ANTI-INFLAMMATORY, ANALGESIC, ULCEROGENIC AND NITRIC OXIDE RELEASING ACTIVITIES OF SOME NOVEL NON-STEROIDAL IBUPROFEN ANALOGS IN ANIMAL MODELS

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Summary

Ibuprofen, widely used NSAID since last 41 years. But even this drug is not an exception to the limitations of gastrointestinal adverse effects associated with the traditional non-selective NSAIDs. The free –COOH group is reported to be the main culprit responsible for GI toxicity of these NSAIDs. Hybrid molecules with nitric oxide releasing group is a new feature in the NSAIDs. Hybrid molecules of ibuprofen like structure, Indomethacin like structure and nitric oxide releasing group were tested in vivo for their anti-inflammatory activity. The compounds, which showed significant analgesic and anti-inflammatory activities comparable to the standard drug Ibuprofen, were screened for their ulcerogenic potential to make sure that designed and synthesized compounds lack ulcerogenecity. The study showed that compound 7a possessed most significant anti-inflammatory activity where as compound 7c and 7d possessed most significant analgesic activity compared to parent drug Ibuprofen. The compound also showed non ulcerogenic action at 12 times the therapeutic dose in animal models. The ulcers in rats were analyzed by histopathological studies. Results showed that compound 7a, 7c, 7d, 7f and control group were unremarkable, and were also devoid of mucosal hemorrhages, mucosal congestion and ulceration compared to that of standard drug Diclofenac also all the synthesized compounds show significant vasodilatory activity.

Key words: Ibuprofen, nitric oxide, anti-inflammatory, analgesic, ulcerogenicity, vasodilatory.

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Introduction

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) are among the most commonly prescribed drugs to reduce pain, inflammation, and fever. Among the most popular NSAIDs, Ibuprofen was introduced 39 years ago and since widely used as compare to other NSAIDs[1].

Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce pain and edema by suppressing the formation of prostaglandins, by inhibiting the activity of the enzyme Cyclooxygenase (COX-1 and COX-2). However, it is now clear that both COX-1 and COX-2 isoforms contribute to mucosal defense. Selective COX-2 inhibitors elicit less GI damage and bleeding than conventional NSAIDs, although the magnitude of this reduction continues to be contested in the literature. As widely reported in the lay-press, the selective COX-2 inhibitors also cause significant adverse effects in the renal and cardiovascular systems, possibly more serious than those caused by conventional NSAIDs. However, their use is limited by their significant side effects upon the stomach and the kidney. Their side effects as well as their therapeutic actions are related to their ability to inhibit cyclooxygenase enzymes involved in the first step of the arachidonic acid cascade [2, 3].

Recent strategies adopted to minimize the side effects of NSAIDs include the use of the dual LOX/COX inhibitors, the use of selective COX-2 inhibitors, and the use of hybrid molecules made up of non-selective or selective COX inhibitors together with a nitric oxide releasing function [4-6]. Recent data revealed serious cardiovascular side effects associated with selective COX-2 inhibitors [5, 7]. In addition, such drugs only minimize the development of new gastric ulcers but do not affect the existing ones [8]. The strategy involving the use of hybrid molecules made up of non-selective COX inhibitors together with a nitric oxide donating moiety constitutes one of the most promising approaches, because nitric oxide supports several endogenous GIT defense mechanisms, including increase in mucus, bicarbonate secretions, increase in mucosal blood flow, and inhibition of the activation of proinflammatory cells.

Moreover, because of the beneficial cardiovascular effects of Nitric Oxide, such drugs are expected to be devoid of the potential adverse cardiovascular effects associated with the use of selective COX-2 inhibitors [6, 9]. Among those Nitric Oxide-NSAIDs that came into clinical trials are nitroaspirin, nitronaproxene, nitroketoprofen, nitroibuprofen, etc. Among the nitric oxide donors adopted to prove the validity of this principle are furoxans, oximes, hydrazides, and organic nitrates [10-12].

In our attempt to discover new, safer and potent agents for treatment of inflammatory diseases, we have synthesized the hybrid molecule having ibuprofen moiety, indomethacin like structure and nitric oxide releasing group order to accentuate potency and reduce GI toxicities associated with the traditional NSAIDs. The compounds designed so, were found to possess much significant analgesic, anti-inflammatory, vasodilatory profile with significant reduction in potential for ulcerogenic toxicities.

Materials and Methods

Experimental Animals:

Swiss albino mice of either sex weighing 20–25 g and wistar rats weighing in the range 140-160g were obtained from National Centre for Cell Sciences (NCCS) , Pune, India. All the animals were housed under standard environmental conditions of temperature (24±2°C) and relative humidity of 30-70 %. A 12:12 h light: dark cycle was maintained. All the animals were allowed to have free access to water and standard pelletized laboratory animal diet. 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 Experiment on Animals (CPCSEA), Government of India.

Chemicals

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

Anti-inflammatory activity: [13]

This activity was performed by the following procedure of Winter et al. on groups of six animals each. A freshly prepared suspension of carrageenan (1.0% w/v, 0.1 ml) was injected in the planter 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 suspended in 1.0% Carboxy Methyl Cellulose (CMC) given orally 1 h before the carrageenan treatment. The volume was measured after 1h, 2 h and 3 h of carrageenan treatment with the help of digital plethysmometer (Panlab LE 7500 SI, Spain). The percent anti-inflammatory activity was calculated at 1 h, 2 h and 3 h according to the following formula:

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

Where *V*t represents the mean increase in paw volume in rats treated with test compounds and *V*c 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: [14]

Compounds were screened for analgesic activity in comparison with Ibuprofen. 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. Ibuprofen 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:

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

Where,

nc = mean number of writhes of control group and

*n*t = 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: [15]

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 a magnifying glass. For each stomach, the mucosal damage was assessed according to the following scoring system:

Score Assignment

0.0 Normal (No injury, bleeding, and latent injury)

0.5 Latent injury or widespread bleeding.

1.0 Slight injury (2 to 3 dotted lines).

2.0 Severe injury (continuous lined injury or 5 to 6 dotted injuries).

3.0 Very severe injury (several continuous lined injuries).

4.0 Widespread lined injury or widened injury.

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 ± S.E.M., 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 is < 0.01.

Histopathology studies: [16, 17]

For the histopathological study, rats were sacrificed 4h after the cold stress and their stomach were removed and put into 10% formalin solution. A longitudinal section of stomach along the greater curvature, which included the ulcer based and both sides of the ulcer margin, was taken and fixed in 4% formalin for 24 h at 4ºC and embedded in paraffin. Morphological examination was performed with haematoxylin and eosin staining for histological changes and examined under light microscope.

Nitric Oxide releasing study:

1. Vasorelaxing activity [18, 19]

To determine a possible vasodilator 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 Norepineohrine (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 in concentration (0.1mg/mL) were added cumulatively.

2. Detection of nitrite [20, 21]

A solution of the appropriate compound (20 μL) in dimethylsulfoxide (DMSO) was added to 2 mL of 1:1 v/v mixture either of 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.

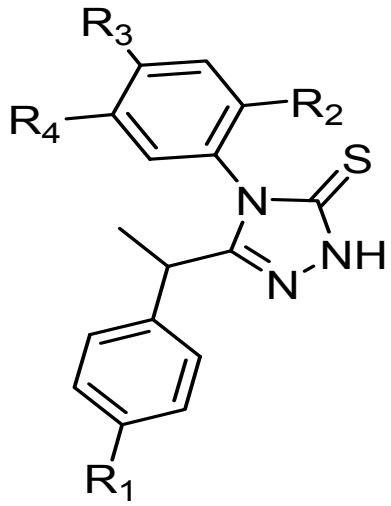
Results

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. 7a, 7c,7d and 7f. 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.



Comp. code	R_1	R_2	R_3	R_4
7a	isobutyl	2-(2-acetylhydrazinyl)-2-oxoethyl nitrate	-H	-H
7b	isobutyl	-H	2-(2-formylhydrazinyl) -2-oxoethyl nitrate	2-(2-formylhydrazinyl)-2-oxoethyl nitrate
7c	isobutyl	-H	3-(2-formylhydrazinyl)-3-oxopropyl nitrate	-H
7d	isobutyl	-H	2-(2-formylhydrazinyl)-2-oxoethyl nitrate	-H
7e	isobutyl	-H	3-(2-formylhydrazinyl)-3-oxopropyl nitrate	3-(2-formylhydrazinyl)-3-oxopropyl nitrate
7f	isobutyl	3-(2-acetylhydrazinyl)-3-oxopropyl nitrate	-H	-H

Comp Code/Dose (mg/kg,p.o)	Change in paw volume in (ml) after drug treatment(±SEM)			Anti-inflammatory activity (% Inhibition)		
	1h	2h	3h	1h	2h	3h
Control	1.65±0.017	1.77±0.02	1.91±0.03	-	-	-
Ibuprofen (20)	0.76±0.072**	0.74±0.009**	0.71±0.02**	53.93	58.19	62.82
7a(49.70)	0.75±0.074**	0.73±0.009**	0.71±0.05**	54.54	58.75	62.82
7b (63.98)	1.04±0.042**	1.06±0.03**	1.11±0.07**	38.78	40.11	41.88
7c (49.70)	0.79±0.009**	0.76±0.01**	0.73±0.01**	52.12	57.06	61.78
7d (48.34)	0.78±0.02**	0.77±0.01**	0.74±0.01**	52.72	56.49	61.25
7e (66.69)	1.19±0.06**	1.24±0.09**	1.30±0.09**	27.87	29.94	31.93
7f (51.06)	0.80±0.03**	0.77±0.02**	0.73±0.01**	51.51	56.49	61.78

Data analyzed by one way ANOVA followed by Dunnett's test, (n=6), *P<0.05, **P<0.01.

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

Acetic acid (0.1 ml, 0.6%) produced 28.83 number of writhing in control group, the number of writhings after administration of acetic acid in various test groups are given in table 2. The number of writhes in Ibuprofen (15 mg/kg) treated group were significantly reduced to 10.83 than that of control group. Dose dependent percentage inhibition of acetic acid induced writhing was observed in group, which was statistically significant compared to the control group, 28.83 ± 0.65 respectively. The results are reported in **Table 2**.

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

Close inspection of the results obtained by ulcerogenecity studies indicate that Ulcerogenic effect of 1a, 1c, 1d , 1f at 12 times the therapeutic dose on stomach was negligible compared to drug Diclofenac acid at the same dose levels **(Table 3)**. Hence it can be said that gastro intestinal tolerance to these compounds is better than that of Diclofenac acid. The results of potential for ulcerogenecity studies by the synthesized compounds are tabulated in the **Table 3**.

Table 2: Analgesic Effect of synthesized compounds in Acetic acid induced writhing model

Compound Code	Dose (mg/kg,p.o)	No of Writhes in 5-15 min. after treatment (Mean ± S.E)	% Inhibition
Control	Acetic acid (1% v/v)	28.83 ± 0.65**	-
Ibuprofen	15	10.83 ± 0.60**	62.43
7a	37.28	11.83 ± 0.94**	58.96
7b	47.98	13.66 ± 0.33**	52.61
7c	37.28	10.16 ± 0.47**	64.75
7d	35.85	10.5 ± 0.42**	63.57
7e	49.46	14.83 ± 0.60**	48.56
7f	37.87	12.66 ± 0.33**	56.08

Data analyzed by one way ANOVA followed by Dunnett's test, (n=6), *P<0.05, **P<0.01.

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

Compound code	Dose (mg/kg, p.o)	Ratio of ulcerated animals	Ulcer index (mean±SE)
Diclofenac	24	6/6	2.3±0.3
7a	41.77	Nil	-
7c	41.77	Nil	-
7d	40.63	Nil	-
7f	42.92	Nil	-

Data analyzed by one way ANOVA followed by Dunnett's test, (n=6), *P<0.05, **P<0.01.

Nitric oxide releasing study.

In isolated Wistar rat aorta rings, compounds **7a-7f** 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 response curves. In order to prove the involvement of nitric oxide in the relaxation process, nitric oxide releasing properties of synthesized compounds were assessed 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-7f).

Comp. Code	EC_{50}	% NO release[b]
7a	55.26	0.35%
7b	38.68	0.42%
7c	30.52	0.39%
7d	42.78	0.48%
7e	34.88	0.57%
7f	58.56	0.32%

[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:

Histopathological analysis showed characteristic features of ulceration in standard drug Diclofenac. After inducing ulcers the tissue samples, isolated from the control group rat stomach, consisted of fibroblasts, macrophages and proliferating endothelial cells forming micro vessels. The sample tissue of Diclofenac treated rat stomach showed some epithelial cells proliferated in the ulcer margin and these epithelial cells were found to be migrated over and into the ulcer crater, which was strongly infiltrated by inflammatory cells, fibroblasts and endothelial cells.

The typical representative examples of ulcerated tissues by Diclofenac and nonulcerated tissue samples by control and drug treated tissue samples are shown in

Fig 1.

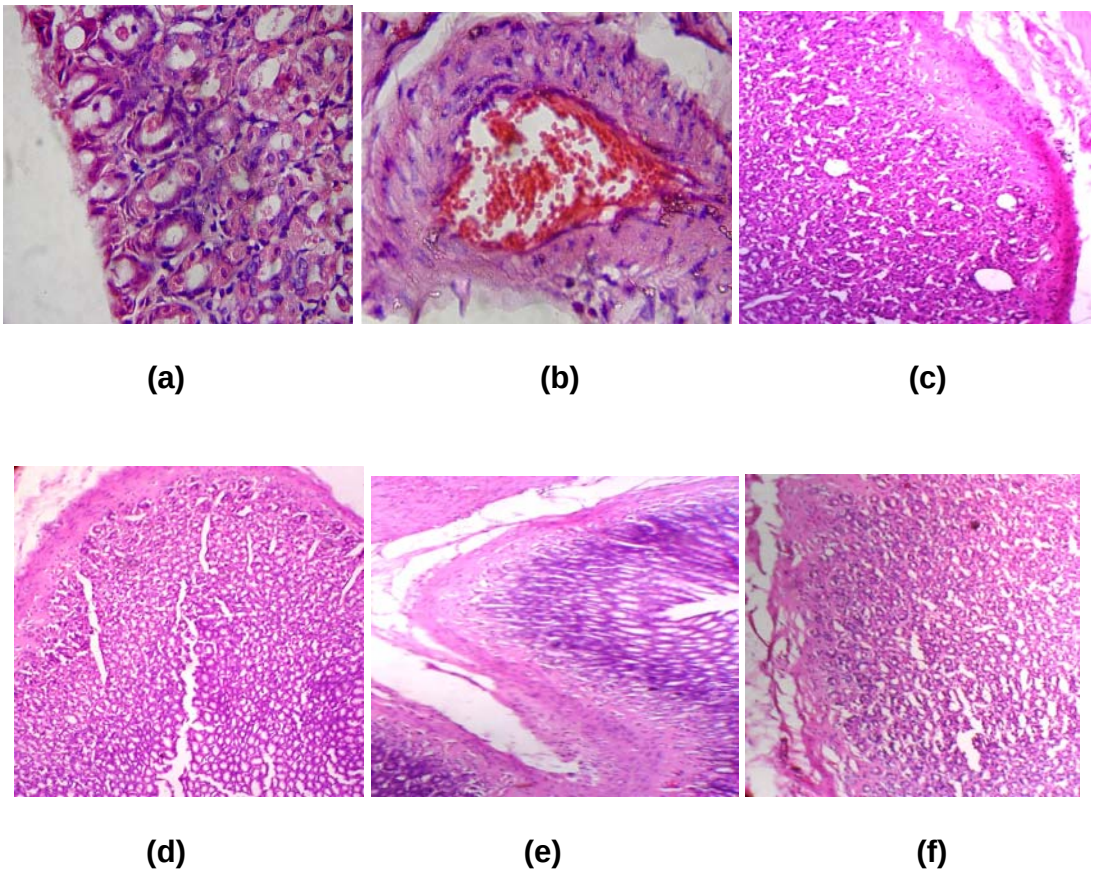


Fig 1: Haematoxylin and eosin Immunohistochemical staining of gastric ulcers after ulcer induction in rats. Intact Mucous membrane in control treated rat (a) showing granulation tissue composed of macrophages, fibroblasts and and endothelial cells forming microvessels. Congestion of mucosal blood vessels in Diclofenac (b). No damage was seen to mucosa of rat treated with synthesized derivatives 7a (c), 7c (d), 7d (e) and 7f (f).Original magnification x 200.

Discussion

The synthesized compounds were tested and compared with the standard drug Ibuprofen. The tested compound showed anti-inflammatory activity ranging from 31.93% to 62.82% at 3 h. (Table 1), where as the standard drug Ibuprofen showed 62.82% edema inhibition at 3 h after drug treatment. The compound 7a, 7c, 7d and 7f showed the significant activity. The maximum activity (62.82%) was shown by 7a. Effect of Ibuprofen and test compounds on percent inhibition of paw edema in rats at various time intervals (1 h and 3 h) is shown below (Fig 2).

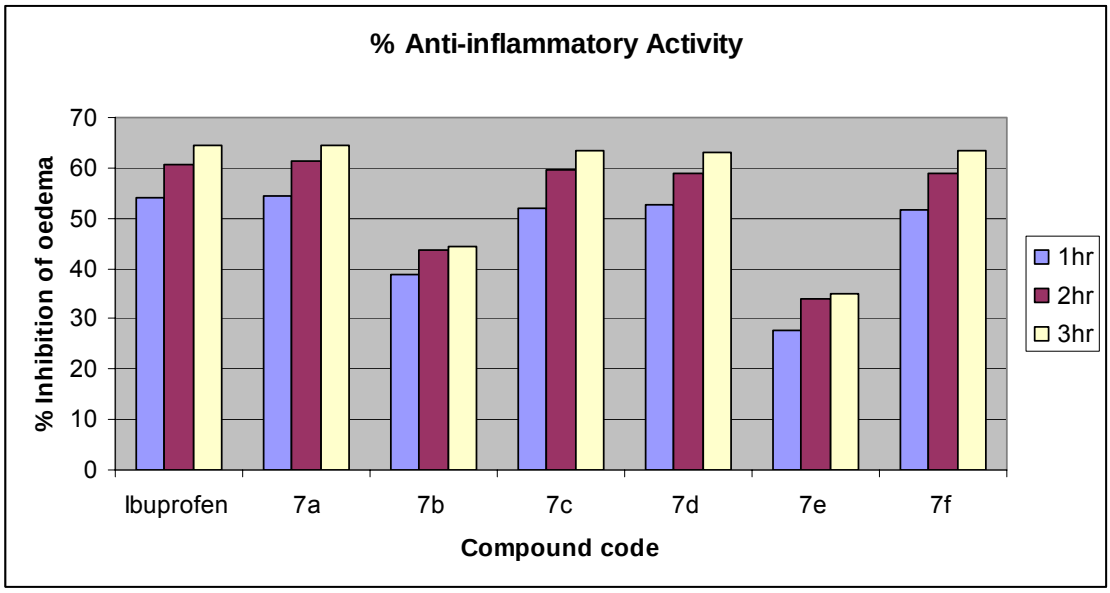


Fig 2: Comparison of % anti-inflammatory action of test compounds at 3 hr.

All the synthesized compounds showed analgesic activity ranging from 48.56% to 64.75%. The compound 7c (64.75%) and 7d (63.57%) showed better analgesic activity than the standard drug Ibuprofen (62.43%).

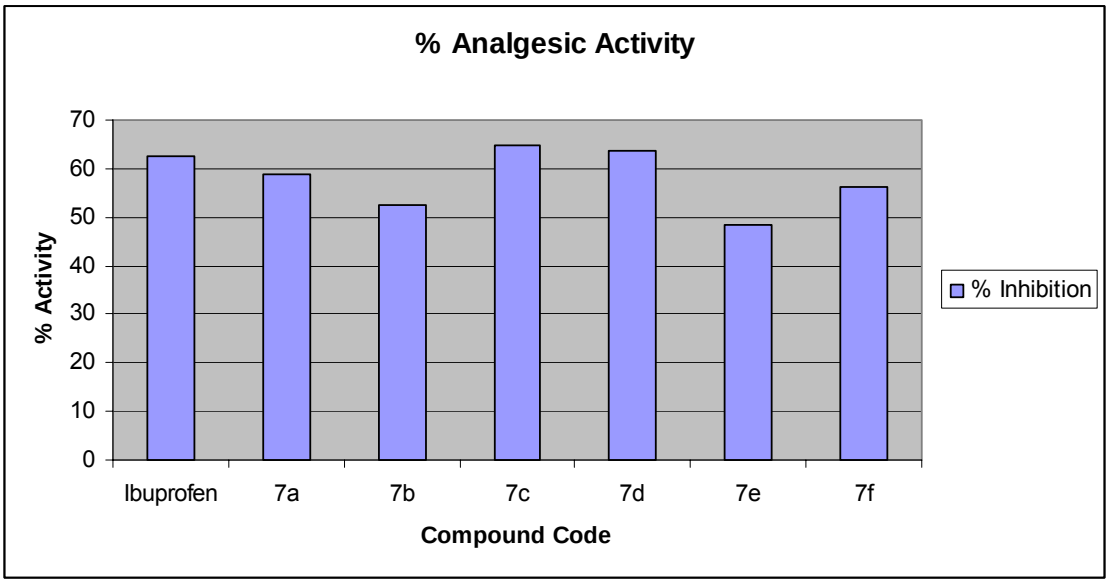


Fig 3: Comparison of % analgesic activity of test compounds with Ibuprofen.

The compound which showed anti-inflammatory activity comparable to that of standard drug Ibuprofen and also showed high analgesic activity were screened for potential for ulcerogenecity. Histopathological analysis showed no ulcerative features in rats treated with 7a, 7c, 7d, and 7f 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, 7c, 7d and 7f 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, 7c, 7d, and 7f 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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