactions were carried out with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. Melting points were determined by open capillary tubes and were uncorrected. FTIR spectra of the powdered compounds were recorded using KBr on a Jasco FTIR V 430+ spectrophotometer using Diffuse Reflectance Attachment and are reported in cm^{-1} and ^1H NMR spectra were recorded on a Varian Mercury YH300 (300 MHz FT NMR) spectrophotometer using TMS as an internal reference (Chemical shift represented in δ ppm). Mass spectra were recorded on GC–MS QP5050A System (benchtop quadrupole mass spectrophotometer). Purity of the compounds was checked on TLC plates using silica gel G as stationary phase and was visualised using iodine vapours or under UV chambers.

5.2. General procedure for the preparation of α-acylated (i) ethyl acetoacetate derivatives (**2a**–**2e**) (ii) diethylmalonate derivatives (**2f**–**2j**) [30]

A mixture of ethyl acetoacetate (**1a**, 12.0 mmol) or diethylmalonate (**1b**, 12.0 mmol), magnesium (12.1 mmol), ethanol (40.0 mmol), CCl_4 (0.3 mL) and anhydrous toluene (30 mL) was stirred at room temperature for 30 min. The mixture was refluxed for 1 h, and then cooled to 0–5 °C. The acylating agent (12.1 mmol) was added dropwise to the solution at 0–5 °C over 30 min, and the reaction mixture was stirred at room temperature for 1 h. The resulting mixture was re-cooled to 0–5 °C and washed with cold 5% aq. HCl solution (25 mL),

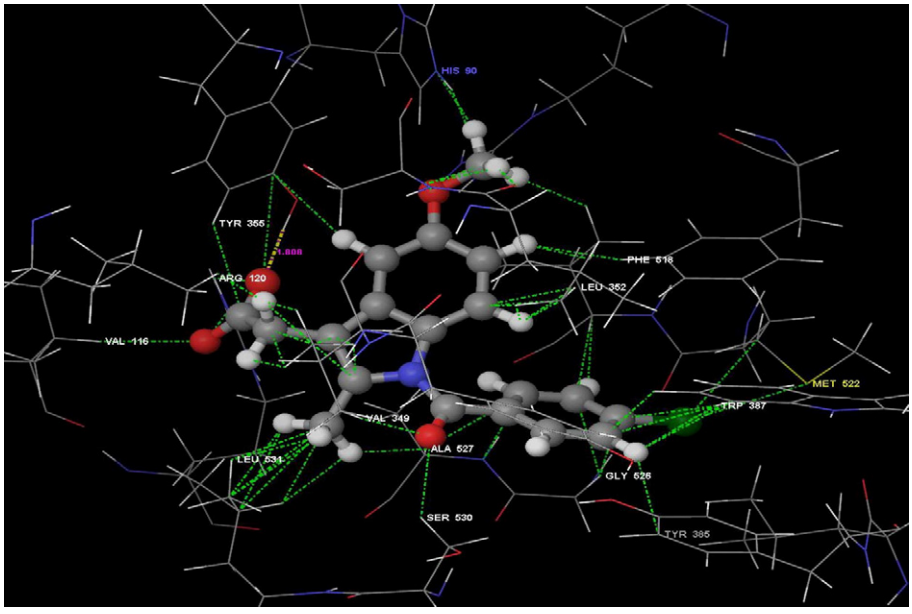


Fig. 3. Molecule Indomethacin docked into COX-2 binding pocket.

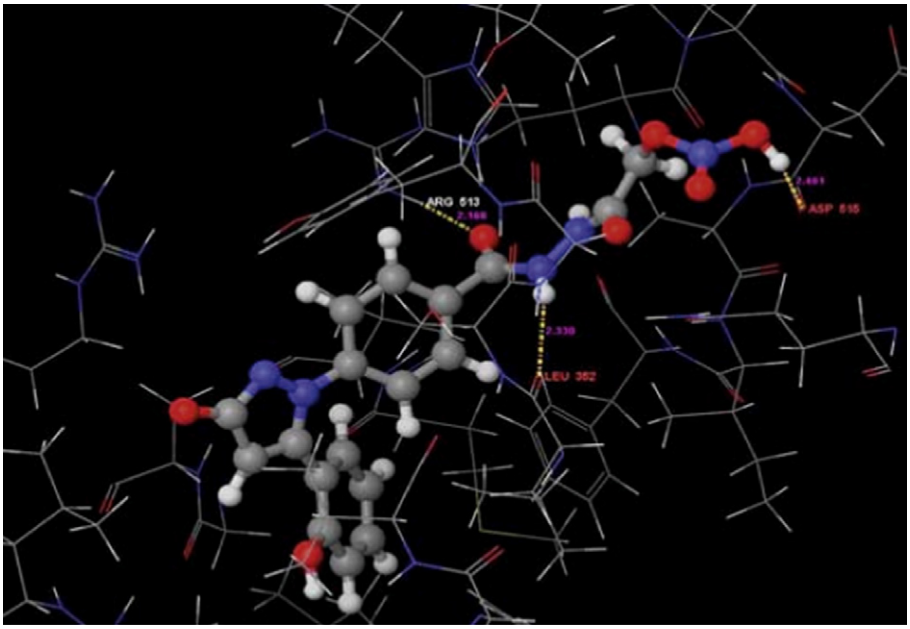


Fig. 4. Molecule **7c** docked into COX-2 binding pocket (showing H-bond interaction with the amino acid residues).

satd $NaHCO_3$ solution (25 mL) and brine (25 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give pale yellow liquids (**2a**–**2j**).

*5.2.1. Ethyl-3-hydroxy-2-(4-nitrobenzoyl)but-2-enoate (**2a**)*

bp 205–207 °C; yield 85%; FTIR (KBr) cm^{-1}: 3498 (Aliphatic OH), 1737 (C=O of ketone), 1798 (C=O, ester), 1315, 1456 (NO_2), 2925 (CH alkane), 3035 (CH, Ar); mass: *m/z* 279 (M^+), 280 (M + 1, 15.3%), 281 (M + 2, 2.2%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.65 (s, 3H, CH_3), 14.12 (s, 1H, OH), 4.19 (q, 2H, CH_2), 1.7 (t, 3H, CH_3), 7.32 (d, 2H, ArH), 8.07 (d, 2H, ArH).

*5.2.2. Ethyl-3-hydroxy-2-(2-methoxybenzoyl)but-2-enoate (**2b**)*

bp 210–212 °C; yield 87%; FTIR (KBr) cm^{-1}: 3487 (Aliphatic OH), 1739 (C=O of ketone), 1729 (C=O, ester), 2947 (CH alkane), 3085 (CH, Ar),; mass: *m/z* 264 (M^+), 265 (M + 1, 15.3%), 266 (M + 2, 11%); ^1H NMR (300 MHz, $CDCl_3$), 1.75 (s, 3H, CH_3), 14.88 (s, 1H, OH), 3.21 (q, 2H, CH_2), 1.41 (t, 3H, CH_3), 7.32–7.63 (m, 4H, ArH), 3.73 (s, 3H, OCH_3).

*5.2.3. Ethyl-3-hydroxy-2-(2-hydroxybenzoyl)but-2-enoate (**2c**)*

bp 245–247 °C; yield 78%; FTIR (KBr) cm^{-1}: 3476 (Aliphatic OH), 1745 (C=O of ketone), 1753 (C=O, ester), 2930 (CH alkane), 3050 (CH, Ar), 3542 (Phenolic OH); mass: *m/z* 250 (M^+), 251 (M + 1, 14%), 252 (M + 2, 4%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.71 (s, 3H, CH_3), 15.01 (s, 1H, OH), 4.19 (q, 2H, CH_2), 1.5 (t, 3H, CH_3), 7.02–7.47 (m, 4H, ArH) 8.98 (s, 1H, Phenolic OH).

*5.2.4. Ethyl-3-hydroxy-2-(4-chlorobenzoyl)but-2-enoate (**2d**)*

bp 250–252 °C; yield 67%; FTIR (KBr) cm^{-1}: 3480 (Aliphatic OH), 1791 (C=O of ketone), 1711 (C=O, ester), 2895 (CH alkane), 3044

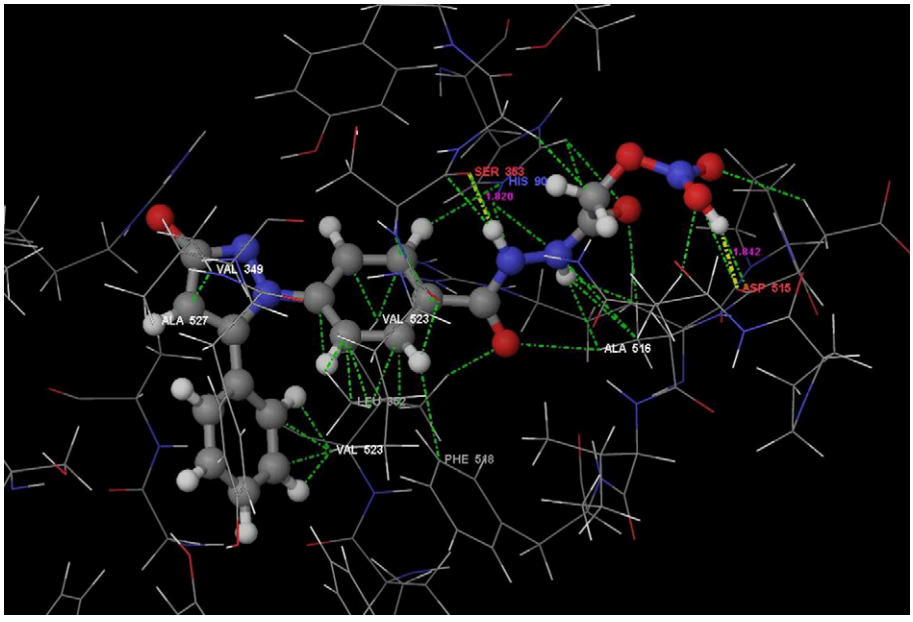


Fig. 5. Molecule **7e** docked into COX-2 binding pocket.

(CH, Ar), 669 (C–Cl); mass: *m/z* 268.5 (M^+), 269.5 (M + 1, 14.2%), 270.5 (M + 2, 32.8%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 1.84 (s, 3H, CH_3), 14.34 (s, 1H, OH), 3.79 (q, 2H, CH_2), 1.9 (t, 3H, CH_3), 7.28 (d, 2H, ArH), 7.51 (d, 2H, ArH).

*5.2.5. Ethyl-2-benzoyl-3-hydroxy but-2-enoate (**2e**)*

bp 279–281 °C; yield 90%; FTIR (KBr) cm^{-1}: 3499 (Aliphatic OH), 1711 (C═O of ketone), 1735 (C═O, ester), 2873 (CH alkane), 3077 (CH, Ar), 1022 (C–O); mass: *m/z* 234 (M^+), 235 (M + 1, 4%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 1.54 (s, 3H, CH_3), 14.77 (s, 1H, OH), 3.7 (q, 2H, CH_2), 1.8 (t, 3H, CH_3), 7.49–7.81 (m, 5H, ArH).

*5.2.6. Ethyl-3-ethoxy-3-hydroxy-2-(4-nitrobenzoyl)acrylate (**2f**)*

bp 270–272 °C; yield 92%; FTIR (KBr) cm^{-1}: 3489 (Aliphatic OH), 1739 (C═O of ketone), 1729 (C═O, ester), 1175 (C–O), 2917 (CH alkane), 3054 (CH, Ar), 1455, 1328 (NO_2); mass: *m/z* 309 (M^+), 310 (M + 1, 15%), 311 (M + 2, 2%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 1.33 (t, 3H, CH_3), 4.12 (q, 2H, CH_2), 15.0 (s, 1H, OH), 3.9 (q, 2H, CH_2), 2.1 (t, 3H, OCH_3), 7.49–7.81 (m, 4H, ArH).

*5.2.7. Ethyl-3-ethoxy-3-hydroxy-2-(2-methoxybenzoyl)acrylate (**2g**)*

bp 277–279 °C; yield 94%; FTIR (KBr) cm^{-1}: 3508 (Aliphatic OH), 1739 (C═O of ketone), 1729 (C═O, ester), 1066 (C–O), 2908 (CH alkane), 3087 (CH, Ar); mass: *m/z* 294 (M^+), 295 (M + 1, 16%), 296 (M + 2, 1.3%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 15.5 (s, 1H, OH), 3.89 (q, 2H, CH_2), 1.43 (t, 3H, CH_3), 6.96–7.47 (m, 4H, ArH), 3.77 (s, 3H, OCH_3), 1.23 (t, 3H, CH_3), 4.11 (q, 2H, CH_2).

*5.2.8. Ethyl-3-ethoxy-3-hydroxy-2-(2-hydroxybenzoyl)acrylate (**2h**)*

bp 217–219 °C; yield 86%; FTIR (KBr) cm^{-1}: 3482 (Aliphatic OH), 1709 (C═O of ketone), 1749 (C═O, ester), 1076 (C–O), 3533, 3509, (Phenolic OH), 2918 (CH alkane), 3066 (CH, Ar); mass: *m/z* 280 (M^+), 281 (M + 1, 15%), 282 (M + 2, 2.4%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 15.56 (s, 1H, OH), 3.92 (q, 2H, CH_2), 1.43 (t, 3H, CH_3), 6.96–7.47 (m, 4H, ArH), 8.73 (s, 1H, Phenolic OH), 1.23 (t, 3H, CH_3), 4.11 (q, 2H, CH_2).

*5.2.9. Ethyl-3-ethoxy-3-hydroxy-2-(4-chlorobenzoyl)acrylate (**2i**)*

bp 236–238 °C; yield 80%; FTIR (KBr) cm^{-1}: 3458 (Aliphatic OH), 1719 (C═O of ketone), 1732 (C═O, ester), 1116 (C–O), 2948 (CH alkane), 3032 (CH, Ar), 683 (C–Cl); mass: *m/z* 298.5 (M^+), 299.5 (M + 1, 15.1%), 300.5 (M + 2, 32%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 1.60 (t, 3H, CH_3), 3.26 (q, 2H, CH_2), 14.60 (s, 1H, OH), 4.08 (q, 2H, CH_2), 1.76 (t, 3H, CH_3), 6.91–7.36 (m, 4H, ArH).

*5.2.10. Ethyl-2-benzoyl-3-ethoxy-3-hydroxyacrylate (**2j**)*

bp 291–294 °C; yield 85%; FTIR (KBr) cm^{-1}: 3439 (Aliphatic OH), 1703 (C═O of ketone), 1746 (C═O, ester), 1106 (C–O), 2938 (CH alkane), 3082 (CH, Ar) mass: *m/z* 264 (M^+), 265 (M + 1, 15.3%), 266 (M + 2, 1.1%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 1.56 (t, 3H, CH_3), 3.65 (q, 2H, CH_2), 15.19 (s, 1H, OH), 4.13 (q, 2H, CH_2), 1.76 (t, 3H, CH_3), 7.09–7.86 (m, 5H, ArH).

*5.3. General procedure for the preparation of 5-phenyl 1,2-dihydropyrazol-3-one (**3a**–**3j**) [30]*

To a stirred solution of the α-acylated ethyl acetoacetate (5.0 mmol) in ethanol (10 mL), hydrazine 0.38 g (7.5 mmol, 98%) was added dropwise at room temperature. The reaction mixture was stirred at room temperature for 16 h. The mixture was concentrated under reduced pressure, and the residue was treated with diethyl ether (10 mL). The resulting solid was filtered and washed with diethyl ether and recrystallized from 10% ethanol in diethyl ether to give white crystals.

*5.3.1. 5-(4-Nitrophenyl)-1,2-dihydropyrazol-3-one (**3a**)*

mp 221–223 °C; yield 80%; FTIR (KBr) cm^{-1}: 3385 (NH), 1629 (C═O,), 3087 (CH, Ar), 1453, 1345 (NO_2); mass: *m/z* 205 (M^+), 206 (M + 1, 11.2%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 8.3 (s, 1H, NH), 7.81 (s, 1H, NH), 5.86 (s, 1H, CH), 7.59–7.86 (m, 4H, ArH).

*5.3.2. 5-(2-Methoxyphenyl)-1,2-dihydropyrazol-3-one (**3b**)*

mp 170–171 °C; yield 82%; FTIR (KBr) cm^{-1}: 3356 (NH), 1759 (C═O,), 3062 (CH, Ar); mass: *m/z* 190 (M^+), 191 (M + 1, 11.2%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 8.0 (s, 1H, NH), 7.87 (s, 1H, NH), 5.36 (s, 1H, CH), 7.59–7.86 (m, 4H, ArH), 3.73 (s, 3H, OCH_3).

*5.3.3. 5-(2-Hydroxyphenyl)-1,2-dihydropyrazol-3-one (**3c**)*

mp 147–149 °C; yield 66%; FTIR (KBr) cm^{-1}: 3387 (NH), 3545 (Phenolic OH), 1665 (C═O), 3021 (CH, Ar); mass: *m/z* 176 (M^+), 177 (M + 1, 10.6%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 7.98 (s, 1H, NH), 7.80 (s, 1H, NH), 5.53 (s, 1H, CH), 6.57–7.16 (m, 4H, ArH), 8.73 (s, 1H, Phenolic OH).

*5.3.4. 5-(4-Chlorophenyl)-1,2-dihydropyrazole-3-one (**3d**)*

mp 227–229 °C; yield 65%; FTIR (KBr) cm^{-1}: 3379 (NH), 1703 (C═O), 3087 (CH, Ar) 747 (C–Cl); mass: *m/z* 194.5 (M^+), 195.5 (M + 1, 9.9%), 196.5 (M + 2, 32%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 8.17 (s, 1H, NH), 7.93 (s, 1H, NH), 5.21 (s, 1H, CH), 7.22–7.36 (m, 4H, ArH).

*5.3.5. 5-Phenyl-1,2-dihydropyrazol-3-one (**3e**)*

mp 187–189 °C; yield 70%; FTIR (KBr) cm^{-1}: 3369 (NH), 1613 (C═O), 3067 (CH, Ar); mass: *m/z* 160 (M^+), 170 (M + 1, 9.9%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 7.90 (s, 1H, NH), 7.73 (s, 1H, NH), 4.98 (s, 1H, CH), 7.30–7.52 (m, 5H, ArH).

*5.3.6. Ethyl-5-(4-nitrophenyl) 3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**3f**)*

mp 170–171 °C; yield 88%; FTIR (KBr) cm^{-1}: 3364 (NH), 1613 (C═O), 2943 (CH, Aliphatic), 3067 (CH, Ar), 1076 (C–O); mass: *m/z* 277 (M^+), 278 (M + 1, 14.3%), 279 (M + 2, 1.2%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 8.09 (s, 1H, NH), 7.79 (s, 1H, NH), 7.33–7.82 (m, 4H, ArH), 4.19 (q, 2H, CH_2), 1.73 (t, 3H, CH_3).

*5.3.7. Ethyl-5-(2-methoxyphenyl) 3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**3g**)*

mp 217–219 °C; yield 84%; FTIR (KBr) cm^{-1}: 3345 (NH), 1643 (C═O), 2927 (CH, Aliphatic), 3036 (CH, Ar), 1177 (C–O); mass: *m/z* 262 (M^+), 263 (M + 1, 14.4%), 264 (M + 2, 1.9%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 8.1 (s, 1H, NH), 7.90 (s, 1H, NH), 6.72–7.23 (m, 4H, ArH), 3.29 (q, 2H, CH_2), 1.53 (t, 3H, CH_3), 3.59 (s, 3H, OCH_3).

*5.3.8. Ethyl-5-(2-hydroxyphenyl) 3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**3h**)*

mp 223–225 °C; yield 76%; FTIR (KBr) cm^{-1}: 3348 (NH), 1613 (C═O), 2937 (CH, Aliphatic), 3067 (CH, Ar), 1123 (C–O); mass: *m/z* 248 (M^+), 249 (M + 1, 13.2%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 8.03 (s, 1H, NH), 7.83 (s, 1H, NH), 6.72–7.23 (m, 4H, ArH), 3.29 (q, 2H, CH_2), 1.53 (t, 3H, CH_3), 9.53 (s, 1H, Phenolic OH).

*5.3.9. Ethyl-5-(4-chlorophenyl) 3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**3i**)*

mp 288–290 °C; yield 84%; FTIR (KBr) cm^{-1}: 3341 (NH), 1673 (C═O), 2951 (CH, Aliphatic), 3091 (CH, Ar), 1158 (C–O), 747 (C–Cl); mass: *m/z* 266.5 (M^+), 267.5 (M + 1, 13.2%), 268.5 (M + 2, 32%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 8.21 (s, 1H, NH), 7.89 (s, 1H, NH), 7.22–7.24 (m, 4H, ArH), 3.29 (q, 2H, CH_2), 1.53 (t, 3H, CH_3).

*5.3.10. Ethyl-3-oxo-5-phenyl-2,3-dihydro-1H-pyrazol-4-carboxylate (**3j**)*

mp 257–259 °C; yield 84%; FTIR (KBr) cm^{-1}: 3385, (NH), 1711 (C=O), 1094 (C–O), 2940 (CH, Aliphatic), 3033 (CH, Ar); mass: *m/z* 232 (M^+), 233 (M + 1, 13.2%), 234 (M + 2, 1.5%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 7.99 (s, 1H, NH), 7.68 (s, 1H, NH), 7.30–7.53 (m, 5H, ArH), 3.29 (q, 2H, CH_2), 1.53 (t, 3H, CH_3).

*5.4. General procedure for the preparation of 4-(3-oxo-5-phenyl-2,3-dihydropyrazol-1-yl)benzoic acid (**4a**–**4j**) [31]*

Equimolar amounts of intermediate **3** (1 g, 0.003 M) and 4-chloro benzoic acid (0.46 g 0.003 M) were refluxed in ethanol (10 mL) for 4 h. After completion of 3 h from starting of reflux $NaHCO_3$ (0.252 g, 0.003 M) was added in reaction mixture to increase rate of forward reaction. After 4 h the reaction mixture was cooled, ethanol was evaporated to yield product **4**.

*5.4.1. 4-(5-(4-Nitrophenyl)-3-oxo-2,3-dihydropyrazol-1-yl)benzoic acid (**4a**)*

mp. 207–209 °C; yield 78%; FTIR (KBr) cm^{-1}: 3299, 826 (NH), 1763 (C=O), 1590 (C=N), 1090 (C–O), 3410 (OH), 1491, 1374 (NO_2), 3033 (CH, Ar), 1702 (acid C=O); mass: *m/z* 325 (M^+), 326 (M + 1, 18.6%), 327 (M + 2, 1.5%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 8.03 (s, 1H, NH), 7.21–8.1 (m, 8H, ArH), 12.79 (s, 1H, acid OH).

*5.4.2. 4-(5-(2-Methoxyphenyl)-3-oxo-2,3-dihydropyrazol-1-yl)benzoic acid (**4b**)*

mp. 222–224 °C; yield 80%; FTIR (KBr) cm^{-1}: 3267, 816 (NH), 1756 (C=O), 1564 (C=N), 1120 (C–O), 3437 (OH), 3052 (CH, Ar), 1702 (acid C=O); mass: *m/z* 310 (M^+), 311 (M + 1, 18.7%), 312 (M + 2, 2.6%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 8.13 (s, 1H, NH), 7.32–8.19 (m, 8H, ArH), 11.65 (s, 1H, acid OH), 3.12 (s, 1H, OCH_3).

*5.4.3. 4-(5-(2-Hydroxyphenyl)-3-oxo-2,3-dihydropyrazol-1-yl)benzoic acid (**4c**)*

mp. 233–235 °C; yield 68%; FTIR (KBr) cm^{-1}: 3333, 842 (NH), 1655 (C=O), 1582 (C=N), 1220 (C–O), 3489 (OH), 3081 (CH, Ar), 1725 (acid C=O), 3540, 3558, (OH); mass: *m/z* 296 (M^+), 297 (M + 1, 18.2%), 298 (M + 2, 1.5%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.68 (s, 1H, CH), 7.98 (s, 1H, NH), 6.78–7.67 (m, 8H, ArH), 8.83 (s, 1H, Phenolic OH), 11.57 (s, 1H, acid OH).

*5.4.4. 4-(5-(4-Chlorophenyl)-3-oxo-2,3-dihydropyrazol-1-yl) benzoic acid (**4d**)*

mp. 236–238 °C; yield 63%; FTIR (KBr) cm^{-1}: 3371, 828 (NH), 1740 (C=O), 1594 (C=N), 1204 (C–O), 3502 (OH), 3052 (CH, Ar), 1718 (acid C=O), 645 (C–Cl); mass: *m/z* 314.5 (M^+), 315.5 (M + 1, 17.5%), 316.5 (M + 2, 32%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.85 (s, 1H, CH), 8.11 (s, 1H, NH), 6.87–7.69 (m, 8H, ArH), 8.83, 11.21 (s, 1H, acid OH).

*5.4.5. 4-(3-Oxo-5-phenyl-2,3-dihydropyrazol-1-yl) benzoic acid (**4e**)*

mp. 242–245 °C; yield 68%; FTIR (KBr) cm^{-1}: 3367, 816 (NH), 1756 (C=O), 1564 (C=N), 1120 (C–O), 3427 (OH), 3052 (CH, Ar), 1702 (acid C=O); mass: *m/z* 380 (M^+), 381 (M + 1, 17.6%), 382 (M + 2, 2.2%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.71 (s, 1H, CH), 7.88 (s, 1H, NH), 6.31–7.59 (m, 9H, ArH), 11.38 (s, 1H, acid OH).

*5.4.6. 4-(5-(4-Nitrophenyl)-4-(ethoxycarbonyl)-3-oxo-2,3-dihydropyrazol-1-yl) benzoic acid (**4f**)*

mp. 283–286 °C; yield 85%; FTIR (KBr) cm^{-1}: 3343, 811 (NH), 1732 (C=O), 1578 (C=N), 1020 (C–O), 3410 (OH), 2961 (CH, Alkyl), 3022 (CH, Ar), 1720 (acid C=O), 1376, 1498 (NO_2); mass: *m/z* 397 (M^+), 398 (M + 1, 21.7%), 399 (M + 2, 3.5%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 4.11 (q, 2H, CH_2), 1.43 (t, 3H, CH_3), 7.92 (s, 1H, NH), 6.61–7.81 (m, 8H, ArH), 11.86 (s, 1H, acid OH).

*5.4.7. 4-(5-(2-Methoxyphenyl)-4-(ethoxycarbonyl)-3-oxo-2,3-dihydropyrazol-1-yl) benzoic acid (**4g**)*

mp. 280–282 °C; yield 82%; FTIR (KBr) cm^{-1}: 3338, 879 (NH), 1736 (C=O), 1529 (C=N), 1027 (C–O), 3543 (OH), 2988 (CH, Alkyl), 3011 (CH, Ar), 1719 (acid C=O); mass: *m/z* 382 (M^+), 383 (M + 1, 22.1%), 384 (M + 2, 3.7%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 3.93 (q, 2H, CH_2), 1.62 (t, 3H, CH_3), 8.07 (s, 1H, NH), 6.39–7.52 (m, 8H, ArH), 12.02 (s, 1H, acid OH), 3.22 (s, 1H, OCH_3).

*5.4.8. 4-(5-(2-Hydroxyphenyl)-4-(ethoxycarbonyl)-3-oxo-2,3-dihydropyrazol-1-yl) benzoic acid (**4h**)*

mp. 215–217 °C; yield 75%; FTIR (KBr) cm^{-1}: 3313, 815 (NH), 1729 (C=O), 1534 (C=N), 1170 (C–O), 3511 (OH), 2952 (CH, Alkyl), 3045 (CH, Ar), 1719 (acid C=O), 3505, 3158 (OH); mass: *m/z* 368 (M^+), 369 (M + 1, 21.3%), 370 (M + 2, 3.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 4.32 (q, 2H, CH_2), 1.53 (t, 3H, CH_3), 8.15 (s, 1H, NH), 6.97–7.70 (m, 8H, ArH), 12.32 (s, 1H, acid OH), 9.23 (s, 1H, Phenolic OH).

*5.4.9. 4-(5-(2-Chlorophenyl)-4-(ethoxycarbonyl)-3-oxo-2,3-dihydropyrazol-1-yl) benzoic acid (**4i**)*

mp. 289–291 °C; yield 74%; FTIR (KBr) cm^{-1}: 3387, 821 (NH), 1752 (C=O), 1544 (C=N), 1186 (C–O), 3468 (OH), 2943 (CH, Alkyl), 3033 (CH, Ar), 1724 (acid C=O), 709 (C–Cl); mass: *m/z* 386.5 (M^+), 387.5 (M + 1, 20.9%), 388.5 (M + 2, 32%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 3.92 (q, 2H, CH_2), 1.53 (t, 3H, CH_3), 7.96 (s, 1H, NH), 6.81–7.70 (m, 8H, ArH), 12.19 (s, 1H, acid OH).

*5.4.10. 4-(4-(Ethoxycarbonyl)-3-oxo-5-phenyl-2-3-dihydropyrazol-1-yl) benzoic acid (**4j**)*

mp. 277–279 °C; yield 87%; FTIR (KBr) cm^{-1}: 3385, 826 (NH), 1756 (C=O), 1581 (C=N), 1090 (C–O), 3498 (OH), 2987 (CH, Alkyl), 3032 (CH, Ar), 1721 (acid C=O); mass: *m/z* 352 (M^+), 353 (M + 1, 20.9%), 354 (M + 2, 3.2%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 4.03 (q, 2H, CH_2), 1.72 (t, 3H, CH_3), 8.19 (s, 1H, NH), 6.31–7.64 (m, 9H, ArH), 11.96 (s, 1H, acid OH).

*5.5. General procedure for the preparation of 4-(3-oxo-5-phenyl-2,3-dihydropyrazol-1-yl)benzohydrazides (**5a**–**5j**)*

The compounds (**5a**–**5j**) were prepared as per procedure reported in the literature [18].

*5.5.1. 4-(5-(4-Nitrophenyl)-3-oxo-2,3-dihydropyrazol-1-yl)benzohydrazide (**5a**)*

mp. 201–203 °C; yield 76%; FTIR (KBr) cm^{-1}: 3367, 829 (NH), 1754 (C=O), 1564 (C=N), 1120 (C–O), 3052 (CH, Ar), 1302, 1436 (NO_2); mass: *m/z* 339 (M^+), 340 (M + 1, 17.6%), 341 (M + 2, 2.6%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.04 (s, 1H, CH), 8.08 (s, 1H, NH), 8.21 (s, 1H, NH), 4.35 (s, 2H, NH_2), 7.02–7.95 (m, 8H, ArH).

*5.5.2. 4-(5-(2-Methoxyphenyl)-3-oxo-2,3-dihydropyrazol-1-yl)benzohydrazide (**5b**)*

mp. 212–215 °C; yield 78%; FTIR (KBr) cm^{-1}: 3355, 836 (NH), 1739 (C=O), 1555 (C=N), 1026 (C–O), 3066 (CH, Ar); mass: *m/z* 324 (M^+), 325 (M + 1, 18.7%), 326 (M + 2, 2.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.04 (s, 1H, CH), 8.11 (s, 1H, NH), 8.38 (s, 1H, NH), 4.35 (s, 2H, NH_2), 3.77 (s, 3H, OCH_3), 6.84–7.70 (m, 8H, ArH).

*5.5.3. 4-(5-(2-Hydroxyphenyl)-3-oxo-2,3-dihydropyrazol-1-yl)benzohydrazide (**5c**)*

mp. 219–221 °C; yield 64%; FTIR (KBr) cm^{-1}: 3358, 812 (NH), 1656 (C═O), 1544 (C═N), 1098 (C–O), 3084 (CH, Ar), 3531, 3504 (OH); mass: *m*/*z* 310 (M^+), 311 (M + 1, 17.6%), 312 (M + 2, 2.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.04 (s, 1H, CH), 8.17 (s, 1H, NH), 8.56 (s, 1H, NH), 4.35 (s, 2H, NH_2), 8.75 (s, 3H, Phenolic OH), 7.12–7.91 (m, 8H, ArH).

*5.5.4. 4-(5-(4-Chlorophenyl)-3-oxo-2,3-dihydropyrazol-1-yl)benzohydrazide (**5d**)*

mp. 224–226 °C; yield 61%; FTIR (KBr) cm^{-1}: 3367, 829 (NH), 1754 (C═O), 1564 (C═N), 1120 (C–O), 3052 (CH, Ar), 702 (C–Cl); mass: *m*/*z* 328.5 (M^+), 329.5 (M + 1, 17.5%), 330.5 (M + 2, 32.2%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.04 (s, 1H, CH), 8.0 (s, 1H, NH), 8.29 (s, 1H, NH), 4.35 (s, 2H, NH_2), 6.89–7.67 (m, 8H, ArH).

*5.5.5. 4-(3-Oxo-5-phenyl-2,3-dihydropyrazol-1-yl)benzohydrazide (**5e**)*

mp. 231–233 °C; yield 66%; FTIR (KBr) cm^{-1}: 3367, 829 (NH), 1754 (C═O), 1564 (C═N), 1120 (C–O), 3052 (CH, Ar); mass: *m*/*z* 294 (M^+), 295 (M + 1, 17.5%), 296 (M + 2, 1.9%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.04 (s, 1H, CH), 8.11 (s, 1H, NH), 8.51 (s, 1H, NH), 4.35 (s, 2H, NH_2), 6.98–7.87 (m, 8H, ArH).

*5.5.6. Ethyl-1-(4-(hydrazinecarbonyl)phenyl)-5-(4-nitrophenyl)-3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**5f**)*

mp. 211–213 °C; yield 83%; FTIR (KBr) cm^{-1}: 3267, 829 (NH), 1754 (C═O), 1564 (C═N), 1120 (C–O), 2910 (CH, Alkyl), 3052 (CH, Ar), 1387, 1532 (NO_2); mass: *m*/*z* 411 (M^+), 412 (M + 1, 21%), 413 (M + 2, 3.6%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.14 (t, 3H, CH_3), 3.34 (q, 2H, CH_2), 8.07 (s, 1H, NH), 8.48 (s, 1H, NH), 4.35 (s, 2H, NH_2), 7.09–7.88 (m, 8H, ArH).

*5.5.7. Ethyl-1-(4-(hydrazinecarbonyl)phenyl)-5-(4-methoxyphenyl)-3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**5g**)*

mp. 254–256 °C; yield 80%; FTIR (KBr) cm^{-1}: 3341, 831 (NH), 1698 (C═O), 1592 (C═N), 1098 (C–O), 2929 (CH, Alkyl), 3090 (CH, Ar); mass: *m*/*z* 396 (M^+), 397 (M + 1, 22.1%), 398 (M + 2, 3.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.49 (t, 3H, CH_3), 3.83 (q, 2H, CH_2), 7.79 (s, 1H, NH), 8.37 (s, 1H, NH), 4.35 (s, 2H, NH_2), 6.89–7.73 (m, 4H, ArH), 3.12 (s, 3H, OCH_3).

*5.5.8. Ethyl-1-(4-(hydrazinecarbonyl)phenyl)-5-(4-hydroxyphenyl)-3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**5h**)*

mp. 243–245 °C; yield 74%; FTIR (KBr) cm^{-1}: 3354, 867 (NH), 1798 (C═O), 1590 (C═N), 1165 (C–O), 2976 (CH, Alkyl), 3087 (CH, Ar), 3540, 3530 (OH); mass: *m*/*z* 382 (M^+), 383 (M + 1, 20.9%), 384 (M + 2, 3.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.51 (t, 3H, CH_3), 3.82 (q, 2H, CH_2), 8.13 (s, 1H, NH), 8.43 (s 1H, NH), 4.35 (s, 2H, NH_2), 7.02–7.92 (m, 8H, ArH), 9.12 (s, 1H, Phenolic OH).

*5.5.9. Ethyl-5-(4-chlorophenyl)-1-(4-(hydrazinecarbonyl)phenyl)-3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**5i**)*

mp. 239–241 °C; yield 71%; FTIR (KBr) cm^{-1}: 3363, 808 (NH), 1707 (C═O), 1586 (C═N), 1097 (C–O), 2954 (CH, Alkyl), 3090 (CH, Ar), 708 (C–Cl); mass: *m*/*z* 400.5 (M^+), 401.5 (M + 1, 20.9%), 402.5 (M + 2, 32.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.59 (t, 3H, CH_3), 3.82 (q, 2H, CH_2), 8.1 (s, 1H, NH), 8.29 (s, 1H, NH), 4.35 (s, 2H, NH_2), 6.82–7.71 (m, 8H, ArH).

*5.5.10. Ethyl-1-(4-(hydrazinecarbonyl)phenyl)-3-oxo-5-phenyl-2,3-dihydro-1H-pyrazol-4-carboxylate (**5j**)*

mp. 261–263 °C; yield 84%; FTIR (KBr) cm^{-1}: 3376, 829 (NH), 1754 (C═O), 1564 (C═N), 1120 (C–O), 2987 (CH, Alkyl), 3052 (CH, Ar); mass: *m*/*z* 366 (M^+), 367 (M + 1, 20.9%), 368 (M + 2, 2.9%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.49 (t, 3H, CH_3), 3.87 (q, 2H, CH_2), 8.08 (s, 1H, NH), 8.41 (s, 1H, NH), 4.35 (s, 2H, NH_2), 6.68–7.53 (m, 8H, ArH).

*5.6. General procedure for the preparation of N′-(2-chloroacetyl)-4-(3-oxo-5-phenyl-2,3-dihydropyrazol-1-yl) benzohydrazides (**6a**–**6j**)*

The compounds (**6a**–**6j**) were prepared as per procedure reported in the literature [18].

*5.6.1. N′-(2-chloroacetyl)-4-(5-(4-nitrophenyl)-3-oxo-5-phenyl-2,3-dihydropyrazol-1-yl) benzohydrazide (**6a**)*

mp. 288–290 °C; yield 74%; FTIR (KBr) cm^{-1}: 3367, 837 (NH), 1756 (C═O), 1576 (C═N), 1130 (C–O), 3032 (CH, Ar), 2859 (CH, alkane), 1376, 1402 (NO_2), 707 (C–Cl); mass: *m*/*z* 415.5 (M^+), 416.5 (M + 1, 21.7%), 417.5 (M + 2, 33.4%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.29 (s, 2H, NH), 8.05 (s, 1H, NH), 6.89–7.71 (m, 8H, ArH).

*5.6.2. N′-(2-chloroacetyl)-4-(5-(2-methoxyphenyl)-3-oxo-2,3-dihydro-1H-pyrazol-1-yl)benzohydrazide (**6b**)*

mp. 264–266 °C; yield 75%; FTIR (KBr) cm^{-1}: 3377, 877 (NH), 1765 (C═O), 1572 (C═N), 1090 (C–O), 3088 (CH, Ar), 2879 (CH, alkane), 747 (C–Cl); mass: *m*/*z* 400.5 (M^+), 401.5 (M + 1, 20.9%), 402.5 (M + 2, 32.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.31 (s, 2H, NH), 8.09 (s, 1H, NH), 6.82–7.58 (m, 8H, ArH), 3.54 (s, 3H, OCH_3).

*5.6.3. N′(2-chloroacetyl)-4-(5-(2-hydroxyphenyl)-3-oxo-2,3-dihydro-1H-pyrazol-1-yl)benzohydrazide (**6c**)*

mp. 267–269 °C; yield 63%; FTIR (KBr) cm^{-1}: 3354, 828 (NH), 1736 (C═O), 1582 (C═N), 1030 (C–O), 3045 (CH, Ar), 2898 (CH, alkane), 5437, 5402 (OH), 723 (C–Cl); mass: *m*/*z* 386.5 (M^+), 387.5 (M + 1, 21.3%), 388.5 (M + 2, 34.9%) ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.19 (s, 2H, NH), 8.11 (s, 1H, NH), 6.93–7.63 (m, 4H, ArH), 9.56 (s, 1H, Phenolic OH).

*5.6.4. N′-(2-chloroacetyl)-4-(5-(2-chlorophenyl)-3-oxo-2,3-dihydro-1H-pyrazol-1-yl)benzohydrazide (**6d**)*

mp. 252–254 °C; yield 59%; FTIR (KBr) cm^{-1}: 3388, 833 (NH), 1706 (C═O), 1596 (C═N), 1139 (C–O), 3092 (CH, Ar), 2873 (CH, alkane), 747, 664 (C–Cl); mass: *m*/*z* 405.5 (M^+), 406.5 (M + 1, 64.2%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.52 (s, 2H, NH), 7.97 (s, 1H, NH), 6.93–7.59 (m, 8H, ArH).

*5.6.5. N′-(2-chloroacetyl)-4-(3-oxo-5-phenyl-2,3-dihydro-1H-pyrazol-1-yl)benzohydrazide (**6e**)*

mp. 189–190 °C; yield 65%; FTIR (KBr) cm^{-1}: 3387, 839 (NH), 1706 (C═O), 1581 (C═N), 1098 (C–O), 3032 (CH, Ar), 2869 (CH, alkane), 713 (C–Cl); mass: *m*/*z* 370.5 (M^+), 371.5 (M + 1, 19.8%), 372.5 (M + 2, 32.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.42 (s, 2H, NH), 8.13 (s, 1H, NH), 6.98–7.67 (m, 9H, ArH).

*5.6.6. Ethyl-1-(4-(2-(2-chloroacetyl)hydrazinecarbonyl)phenyl)-5-(4-nitrophenyl)3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**6f**)*

mp. 265–267 °C; yield 81%; FTIR (KBr) cm^{-1}: 3369, 837 (NH), 1756 (C═O), 1576 (C═N), 1130 (C–O), 3032 (CH, Ar), 2859 (CH, alkane), 1387, 1498 (NO_2), 747 (C–Cl); mass: *m*/*z* 459.5 (M^+), 460.5 (M + 1, 22.1%), 461.5 (M + 2, 32.4%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.42 (t, 3H, CH_3), 3.84 (q, 2H, CH_2), 8.07 (s, 1H, NH), 8.24 (s, 2H, NH), 7.01–7.84 (m, 8H, ArH).

*5.6.7. Ethyl-1-(4-(2-(2-chloroacetyl)hydrazinecarbonyl)phenyl)-5-(2-methoxyphenyl)3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**6g**)*

mp. 168–160 °C; yield 78%; FTIR (KBr) cm^{-1}: 3382, 807 (NH), 1706 (C=O), 1566 (C=N), 1130 (C–O), 3032 (CH, Ar), 2923 (CH, alkane), 689 (C–Cl); mass: *m*/*z* 444.5 (M^+), 445.5 (M + 1, 24.4%), 446.5 (M + 2, 33.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.69 (t, 3H, CH_3), 3.92 (q, 2H, CH_2), 8.10 (s, 1H, NH), 8.43 (s, 2H, NH), 6.87–7.62 (m, 8H, ArH), 3.65 (s, 3H, OCH_3).

*5.6.8. Ethyl-1-(4-(2-(2-chloroacetyl)hydrazinecarbonyl)phenyl)-5-(2-hydroxyphenyl)3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**6h**)*

mp. 176–178 °C; yield 72%; FTIR (KBr) cm^{-1}: 3387, 887 (NH), 1745 (C=O), 1596 (C=N), 1180 (C–O), 3091 (CH, Ar), 2846 (CH, alkane), 3548 (OH), 697 (C–Cl); mass: *m*/*z* 430.5 (M^+), 431.5 (M + 1, 22%), 432.5 (M + 2, 32.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.39 (t, 3H, CH_3), 4.04 (q, 2H, CH_2), 8.07 (s, 1H, NH), 8.38 (s, 2H, NH), 6.92–7.62 (m, 8H, ArH), 9.79 (s, 1H, Phenolic OH).

*5.6.9. Ethyl-1-(4-(2-(2-chloroacetyl)hydrazinecarbonyl)phenyl)-5-(4-chlorophenyl)3-oxo-2,3-dihydro-1H-pyrazol-4-carboxylate (**6i**)*

mp. 188–190 °C; yield 70%; FTIR (KBr) cm^{-1}: 3372, 817 (NH), 1716 (C=O), 1584 (C=N), 1131 (C–O), 3082 (CH, Ar), 2859 (CH, alkane), 689, 747 (C–Cl); mass: *m*/*z* 448.5 (M^+), 449.5 (M + 1, 23.3%), 450.5 (M + 2, 65.1%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.69 (t, 3H, CH_3), 3.97 (q, 2H, CH_2), 8.01 (s, 1H, NH), 8.2 (s, 2H, NH), 6.99–7.71(m, 8H, ArH).

*5.6.10. Ethyl-1-(4-(2-(2-chloroacetyl)hydrazinecarbonyl)phenyl)-3-oxo-5-phenyl-2,3-dihydro-1H-pyrazol-4-carboxylate (**6j**)*

mp. 196–198 °C; yield 82%; FTIR (KBr) cm^{-1}: 3342, 839 (NH), 1726 (C=O), 1587 (C=N), 1142 (C–O), 3042 (CH, Ar), 2888 (CH, alkane), 667 (C–Cl); mass: *m*/*z* 414.5 (M^+), 415.5 (M + 1, 23.3%), 416.5 (M + 2, 33.1%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.74 (t, 3H, CH_3), 3.82 (q, 2H, CH_2), 8.12 (s, 1H, NH), 8.39 (s, 2H, NH), 6.90–7.68 (m, 9H, ArH).

*5.7. General procedure for the preparation of 2-oxo-2-(2-(4-(3-oxo-5-phenyl-2,3-dihydropyrazol-1-yl) benzoyl)hydrazinyl)ethyl nitrate (**7a**–**7j**)*

The compounds (**7a**–**7j**) were prepared as per procedure reported in the literature [18].

*5.7.1. 2-(2-(4-(5-(4-Nitrophenyl)-3-oxo-2,3-dihydropyrazol-1-yl)-benzoyl)hydrazinyl)-2-oxoethylnitrate (**7a**)*

mp. 210–212 °C; yield 47%; FTIR (KBr) cm^{-1}: 3399, 826 (NH), 1763 (C=O), 1590 (C=N), 1090 (C–O), 1491, 1374 (NO_2); mass: *m*/*z* 442 (M^+), 443 (M + 1, 19.9%), 444 (M + 2, 3.9%); ^1H NMR (300 MHz, $CDCl_3$), δ ppm, 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.42 (s, 2H, NH), 8.05 (s, 1H, NH), 6.92–7.68 (m, 8H, ArH). Anal. Calcd for $C_{18}H_{14}N_6O_8$: C, 48.87; H, 3.19; N, 19.00. Found: C, 48.84; H, 3.11; N, 18.9.

*5.7.2. 3-(2-(4-(5-(2-Methoxyphenyl)-3-oxo-2,3-dihydropyrazol-1-yl)-benzoyl)hydrazinyl)-2-oxoethylnitrate (**7b**)*

mp. 192–194 °C; yield 63%; FTIR (KBr) cm^{-1}: 3385, 840 (NH), 1677 (C=O), 1598 (C=N), 1098 (C–O), 1491, 1374 (NO_2); mass: *m*/*z* 427 (M^+), 428 (M + 1, 21%), 429 (M + 2, 3.5%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.18 (s, 2H, CH_2), 8.32 (s, 2H, NH), 8.12 (s, 1H, NH), 7.09–7.89 (m, 8H, ArH), 3.54 (s, 3H, OCH_3). Anal. Calcd for $C_{19}H_{17}N_5O_7$: C, 53.40; H, 4.01; N, 16.39. Found: C, 53.28; H, 3.99; N, 16.37.

*5.7.3. 3-(2-(4-(5-(2-Hydroxyphenyl)-3-oxo-2,3-dihydropyrazol-1-yl)-benzoyl)hydrazinyl)-2-oxoethylnitrate (**7c**)*

mp. 190–192 °C; yield 71.83%; FTIR (KBr) cm^{-1}: 3375, 848 (NH), 1684 (C=O), 1595 (C=N), 1091 (C–O), 1488, 1302 (NO_2), 3540, 3500, 3158 (Phenolic OH); mass: *m*/*z* 413 (M^+), 414 (M + 1, 19.9%), 415 (M + 2, 3.6%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.47 (s, 2H, NH), 8.0 (s, 1H, NH), 6.79–7.59 (m, 8H, ArH), 9.56 (s, 1H, Phenolic OH). Anal. Calcd for $C_{18}H_{15}N_5O_7$: C, 52.30; H, 3.66; N, 16.94. Found: C, 52.43; H, 3.73; N, 17.12.

*5.7.4. 3-(2-(4-(5-(4-Chlorophenyl)-3-oxo-2,3-dihydropyrazol-1-yl)-benzoyl)hydrazinyl)-2-oxoethylnitrate (**7d**)*

mp. 218–220 °C; yield 55%; FTIR (KBr) cm^{-1}: 3327, 842 (NH), 1783 (C=O), 1598 (C=N), 1092 (C–O), 1412, 1262 (NO_2), 664 (C–Cl); mass: *m*/*z* 431.5 (M^+), 432.5 (M + 1, 19.9%), 433.5 (M + 2, 32.3%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 3.98 (s, 2H, CH_2), 8.29 (s, 2H, NH), 7.79 (s, 1H, NH), 6.89–7.70 (m, 8H, ArH). Anal. Calcd for $C_{18}H_{14}ClN_5O_6$: C, 50.07; H, 3.27; N, 16.22. Found: C, 50.13; H, 3.31; N, 16.31.

*5.7.5. 2-Oxo-2-(2-(4-(3-oxo-5-phenyl-2,3-dihydropyrazol-1-yl)-benzoyl)hydrazinyl)ethylnitrate (**7e**)*

mp. 208–210 °C; yield 73%; FTIR (KBr) cm^{-1}: 3397, 844 (NH), 1692 (C=O), 1594 (C=N), 1095 (C–O), 1495, 1294 (NO_2); mass: *m*/*z* 397 (M^+), 398 (M + 1, 19.9%), 399 (M + 2, 3.1%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 6.14 (s, 1H, CH), 4.04 (s, 2H, CH_2), 8.3 (s, 2H, NH), 8.05 (s, 1H, NH), 6.92–7.61 (m, 9H, ArH). Anal. Calcd for $C_{18}H_{15}N_5O_6$: C, 54.41; H, 3.81; N, 17.63. Found: C, 54.13; H, 3.69; N, 17.48.

*5.7.6. Ethyl-1-(4-(2-(2-(nitrooxy)acetyl)hydrazinecarbonyl) phenyl)-5-(4-nitrophenyl)-3-oxo-2,3-dihydropyrazol-4-carboxylate (**7f**)*

mp. 240–242 °C; yield 67%; FTIR (KBr) cm^{-1}: 3383, 829 (NH), 1713 (C=O), 1599 (C=N), 1019 (C–O), 1492, 1399 (NO_2); mass: *m*/*z* 514 (M^+), 515 (M + 1, 25.5%), 516 (M + 2, 2.6%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.71 (t, 3H, CH_3), 4.02 (q, 2H, CH_2), 3.79 (s, 2H, CH_2), 8.03 (s, 1H, NH), 8.5 (s, 2H, NH), 7.02–7.81 (m, 8H, ArH). Anal. Calcd for $C_{21}H_{18}N_6O_{10}$: C, 49.03; H, 3.53; N, 16.34. Found: C, 49.1; H, 3.57; N, 16.36.

*5.7.7. Ethyl-1-(4-(2-(2-(nitrooxy)acetyl)hydrazinecarbonyl) phenyl)-5-(2-methoxyphenyl)-3-oxo-2,3-dihydropyrazol-4-carboxylate (**7g**)*

mp. 263–265 °C; yield 73%; FTIR (KBr) cm^{-1}: 3385, 838 (NH), 1677 (C=O), 1598 (C=N), 1098 (C–O), 1491, 1374 (NO_2); mass: *m*/*z* 499 (M^+), 500 (M + 1, 24.4%), 501 (M + 2, 4.7%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.62 (t, 3H, CH_3), 3.98 (q, 2H, CH_2), 4.02 (s, 2H, CH_2), 7.89 (s, 1H, NH), 8.39 (s, 2H, NH), 6.99–7.7 (m, 8H, ArH), 3.65 (s, 3H, OCH_3). Anal. Calcd for $C_{21}H_{19}N_5O_9$: C, 51.96; H, 3.95; N, 14.43. Found: C, 51.92; H, 3.89; N, 14.42.

*5.7.8. Ethyl-1-(4-(2-(2-(nitrooxy)acetyl)hydrazinecarbonyl)phenyl) -5-(2-hydroxyphenyl)-3-oxo-2,3-dihydropyrazol-4-carboxylate (**7h**)*

mp. 230–232 °C; yield 70.25%; FTIR (KBr) cm^{-1}: 3537, 836 (NH), 1678 (C=O), 1599 (C=N), 1163 (C–O), 1512, 1282 (NO_2), 3237 (OH); mass: *m*/*z* 485 (M^+), 486 (M + 1, 25.1%), 487 (M + 2, 4.7%); ^1H NMR (300 MHz, $CDCl_3$, δ ppm), 1.49 (t, 3H, CH_3), 3.90 (q, 2H, CH_2), 4.03 (s, 2H, CH_2), 7.82 (s, 1H, NH), 8.35 (s, 2H, NH), 6.77–7.48 (m, 8H, ArH), 9.12 (s, 1H, Phenolic OH). Anal. Calcd for $C_{22}H_{21}N_5O_9$: C, 52.91; H, 4.24; N, 14.02. Found: C, 52.94; H, 4.25; N, 14.01.

*5.7.9. Ethyl-1-(4-(2-(2-(nitrooxy)acetyl)hydrazinecarbonyl)phenyl) -5-(4-chlorophenyl)-3-oxo-2,3-dihydropyrazol-4-carboxylate (**7i**)*

mp. 218–220 °C; yield 65%; FTIR (KBr) cm^{-1}: 3323, 820 (NH), 1705 (C=O), 1593 (C=N), 1088 (C–O), 1482, 1213 (NO_2), 677 (C–Cl);

mass: *m*/*z* 503.5 (M^+), 504.5 (M + 1, 23.2%), 505.5 (M + 2, 32.4%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 1.59 (t, 3H, CH_3), 3.74 (q, 2H, CH_2), 3.89 (s, 2H, CH_2), 8.03 (s, 1H, NH), 8.21 (s, 2H, NH), 6.99–7.64 (m, 8H, ArH). Anal. Calcd for $C_{21}H_{18}ClN_5O_8$: C, 50.06; H, 3.60; Cl, 7.04; N, 13.90. Found: C, 50.09; H, 3.62; N, 13.94.

*5.7.10. Ethyl-1-(4-(2-(2-(nitrooxy)acetyl)hydrazinecarbonyl) phenyl)-3-oxo-5-phenyl-2,3-dihydro-1*H*-pyrazol-4-carboxylate (**7j**)*

mp. 200–202 °C; yield 87%; FTIR (KBr) cm^{-1}: 3396, 845 (NH), 1687 (C═O), 1590 (C═N), 1088 (C–O), 1475, 1393 (NO_2); mass: *m*/*z* 469 (M^+), 470 (M + 1, 23.2%), 471 (M + 2, 4.2%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm), 1.71 (t, 3H, CH_3), 3.69 (q, 2H, CH_2), 4.0 (s, 2H, CH_2), 7.98 (s, 1H, NH), 8.21 (s, 2H, NH), 7.1–7.82 (m, 9H, ArH). Anal. Calcd for $C_{21}H_{19}N_5O_8$: C, 53.73; H, 4.08; N, 14.92. Found: C, 53.74; H, 4.10; N, 14.96.

6. Molecular docking studies

All the compounds were constructed using standard fragment library of Maestro 8.0 and geometry optimized by Macromodel programme (Schrödinger, LLC) using the Optimized Potentials for Liquid Simulations-all atom (OPLS-AA) force field 2005 [32].

The X-ray crystal structure of COX-2 enzyme (PDB ID: 1CX2) was obtained from the RCSB Protein Data Bank (PDB) was used in order to get the detailed sights of ligand–protein structure in this study. Water molecules of crystallization were removed from the complex and the protein was optimized for docking using the protein preparation wizard and refinement utility provided by Schrödinger LLC 8.0.

All the docking calculations were performed using "Standard Precision" (SP) mode of Glide 4.5 programme and the 2005 implementation of OPLS-AA force field. The binding site, for which the various energy grids were calculated and stored, was defined in terms of two concentric cubes; the binding box, which must contain the center of any acceptable ligand pose, and the enclosing box, which must contain all ligand atoms of an acceptable pose. Cubes with an edge length of 12 Å and centered at the midpoint of the longest atom–atom distance in the respective co-crystallized ligand defined the binding box in the protein. The large enclosing box was also defined in terms of the co-crystallized ligand: an edge length of 20 Å was used. Poses with an RMSD of less than 0.3 Å were used for optimization. The scale factor for van der Waals radii was applied to those atoms with absolute partial charges less than or equal to 0.15 (scale factor of 0.8) and 0.25 (scale factor of 1.0) electrons for ligand and protein, respectively. The best docked structure was chosen using a Glide score (Gscore) function. The Gscore is a modified and extended version of the empirically based Chemscore function. Another scoring function used by Glide is Emodel, which itself derived from a combination of Gscore, energy in coloumbs, van der Waals and the strain energy of the ligand. Besides this energy, contacts which include good, bad and ugly van der Waals interactions were also taken into consideration for the evaluation of the docked complexes. GScore is calculated as given Eq. (1).

$$\text{Gscore} = a \times \text{vdw} + b \times \text{Coul} + \text{Libo} + \text{H-bond} + \text{Metal} + \text{BuryP} + \text{RotB} + \text{Site} \quad (1)$$

where, vdw: van der Waals energy; Coul: Coulomb energy; Lipo: Lipophilic contact term; H-Bond: Hydrogen-bonding term; Metal: Metal-binding term; BuryP: Penalty for buried polar groups; RotB: Penalty for freezing rotatable bonds; Site: Polar interactions at the active site; and the coefficients of vdw and Coul are: $a = 0.065$, $b = 0.130$.

7. Pharmacology

7.1. Animals

Swiss albino mice of either sex weighing 20–25 g and Wistar rats weighing in the range 100–120 g were obtained from, National Center for Cell Science (NCCS), Pune, India. All the animals were housed under standard ambient conditions of temperature (25 ± 2 °C) and relative humidity of 50 ± 5%. A 12:12 h light:dark cycle was maintained. All the animals were allowed to have free access to water and standard palletized laboratory animal diet 24 h prior to pharmacological studies. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of College, Pune, constituted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA/257, CPCSEA/300), Government of India [33].

7.2. Preparation of test compounds

After suspending the test compounds in 1.0% aqueous solution of sodium carboxymethyl cellulose (CMC), test samples were administered to test animals orally. The positive and negative control group animals received the same experimental handling as those of the test groups except that the drug treatment, control group animals received only appropriate volumes of vehicle and of the reference drug, Indomethacin, respectively.

7.3. Anti-inflammatory activity

Anti-inflammatory activity was evaluated using the well known carrageenan induced rat paw oedema model of Winter et al. [34] using groups of six animals each. A freshly prepared aqueous suspension of carrageenan (1.0% w/v, 0.1 mL) was injected in the subplantar region of right hind paw of each rat. One group was kept as control and the animals of the other group were pretreated with the test drugs, 1 h before the carrageenan treatment. The volume was measured before and after carrageenan treatment at the 30 min interval with the help of digital plethysmometer (Panlab LE 7500, USA).

7.4. Analgesic activity

The analgesic activity was evaluated using the acetic acid induced writhing method [35].

7.5. Acute ulcerogenicity studies

Acute ulcerogenicity screening was done according to method reported by Cioli et al. [36]. The mucosal damage was examined by means of an electron microscope. For each stomach specimen, the mucosal damage was assessed according to the following scoring system.

7.5.1. Score description

0.0 Normal (no injury, bleeding and latent injury).
0.5 Latent injury or widespread bleeding (>2 mm).
1.0 Slight injury (2–3 dotted lines).
2.0 Severe injury (continuous lined injury or 5–6 dotted injuries).
3.0 Very severe injury (several continuous lined injuries).
4.0 Widespread lined injury or widened injury/erosion.

The mean score of each treated group minus the mean score of control group was regarded as severity index of gastric mucosal damage. Data are expressed as mean ulcer score ± SEM; data analyzed by one-way ANOVA followed by Dunnett's test to determine the significance of the difference between the standard group and rats treated with the test compounds. The differences in results were considered significant when P was found to be <0.01.

7.6. Histopathological studies [37–40]

For the histopathological study, rats were sacrificed 4 h after the cold stress and their stomach specimens were removed and put into 10% formalin solution. A longitudinal section of stomach along the greater curvature, which included the ulcer base and both sides of the ulcer margin, was taken and fixed in 10% formalin for 24 h at 4 °C and embedded in white solid paraffin. Morphological examination was performed with Haematoxylin and Eosin staining to analyze histological changes and examined under electron microscope. The disturbances in GI epithelial morphology were closely analyzed and recorded in the form of images. The results of the same are depicted in Fig. 1.

7.7. Vasorelaxing activity [41,42]

To determine a possible vasodilatory mechanism of action, the compounds were tested on isolated aortae of male normotensive Wistar rats (250–350 g). The rats were killed by cervical dislocation under light ether anaesthesia and bled. The aortae were immediately excised, freed of extraneous tissues and prepared as multiple-ring preparations. Then the vessels were suspended, under a preload of 2 g, in 10 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4 \cdot 7H_2O$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously bubbled with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (BIOPAC System, Inc, MP 35).

After an equilibration period of 60 min, the endothelial integrity was confirmed by Acetylcholine (ACh) (55 μM) induced relaxation of Norepinephrine (NE. 20 μg/mL) precontracted tissues. A relaxation ≥ 70% of the NE-induced contraction was considered representative of an acceptable presence of the endothelial layer, while the organs, showing a relaxation < 70%, were not used in the experimental procedures. 30–40 min after the confirmation of the endothelial integrity, the aortic preparations were contracted by treatment with a single concentration of NE (20 μg/mL) or KCl (30 mM) and when the contraction reached a stable plateau, the test compounds of concentration 0.1 mg/mL were added cumulatively.

7.8. Assay of in vitro NO release [20,43]

A solution of the appropriate compound (20 μL) in dimethylsulfoxide (DMSO) was added to 2 mL of 1:1 v/v mixture of either 50 mM phosphate buffer (pH 7.4) or of an HCl solution (pH 1) with MeOH, containing 5× of 10^{-4} M L-cysteine. The final concentration of drug was 10^{-4} M. After 1 h at 37 °C, 1 mL of the reaction mixture was treated with 250 μL of Griess reagent [Sulfanilamide (4 g), *N*-naphthylethylenediamine dihydrochloride (0.2 g), 85% phosphoric acid (10 mL) in distilled water (final volume: 100 mL)]. After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 μmol/mL) were used to construct the calibration curve. The results were expressed as the percentage of NO released ($n = 2$) relative to a theoretical maximum release of 1 mol NO/mol of test compound.

7.9. Statistical analyses

Data obtained for each set of anti-inflammatory model were expressed as mean of change in paw volume ± SD and analyzed by one-way ANOVA followed by Dunnett's test. Data from acetic acid induced writhing model were expressed as mean of number of writhes ± SEM and analyzed by one-way ANOVA followed by Dunnett's '*t*' test. Level of significance was set to $P < 0.05$. All statistical calculations were performed using evaluation version of Graph Pad® Prism 3.0 (USA) statistical software [44].

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