Pharmacological Screening of Novel Nitric Oxide Donars Containing 1, 5-Diaryl Pyrazolin-3-One as Non Toxic NSAIDs

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Summary

Various substituted 1,5-diarylpyrazol-3-one derivatives were 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 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. Thus, the rationale used to design the NCEs was found to produce the promising results as anticipated.

Key words: 1,5-diarylpyrazolin-3-one; Nitric oxide; Anti-inflammatory; Analgesic; Non-ulcerogenic; Vasodilatory.

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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 exhibit 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 approach i.e. molecule made up of non-selective or selective COX inhibitors together with a vasodilator (nitric oxide releasing) function. 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 [18].

It's 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) on to COX-2 selective pharmacophore. In this paper we report pharmacological screening of some 1,5-diarylpyrazolin-3-one pharmacophore containing compounds.

Materials and Methods

Experimental 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 [19].

Chemicals:

Carrageenan (Sigma-Aldrich, USA), acetic acid (Spectro. chem. Ltd., Mumbai), Anaesthetic Ether I.P. (TKM Pharma, Hyderabad) were procured. Indomethacin and Diclofenac were obtained locally. All the chemicals were of analytical grade.

Anti-inflammatory Activity:

Anti-inflammatory activity was evaluated using the well known Carrageenan induced rat paw oedema model of Winter et al. [20] using groups of six animals each. A freshly prepared aqueous suspension of carrageenan (1.0% w/v, 0.1 mL) was injected in the subplanter 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 1 h, 2 h and 3 h interval with the help of digital plethysmometer (Panlab LE 7500, USA) by using the following formula:

$$\% \textit{ Anti-inflammatory activity} = (Vc - Vt / Vc) \times 100$$

Where Vt represents the mean increase in paw volume in rats treated with test compounds and Vc represents the mean increase in paw volume in control group of rats. Data are expressed as % anti-inflammatory activity ± S.E.M. and analyzed by one-way ANOVA followed by Dunnett's t test to determine the significance of the difference between the control group and rats treated with the test compounds. The difference in results were considered significant when $P < 0.01$. All statistical calculations were carried out using Graph Pad® Prism 3.0 (USA) statistical software.

Analgesic Activity:

Compounds were screened for analgesic activity in comparison with Indomethacin. The acetic acid induced writhing test was performed by injecting 0.1 ml of 1 % aqueous acetic acid solution intraperitoneally. Animals were divided in a group of 6 each. Mice were housed individually in the test cage, before acetic acid injection and allowed to acclimatize for 30 min. prior to dosing. Analgesic activity was screened at the dose of 10 mg/kg body weight. All the compounds were suspended in aqueous 1% CMC solution. The control group animals received only 1% CMC solution. Indomethacin was used as reference drug to validate the model on experimental animals. After 1 h of drug administration, 0.10 ml of 1% acetic acid solution was injected to mice intraperitoneally. Stretching movements consisting of arching of the back, elongation of body and

extension of hind limbs were counted for 5–15 min after acetic acid injection. The analgesic activity was expressed in terms of % inhibition, and calculated as follows:

$$\% \text{ Analgesic activity} = (nc - nt/nc) \times 100$$

Where,

nc = mean number of writhes of control group and

nt = mean number of writhes of test group.

Data are expressed as mean no. of writhes ± S.E.M., one way ANOVA followed by Dunnetts test was applied to determine the significance of the difference between the control group and mice treated with the test compounds. The difference in results were considered significant when $P < 0.01$. All statistical calculations were performed using Graph Pad® Prism 3.0 (USA) statistical software.

Acute ulcerogenecity studies:

Acute ulcerogenicity screening was done according to method reported by Cioli et al. [21]. Albino rats were divided into different groups of six animals in each group. Potential for ulcerogenecity was evaluated after p.o. administration of test or standard compounds at 12 times the therapeutic doses. Control rats received 1% CMC as vehicle. Animals were fasted for 24 h before dosing, with water ad libitum. In order to induce prominent ulcers, after the drug treatment, the rats were exposed to cold stress at -20ºC for 4 h and then sacrificed by ether inhalation. The animals were sacrificed and dissected along the greater curvature of the stomach. And the stomach specimen were washed with distilled water and cleaned gently by dipping in saline. 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.

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.

Histopathology studies: [22-25]

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 analysed and recorded in the form of images.

Nitric Oxide releasing study:

1. Vasorelaxing activity [26, 27]

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; KC1 2.95; $CaCl_2$ 1.80; $MgSO_4$ $7H_2O$ 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 minutes, 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 minutes 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 0.1mg/mL were added cumulatively.

2. Detection of nitrite: [18, 28]

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.

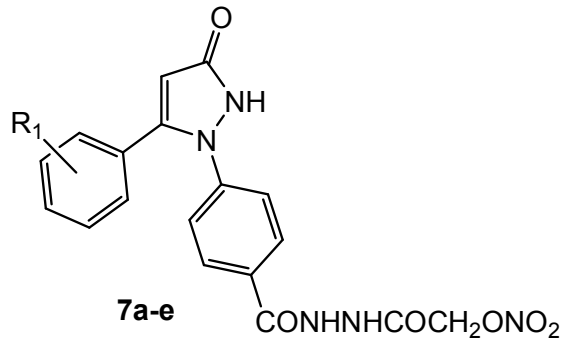
Results

The analgesic and anti-inflammatory activities were evaluated using equimolar doses compared to the standard, Ibuprofen for Albino mice as well as Wistar rats.

Effect of Synthesized derivatives on caraageenan induced rat paw edema.

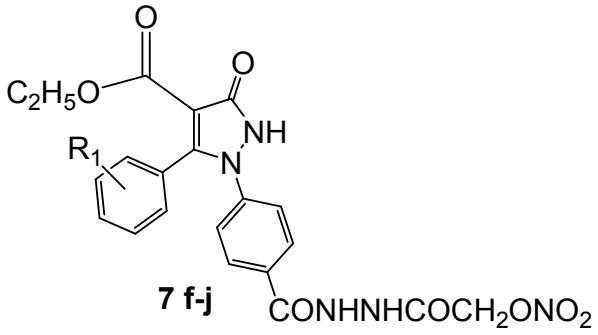
Subplanter injection of carrageenan produced increase in paw volume (inflammation) of all the animals of various groups. The onset of action was evident from one hour in all the groups. The onset of reduction at 1 h. of rat paw volume was shown by only few compounds viz. 7b and 7j. The significant reduction of rat paw edema was observed by the above test compounds at 3 h compared to vehicle treated group. (Table 1)

Table 1: Chemical structure of synthesized derivatives and there anti-inflammatory activity against carrageenan induced rat paw edema.



7a-e

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



7 f-j

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

Mean reduction in paw volume (mL) after treatment with test compounds (Mean ±	**Anti-inflammatory activity (% inhibition)**

SEM)						
Compounds	**1h**	**2h**	**3h**	**1h**	**2h**	**3h**
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.

Results of Analgesic activity for synthesized compounds by acetic acid induced writhing model in Swiss Albino mice:

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 exhbited significant analgesic activity (Table 2). The analgesic effects of **(7j)** (56.86%) were found to be better than that of Indomethacin (54.90%).

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	10.78ns
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	10.78ns
7i	8.44	19 ± 0.83	6.86ns
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.

Gastric Ulcerogenic studies after single oral administration of the compounds under investigation:

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 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.

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 occures. Thus, such functionalization of free –COOH of Diclofenac in to pyrazolinone ring and presence of additional NO releasing function has resulted into more potent and nontoxic New Chemical Entities (NCEs).

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	-	-

Nitric oxide releasing study:

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 was 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).

Table 4: EC_{50} and Nitric Oxide releasing properties of the compounds (7a-7j).

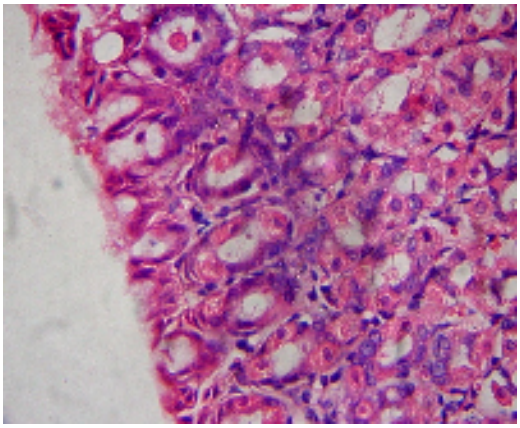
Comp. Code	EC_{50}	% NO release[b]
7a	49.11	0.39%
7b	66.85	0.32%
7c	17.82	0.69%
7d	21.32	0.66%
7e	26.74	0.51%
7f	28.18	0.49%
7g	63.09	0.35%
7h	70.79	0.27%
7i	50.18	0.37%
7j	35.48	0.46%

[b] 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.

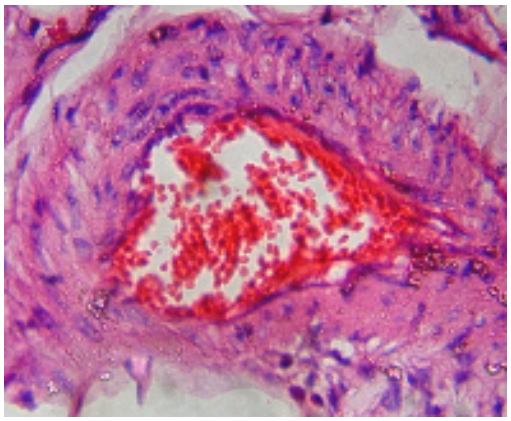
Histopathology studies:

The stomach specimen of Diclofenac acid treated rats were characterized by complete disruption of protective mucosal layer **(Fig. 1** specimen b**).** Histopathological analysis also showed characteristic features of ulceration in Diclofenac acid treated group of animals. The tissue of Diclofenac acid treated rats have 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 **Figure 1**, specimen c-f which is identical to that of the control, specimen a.



a



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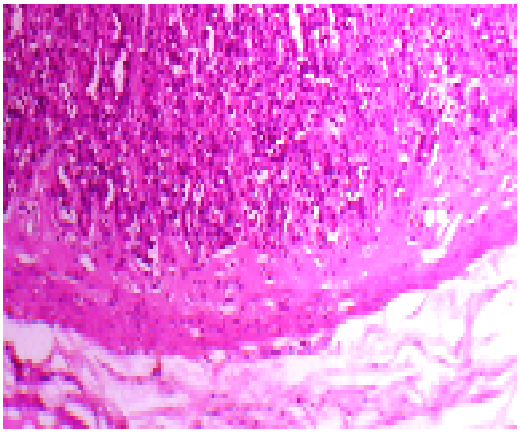

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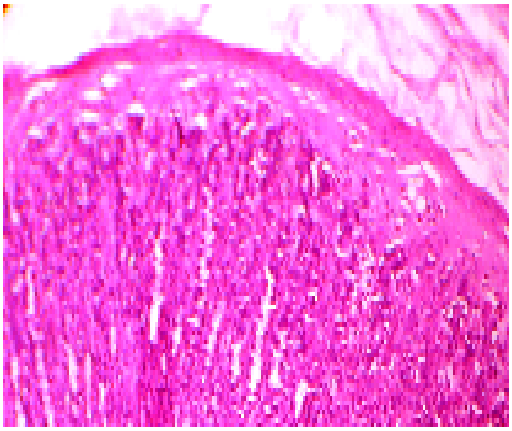

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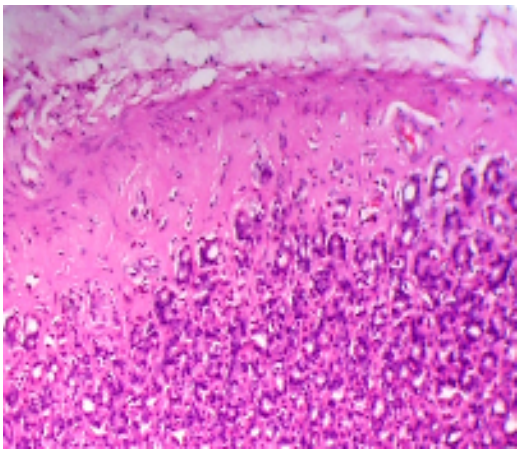

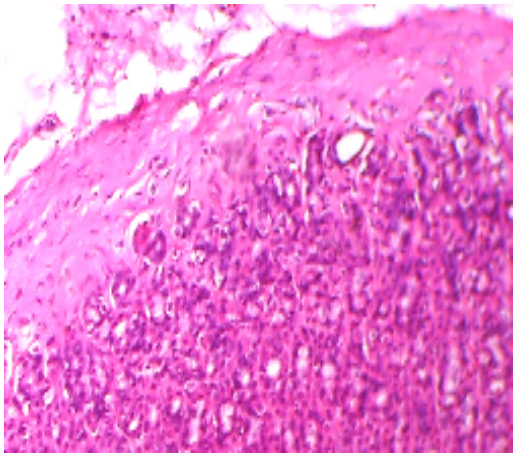

e **f**

Figure 1: Haematoxylin and eosin Immunohistochemical staining of gastric ulcers after ulcer induction in rats. As illustrated in Fig. 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 200X.

Discussion

The synthesized compounds were tested and compared with the standard drug Indomethacin. The tested compound showed anti-inflammatory activity ranging from 5.84% to 59.64% at 3 h. (Table 1), where as the standard drug Indomethacin showed 57.89% edema inhibition at 3 h after drug treatment. The maximum activity (59.64%) was shown by 7j. Effect of Indomethacin and test compounds on percent inhibition of paw edema in rats at various time intervals (1 h and 3 h) is shown below (Figure 2).

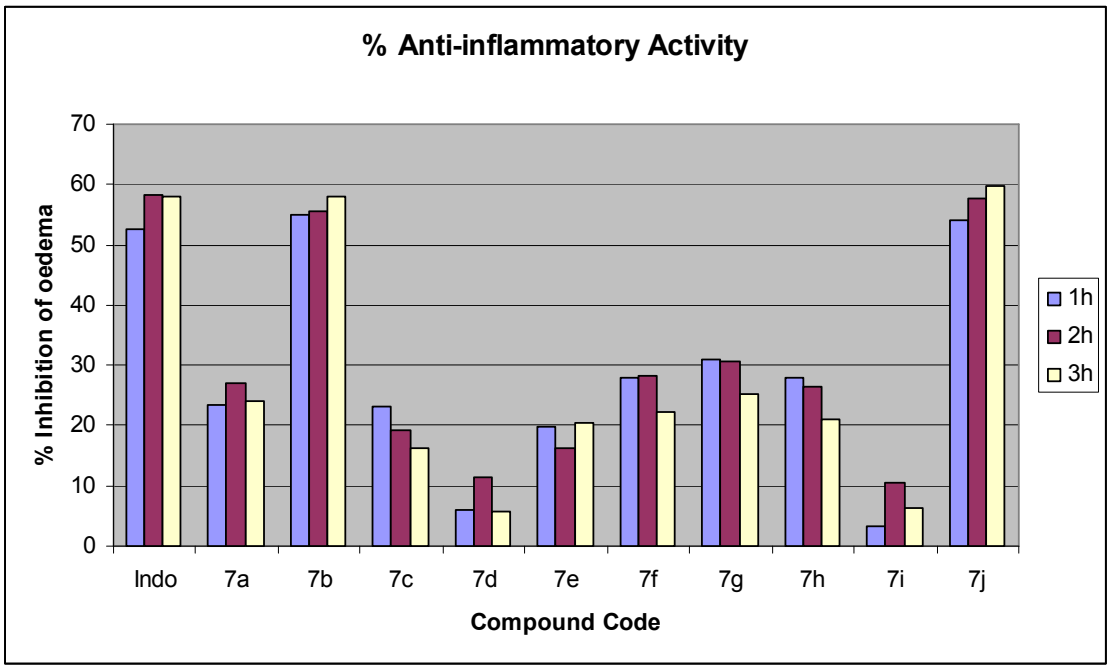

Figure 2: Comparison of % anti-inflammatory action of test compounds at 3 hr.

All the synthesized compounds showed analgesic activity ranging from 6.86% to 56.86%. The compound 7j (56.86%) showed better analgesic activity than the standard drug Indomethacin (54.9%).

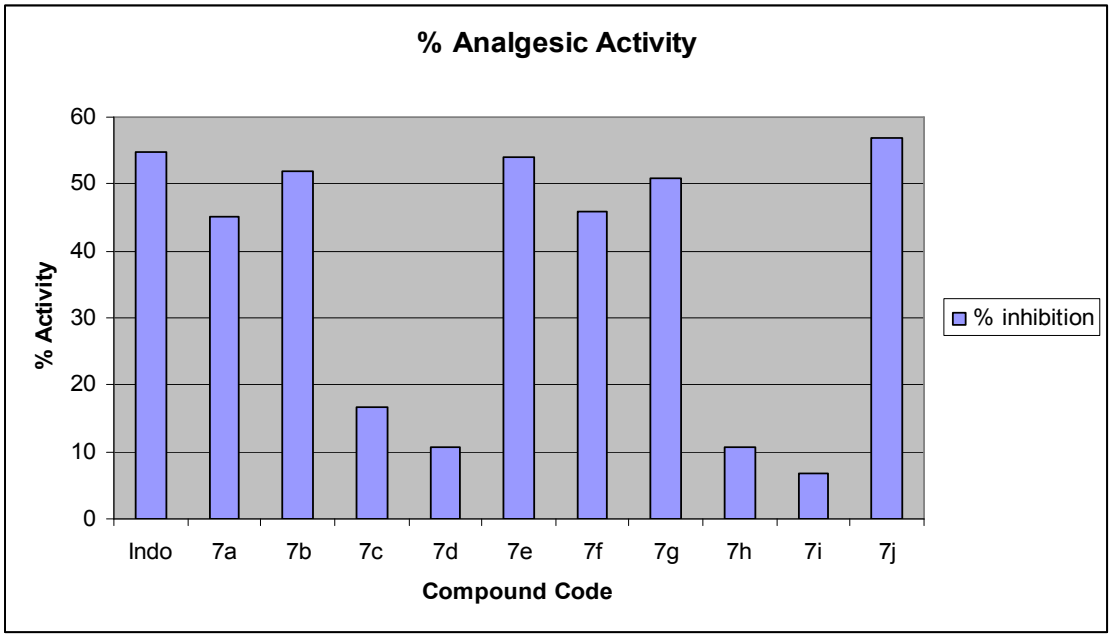

Figure 3: Comparison of % analgesic activity of test compounds with Indomethacin.

The compound which showed anti-inflammatory activity comparable to that of standard drug Indomethacin and also showed high analgesic activity were screened for potential for ulcerogenecity. Histopathological analysis showed no ulcerative features in rats treated with 7a, 7b, 7g, and 7j group, the sections were also devoid of mucosal hemorrhages, and mucosal congestion and ulceration .In the tissue of control group the mucous membrane was intact without any damage to the adjacent cells. Same effect was seen in the group treated with the 7a, 7b, 7g and 7j derivatives. While the group treated with standard drug Diclofenac showed proliferation and migration of epithelial cells in the region of ulcer crater, which was strongly infiltrated by inflammatory cells. It also showed marked evidence of congestion of mucosal blood vessels, mucosal ulceration and mucosal hemorrhages.

In all it can be concluded that compound 7a, 7b, 7g, and 7j are devoid of Ulcerogenic activity at 12 times the therapeutic dose while retaining their anti-inflammatory properties in animal models. The most potent and safer synthesized derivatives can be further subjected to acute and chronic toxicity studies and to clinical studies if found to be nontoxic. Also all the synthesized derivatives exhibited significant nitric oxide releasing property both in-vivo and in-vitro.

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