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Synthesis and Evaluation of Anti-Inflammatory, Analgesic, Ulcerogenicity, and Nitric Oxide Releasing studies of Novel Pyrimidine Analogs as non-Ulcerogenic Derivatives

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A B S T R A C T

Most non-steroidal anti-inflammatory drugs (NSAIDs) suffer from the deadlier gastrointestinal (GI) toxicities. In the present research work, the main objective was to develop new chemical entities as potential anti-inflammatory agents with no GI toxicities. Seven derivatives based on interesting Pyrimidine heterocyclic scaffold viz. 2-[2-(6-methyl-2-oxo/thio-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carbonyl) hydrazinyl]-2-oxoethyl nitrate were synthesized. These compounds were tested *in vivo* for their anti-inflammatory, analgesic, and ulcerogenicproperties, and subjected to histopathological studies. Two compound IV_6) and (IV_7) was the most potent in this series. The compounds that showed significantly reduced GI ulcerogenicity also showed promising results in histopathologicalstudies, and they were found to cause no mucosal injury. All the synthesized compounds were found to exhibit significant nitric oxide releasing activity in an *in vitro* method. In conclusion, the designed hybrid molecules were found to be significantly promising.

Keywords: Anti-inflammatory, Analgesic, NSAIDs.

A R T I C L E I N F O

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1. Introduction

The multifunctional pyrimidine represents a heterocyclic system of remarkable pharmacological efficiency. [1,2,3,4, 5] Keeping in view the biological activity of these molecules, we have reported novel pyrimidine derivative with anti-inflammatory activity. Non-steroidal Anti-Inflammatory Drugs (NSAIDs) are among the most commonly prescribed drugs to reduce pain, inflammation, and fever. These drugs reduce pain and edema by suppressing the formation of prostaglandins, by inhibiting the activity of the enzyme Cyclooxygenase (COX-1 and COX-2). Selective COX-2 inhibitors elicit less or no GI damage and bleeding compared with conventional NSAIDs, although the magnitude of this reduction continues to be debated in the literature. [6,7] 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 hybridmolecules made up of non-selective or selective COX inhibitors together with a nitric oxide-releasing functional group.[8,9,10]

The strategy involving the use of hybrid molecules made up of non-selective COX inhibitors together with a nitric oxide-releasing 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 proinflammatory cells.[11,12,13] Moreover, because of the beneficial cardiovascular effects (vasodilation) of Nitric Oxide, such drugs are expected to be devoid of the cardiovascular adverse effects associated with the use of selective COX-2 inhibitors.[9,12] 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.

Synthetic approaches based on chemical modification have been taken with the aim of improving safety profile and in turn therapeutic window of the resultant NSAIDs. In our attempt to continue to discover new, safer, and potent agents for the treatment of inflammatory diseases, we have synthesized hybrid compounds containing pharmacophore of pyrimidine ring, and nitric oxide-releasing group 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 ulcerogenic toxicities [14,15,16].

2. Experimental

Chemistry

Synthesis of 2-[2-(6-methyl-2-oxo/thio-4-phenyl-1, 2, 3, 4-tetrahydropyrimidine-5-carbonyl) hydrazinyl]-2-oxoethyl nitrate was carried out in 4 steps, as shown in scheme. The first step compound I_1 to $I_7$was synthesize by Biginelli condensation (One pot condensation) reported in literature. In the second step reflux compound I with hydrazine hydrate (99%) in ethanol for 30 hrs. yielding corresponding hydrides In the next refluxing compound II with chloroacetyl chloride in dry benzene and triethylamine for 10 hrsyielded, compound (III_1 to III_7) derivative and finally different nitrate derivative (IV_1-IV_2) prepared by addition of silver nitrate to compound III, chloro function is replace by nitric oxide with stirring in presence of acetonitrile for 2 hrs. The structures of various synthesized compounds were assigned on the basis of results of chromatographic and spectral studies. The physical FTIR analytical data for all the synthesized compounds are given in the experimental protocols. FTIR spectra of title compound shows expected band for characteristics groups present in structure.

2-[2(6-methyl-2-oxo-4-phenyl-1, 2, 3, 4 tetrahydro pyrimidine -5-carbonyl) hydrazinyl] 2-oxoethyl nitrate. (IV_1): Compound was synthesized as per procedure. %Yield 61.23, M.P 212-215°C, FTIR spectra of the compound showed bands at 3470 (N-H), 1725(cyclic CONH), 1520 (NO_2).

2-[2-(6-methyl-4-phenyl-2-thioxo-1, 2, 3, 4-tetrahydro pyrimidine-5-carbonyl) hydrazinyl] 2-oxoethyl nitrate. (IV_2): Compound was synthesized as per procedure. % Yield 64.22, M. P 220-224 °C, FTIR spectra of the compound showed bands at 3467(N-H), 1680 (cyclic CONH), 1520 (NO_2), 1264 (C=S).

2-[2-(6-methyl-4-(3-nitrophenyl)-2-oxo-1, 2, 3, 4 tetra hydropyrimidine-5-carbonyl) hydrazinyl]-2-oxoethyl nitrate. (IV_3): Compound was synthesized as per procedure. % Yield 63.24, M. P 234-236 °C, FTIR spectra of the compound showed bands at 3448(N-H), 1740 (cyclic CONH), 1535 (Ar.NO_2) 1454 (NO_2) .

2-[2-(6-methyl-4-(3-nitrophenyl)-2-thioxo-1, 2, 3, 4-tetra hydro pyrimidine-5-carbonyl) hydrazinyl]-2-oxoethyl nitrate. (IV_4): Compound was synthesized as per procedure. % Yield 67.42, M. P 243-244°C, FTIR spectra of the compound showed bands at 3325 (N-H), 1740 (cyclic CONH), 1465 (NO_2), 1280 (thiourea C=S), 3105 (Ar- C-H).

2-[2-(4-(2-chlorophenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetra hydro pyrimidine-5-carbonyl) hydrazinyl]-2-oxoethyl nitrate. (IV_5): Compound was synthesized as per procedure. % Yield 70.22, M. P 202-204°C, FTIR spectra of the compound showed bands at 3437 (N-H), 1740 (cyclic CONH), 1490 (NO_2), 1050 (C=Cl), 3110 (Aromatic- C-H).

2-[2-(4-(4-methoxyphenyl)-6-methyl-2-oxo-1, 2,3, 4-tetra hydropyrimidine-5-carbonyl) hydrazinyl]-2-oxoethyl nitrate. (IV_6): Compound was synthesized as per procedure. % Yield 69.78, M. P 207-208°C, FTIR spectra of the compound showed bands at 3464 (N-H), 1710 (cyclic CONH), 1505 (NO_2), 1232(Aryl alkyl ether C-O-C), 3046(Aromatic-C-H)

2-[2-(4-(4-fluorophenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetra hydropyrimidine-5-carbonyl) hydrazinyl]-2-oxoethyl nitrate. (IV_7): Compound was synthesized as per procedure. % Yield 68.89, M. P 209-210°C, FTIR spectra of the compound showed bands at 3476 (N-H), 1712 (cyclic CONH), 1505 (NO_2), 1035 (C-F), 2990 (Ar. C-H).

Pharmacology:

The synthesized new chemical entities (NCEs) were subjected to the evaluation of anti-inflammatory activity,

analgesic activity, and acuteulcerogenicity studies as well as investigated for their nitric oxide-releasing properties. Ibuprofen and Diclofenac were used as reference standards. The experiments were performed using albino rats of Wistar strain of either sex, weighing in the range of 140–160 g. The animals were maintained at 25 ± 2_C, 50 ± 5% relative humidity, and 12-h light/dark cycle. All the animals were fasted for 24 h before the experiments, and water was provided ad libitum. The test compounds were suspended in 1% aqueous carboxy methyl cellulose (CMC) solution and administered orally to experimental animals for all the studies.

Anti-inflammatory activity: [20]

Anti-inflammatory activity was evaluated using the well-known carrageenan induced rat paw oedema model 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 compounds, 1 hour before the carrageenan treatment. The volume was measured before and after carrageenan treatment at 60 min. interval with the help of digital plethysmometer (Panlab LE 7500, USA).

Analgesic activity: [21]

The analgesic activity was evaluated using the acetic acid induced writhing method. (Koster et.al 1959) The test compounds at dose levels (10 mg/kg) were administered orally. The reaction time was recorded at 30 min, 1, 2 and 3 h after the treatment. The percent analgesic activity (PAA) was calculated by the following formula:

% Analgesic activity of the drug =

$$\frac{\text{(Average no. of writhes in control group) - (Average no. of writhes in treated group)}}{\text{(No. of writhes in control group)}} \times 100$$

Acute ulcerogenicity studies: [22]

Acute ulcerogenicity screening was done according to method reported. 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

- Normal (no injury, bleeding and latent injury).
- 0.5 Latent injury or widespread bleeding (>2 mm).
- Slight injury (2–3 dotted lines).
- Severe injury (continuous lined injury or 5–6 dotted injuries).
- Very severe injury (several continuous lined injuries).
- 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 ulcer score ±SEM, data analyzed by one way ANOVA followed by Dunnett's't' 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.

Histopathological studies: [23,24]

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^{o}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**.**

Detection of nitrite: [25] (info@RnDSystems.com)

A solution of the appropriate compound (20 μL) in dimethyl sulfoxide (DMSO) was added to 2 ml of 1:1 v/v mixture either of 50 mM phosphate buffer (pH 7.4) or in HCl solution (pH 1) with MeOH, containing 5 × of 10^{-4} M L-cysteine. The final concentration of test compound 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-naphthylethylene diaminedihydrochloride (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.

Statistical analysis

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. (www.graphpad.com)

3. Results and Discussion

Pharmacology:

The designed synthesized compounds were subjected to evaluation of anti-inflammatory activity,analgesic activity, acute ulcerogenicity study and studies to prove their ability to release NO. Diclofenac was used as a reference standard.

1). Anti-inflammatory activity:

The significant ($p < 0.01$) reduction of rat paw oedema was observed for most of the test compounds, at 3 h compared to the control group. (Table2). Out of the synthesized 7 compounds, 3 compounds viz. $IV_{3,}$ $IV_{6,}$ and IV_7 exhibited very significant anti- inflammatory activity compared to standard drug Diclofenac at 3 hrs andNitrate compound having oxo at 2nd carbon (Pyrimidin 2- one) and electron withdrawing groups ($NO_{2,}$ OCH_3, F) substituted phenyl ring at 4th carbon of pyrimidine having good anti-inflammatory activity than other compound Compounds with significant anti-inflammatory profile were subjected to GI-ulcerogenicity potential studies at 12 times the therapeutic doses with additional physical (cold) stress.

2). Analgesic activity: From the results, it was noticed that all compounds possess significant analgesic activity. The analgesic effects of IV_6 (67.24) and IV_7 (64.92**)** were found to be better than that of standard Diclofenac (64.29) and IV_5 (61.16) was comparable to Diclofenac. Similar to anti-inflammatory Nitrate compound having oxo at 2nd carbon (Pyrimidin 2- one) and electron withdrawing groups (NO_2, OCH_3, F) substituted phenyl ring at 4th carbon of pyrimidine having good analgesic activity.

3). Acute ulcerogenecity studies

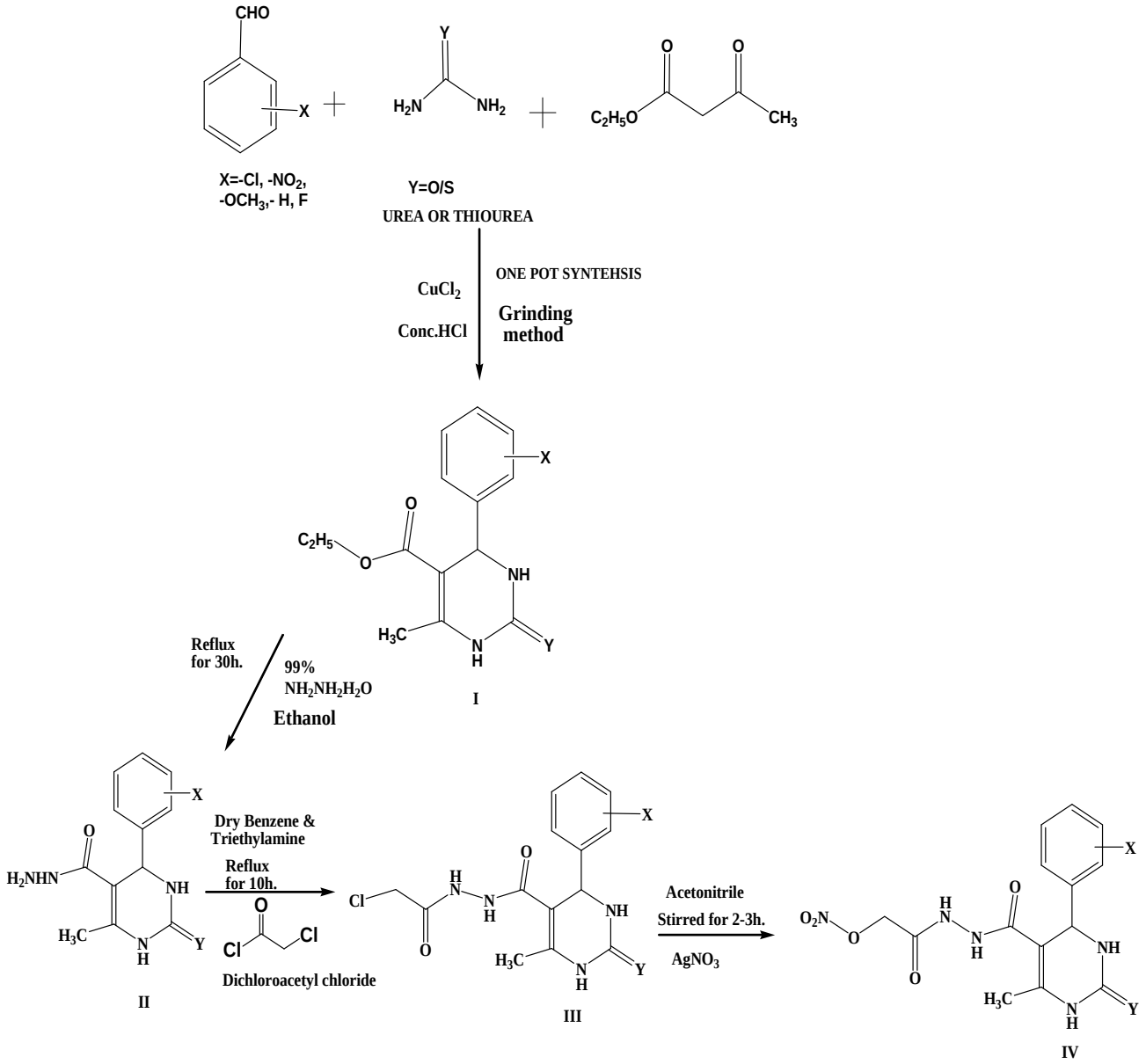
Ulcerogenic effect of IV5, IV6 and IV7 derivatives with best overall profile in animal efficacy model was evaluated for gastric ulcerogenic potential in rat stress model. When compared with Diclofenac, these compounds did not cause any gastric ulceration and disruption of gastric epithelial cells at the mentioned oral doses.

4). Histopathological studies.

The stomach specimen of Diclofenac treated rats was characterized by complete disruption of protective mucosal layer (Fig. 1). Histopathological analysis also showed characteristic features of ulceration in diclofenac treated group of animals. The tissue of Diclofenac treated rats has shown that some epithelial cells in the ulcer margin were proliferated and migrated over and into the ulcer crater, which was indicating complete disruption of gastric epithelial layer. Scanning of stomach specimens using electron microscope revealed that in the rats treated with synthesized derivatives (IV_6 and IV_7), there was no injury observed in stomach mucosa. As illustrated in **Fig 1**, specimen B ($IV_{6)}$andspecimen C(IV_7)which are seen identical to that of the control

5). In vitro Nitric oxide releasing studies (Nitrite test)

The nitric oxide releasing properties of these compounds were assessed in phosphate buffer, pH 7.4, in the presence of L-cysteine, relative to nitric oxide (NO) released from standard sodium nitrite solution. Percentage of NO released (n = 2) relative to a theoretical maximum release of 1 mol NO/mol of test compound.



Scheme 1: Scheme for synthesis [17,18,19]

Table 1: Physiochemical properties of synthesized compounds.

S.No.	Comp. Code	-X	-Y	Molecular Formula	Mol.wt. (g)	Yield (%)	M.P (^{0}C)	Rf value
1.	IV_1	-H	-O	$C_{14}H_{15}N_5O_6$	349	61.23	212 -215	0.50
2.	IV_2	-H	-S	$C_{14}H_{15}N_5O_5S$	365	64.22	220-224	0.53
3.	IV_3	-3-NO_2	-O	$C_{14}H_{14}N_6O_8$	394	63.24	234-236	0.61
4.	IV_4	-3-NO_2	-S	$C_{14}H_{15}N_6O_7S$	410	67.42	243-244	0.64
5.	IV_5	-2-Cl	-O	$C_{14}H_{14}N_5O_6Cl$	383	70.22	202-204	0.60
6.	IV_6	-4-OCH_3	-O	$C_{15}H_{17}N_5O_7$	379	69.78	207-208	0.57
7.	IV_7	-4-F	-O	$C_{14}H_{14}N_5O_6F$	367	68.89	209-210	0.54

Table 2: Results of anti-inflammatory activity of synthesized compounds against Carrageenan-induced rat paw edema model in rats

Comp Code/dose mg/ml	Change in paw volume in (ml) after drug treatment(±SEM)			Anti-inflammatory Activity (% Inhibition)		
	1hr	2hr	3hr	1hr	2hr	3hr
Control	0.506±0.012	0.494±0.0074	0.482±0.038	-	-	-
Diclofenac(10mg/ml)	0.402±0.025**	0.180±0.0348**	0.128±0.021**	20.56**	63.57**	70.45**
IV_1(12.33)	0.410±0.0287**	0.321±0.0286**	0.264±0.0261**	18.97**	35.23**	45.22**
IV_2 (12.87)	0.409±0.0202**	0.236±0.033**	0.280±0.0357**	19.16**	34.01**	41.90**
IV_3(13.85)	0.414±0.0224**	0.288±0.0439**	0.161±0.0243**	18.18**	41.71**	68.45**
IV_4(14.39)	0.453±0.0023	0.278±0.0332**	0.230±0.0269**	10.47	43.72**	52.28**
IV_5(13.47)	0.458±0.01393	0.256±0.0139**	0.192±0.0324**	8.31	37.25**	60.17**
IV_6(13.34)	0.406±0.0136**	0.226±0.0283**	0.125±0.0445**	19.76**	48.18**	70.60**
IV_7(12.93)	0.404±0.0199**	0.311±0.0322**	0.112±0.0358**	20.16**	54.26**	71.12**

Table 3: Results of analgesic activity of synthesized compounds against acetic acid-induced writhing test in mice

Compound	Dose (mg/kg, p.o)	No of writing after treatment (mean±SEM)	% Inhibition
Control	Acetic acid 1%v/v	42.2± 2.421	0
Diclofenac	10mg/kg	15.2±1.72	63.98**
IV_1	(12.33)	23.2±4.363	45.02*
IV_2	(12.87)	21.3±3.776	49.52**
IV_3	(13.85)	18.5±4.576	56.16**
IV_4	(14.39)	22.6±4.308	46.44**
IV_5	(13.47)	16.2±3.597	61.16**
IV_6	(13.34)	13.8±2.337	67.24**
IV_7	(12.93)	14.8±2.915	64.92**

Table 4: 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	10	4/6	1.7±0.2
	30	6/6	2.3±0.3
	50	Not tested	-
IV_5	10	Nil	-
	30	Nil	-
	50	Nil	-
IV_6	10	Nil	-
	30	Nil	-
	50	Nil	-
IV_7	10	Nil	-
	30	Nil	-
	50	Nil	-

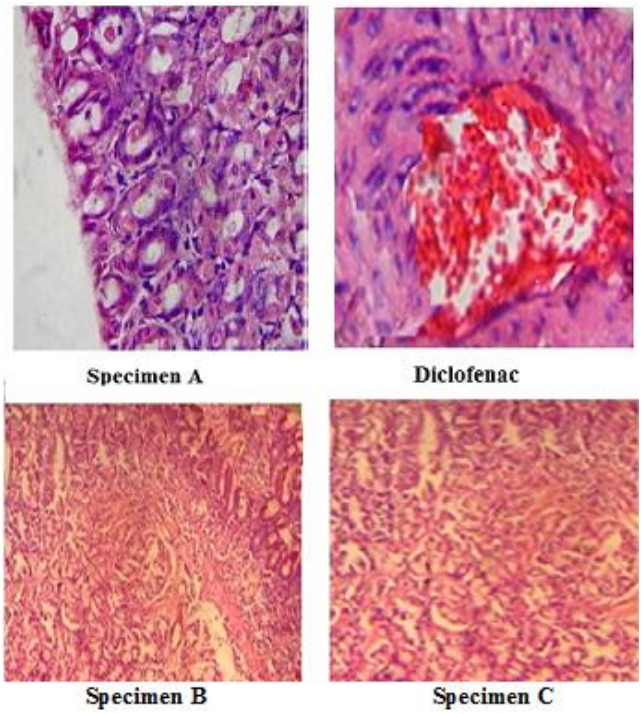


Figure 1: Figure 1: Haematoxylin and Eosin Immunohistochemical staining of gastric ulcers after ulcer induction in rats. As illustrated in Fig 1, specimen A shows intact mucous membrane in treated control rat showing granular tissues composed of macrophages, fibroblasts, and endothelial cells forming microvessels. Congestion of mucosal blood vessels was observed in Diclofenac-treated group, specimen B. No damage was seen to mucosa of rat treated with test compound, IV_6 and specimen C were identical to that of the control.

Figure 5: *In-vitro* Nitric oxide releasing studies (Nitrite test)

Comp. Code	% NO release[b]
IV_1	0.35%
IV_2	0.42%
IV_3	0.39%
IV_4	0.48%
IV_5	0.57%
IV_6	0.32%
IV_7	0.41%

4. Conclusion

Various substituted pyrimidine derivatives were synthesized and screened for anti-inflammatory, analgesic, ulcerogenic potential and Nitric oxide releasing ability. Most compounds exhibited significant analgesic and anti-inflammatory activity. It was interesting to note that out of seven compound IV_6(70.60%) and IV_7 (71.12%) was found to have anti-inflammatory activity better than that of standard drug Diclofenac (70.45%), whereas compound IV_3(68.45%) was found to have comparable to that of standard drug. It was interesting to note that presence of 4-methoxy, 4-fluro and Nitro (Electron win drawing) groups on phenyl ring at 4^{th} of pyrimidine led to enhanced anti-inflammatory activity means that 4- substituted phenyl group important for anti-inflammatory activity. However only a phenyl substituted derivative found to have most potent activity. And also on pyrimidine ring O atom at second carbon substituted compound shows good overall activity than thio(S) atom. Similarly, when we calculated analgesic activity the compound IV_6 (64.27%) and IV_7 (64.92%) which is a phenyl substituted moiety showed strong analgesia in acetic acid induced writhing tests when compared to standard drug Diclofenac (63.98%). Compound having 4-substituted phenyl ring showed good analgesic activity. After detailed analysis of the results of all pharmacological studies, it was concluded that the synthesized compounds have not only retained the anti-inflammatory profile of Diclofenac but have helped in enhancing the anti-inflammatory activity and are devoid of the deadlier gastrointestinal toxicities.

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