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DESIGN, SYNTHESIS AND PHARMACOLOGICAL SCREENING OF A 5-CYANO-6-METHYLMERCAPTO-2,4-DI[(SUBSTITUTED ARYL)AMINO]PYRIMIDINES AS POTENT ANTI-INFLAMMATORY AGENTS (SELECTIVE COX-II INHIBITORS)

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ABSTRACT

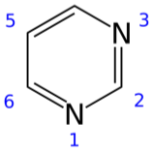
The design, synthesis, and development of novel non-steroidal anti-inflammatory drugs (NSAIDs) with better activity and lower side effects are respectable area of research. Novel Pyrimidine derivatives were designed and synthesized, and their respective chemical structures were deduced using various spectral tools (IR, ^{1}H NMR and MS). The compounds were synthesized via Dimroth rearrangement reaction. The reaction involves nucleophilic attack by amine of S,N acetal at the amidine carbon of methylisothiourea ether with concomitant loss of methyl mercaptan. The resultant guanidine intermediate undergoes intramolecular cyclisation to afford the condensed 4-iminopyrimidine, the designed series. The anti-inflammatory activity of all synthesized compounds was investigated applying the Carrageenan-induced paw edema model against Celecoxib as positive control. Percentage inhibition of edema indicated that compounds were exhibiting a significant anti-inflammatory activity. Ulcerogenic potency of most potent compound among all was evaluated using: Development of hemorrhages and ulcer after treatment with drug technique. Further same compound was screened for COX-I binding capacity using Inhibition of RBC aggregation method. The selected compound showed law ulcerogenic potency as compared to Aspirin and had law COX-I binding capacity, which help us to postulate our compounds to be selective COX-II inhibitors.

KEYWORDS: NSAIDs, Inflammation, Ulcer, COX, Pyrimidine.

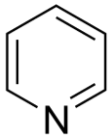
1. INTRODUCTION

Inflammation is an essential protective response to any noxious stimuli triggered by dust, thermal and electric shock, some drugs, and microorganisms.[1] Inflammatory disorder such as rheumatoid arthritis (RA) imposes a substantial socioeconomic burden on both individuals and society. Despite the huge prescription of NSAIDs, most of them exhibit shared set of adverse effects including gastrointestinal complications[2–3] that are generally attributed to the primary local irritation following the direct contact of carboxylic acid functionality of NSAID with GI mucosal cells and the reduced cytoprotection effect resulting from decreased tissue prostaglandin production[4]; consequently further studies were carried out in order to achieve innovative compounds with lower side effects. Cyclooxygenases are enzymes that take part in a complex biosynthetic cascade that results in the conversion of polyunsaturated fatty acids to prostaglandins and thromboxane(s).[5] Their main role is to catalyze the transformation of arachidonic acid into the intermediate prostaglandin H2, which is the precursor of a variety of prostanoids with diverse and potent biological actions.[6] Cyclooxygenases have two main isoforms that are called COX-1 and COX-2 (as well as a COX-3). COX-1 is responsible for the synthesis of prostaglandin and thromboxane in many types of cells, including the gastro-intestinal tract and blood platelets. COX-2 plays a major role in prostaglandin biosynthesis in inflammatory cells and in the central nervous system. Prostaglandin synthesis in these sites is a key factor in the development of inflammation and hyperalgesia.[7] COX-2 inhibitors have analgesic and anti-inflammatory activity by blocking the transformation of arachidonic acid into prostaglandin H2 selectively.[8] The impetus for development of selective COX-2 inhibitors was the adverse gastrointestinal side-effects of NSAIDs. Soon after the discovery of the mechanism of action of NSAIDs, strong indications emerged for alternative forms of COX.[9] Most selective COX-2 inhibitors, including the recently approved drugs celecoxib[10] and rofecoxib[11], belong to the diarylheterocycle class of compounds.[12-14] Diarylheterocycles have been investigated extensively as COX-2 inhibitors since the description of the 2,3-diarylthiophene, DuP 697, as a nonulcerogenic anti-inflammatory agent.[15] In contrast, relatively few reports document structural modifications of NSAIDs into selective COX-2 inhibitors. Indomethacin[16-17], zomepirac[18], aspirin[19,20], and flurbiprofen[21] are the only examples of NSAIDs that have been transformed successfully into COX-2-selective inhibitors. However, the methodologies used in their modifications are not general and required extensive structure–activity relationship (SAR) studies on individual compounds. For instance, replacement of the 4-chlorobenzoyl group in indomethacin with a 4-bromobenzyl moiety generates a COX-2-selective inhibitor.[16] In contrast, exchanging the

carboxylate moiety in aspirin with alkyl sulfide functionalities affords specific COX-2 inhibitors.[19,20]



1a Pyrimidine



1b Pyridine

Pyrimidine (1a) is an aromatic heterocyclic compound analogous to pyridine (1b) It is one of the three diazines (unsaturated six-membered ring containing two nitrogen atoms) that has two nitrogen atoms at positions-1 and -3 in the ring. Heterocyclic compounds carrying pyrimidine rings are of enormous importance because they represent a vital family of natural and synthetic products, several of which display valuable clinical applications and bioactivities.[22] Substituted pyrimidines and purines are extensively found in living things and are among the leading compounds investigated by chemists. Literature survey indicates that pyrimidine derivatives demonstrate a variety of pharmacological activities comprising antifungal[23], antibacterial[24,25], analgesic[26], antileishmanial[27], antihypertensive[28], antiviral[29], antipyretic[30] antidiabetic[31] antioxidant[32], anticonvulsant[33], antihistaminic[34] and anti-inflammatory.[35] Several methods are reported for synthesis of pyrimidine derivatives.[36-39]

II AIM OF PRESENT WORK

Several Pyrimidine derivatives have been synthesized and reported as selective COX II Inhibitor.[40-43] So, it was thought of interest to design and synthesize substituted pyrimidine for selective COX-II inhibitor activity.

III. MATERIALS AND METHODS

III A. Chemistry

Melting points of all the compounds were determined in open capillary and are uncorrected. Infrared spectra were recorded in potassium bromide disc on Perkin-Elmer Model-841 spectrophotometer. spectra were recorded on Shimadzu 640-A UV-Visible spectrophotometer. Nuclear magnetic spectra were taken on Varian A-60 Spectrophotometer at 60 MHz and the chemical shifts are given in parts per million (δ), down field from Tetramethyl silane (TMS) as internal standard. Splitting patterns are designated as follow. s = singlet, d = doublet, t = triplet, q = quartet and m = muptiplet. Mass spectra were obtained on

Perkin Elmer LC-MS PE SCIEX API 165 spectrophotometer. The thin layer chromatography was performed on microscopic slides (2 x 7.5 cms.) coated with silica gel G and spots were visualized by UV radiation and exposure to iodine.

Synthetic grade chemicals were used.

Synthesis of 5-Cyano-6-methylmercapto-2,4-di[(substituted aryl)amino]pyrimidines

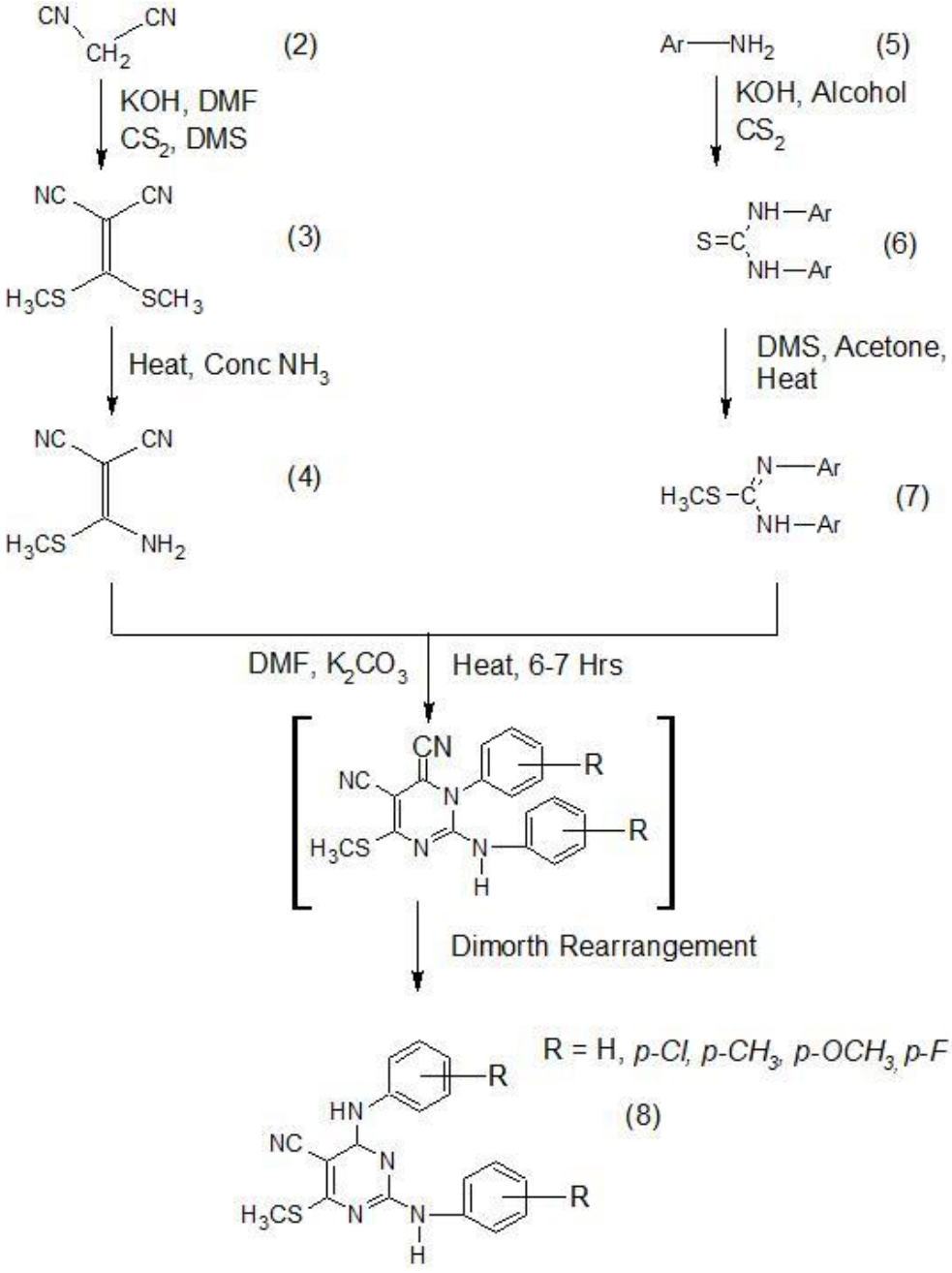


Figure 1: Scheme for synthesis of designed series.

Sarting materials (3), (4), (6), and (7) were prepared as reported method.

Procedure for Synthesis of Di(methylthio)methelenemalenonitrile (3),

To an ice-old solution of 13.2 gram (0.2 mole) of potassium hydroxide (85%) in 10 ml of water, 30 ml of dimethylformamide was added with cooling and stirring, 11.3 ml (0.1 mole) of malenonitrile followed by 7.6 gm (0.1 mole) of carbon disulfide. The mixture was stirred for one hour at room temperature, cooled and treated dropwise with 25.2 gm (0.2mole) of dimethylsulphate maintaining the temperature at 20^{o} C. The reaction mixture was allowed to stand at room temperature for 12 hours and poured inro ice water. The solid obtained was filtered, washed with water and dried. Recrystallised from methanol to get pale yellow crystalline product.

Procedure for Synthesis of 3-amino-2-cyano-3-methylthioacrylonitrile (4)

To the solution of Ethyl Di(methylthio)methelenemalenonitrile (3) (10 gm, 0.04 mole) in 10-15 ml of rectified spirit, was added 50ml (1 mole) of concentrated ammonia solution. The mixture was stirred for 12 hours, and then poured in ice-cold water. The crystals separated were filtered, washed with alcohol and dried. The crude product was recrystallized from ethanol-chloroform mixture.

General procedure for Synthesis of N,N-diarylisothioureas (6)

To a solution of potassium hydroxide (5.6 gm, 0.1 mole) in ethanol (30 ml, 100%), was added the appropriate monoarylamine (0.2 mole) and carbondisulphide (20 ml). The mixture was refluxed on a steam bath until the crystals of N,N-diarylisothiourea starts separating out. Excess of organic solvent was removed by distillation under reduced pressure. The residue was washed with HCl (10% v/v) and water, dried and recrystallised from ethanol.

General procedure for Synthesis of S-methyldiarylisothioureas (7)

To the ice-cold suspension of the appropriate diarylisothiourea (6), (0.1 mole) in minimum quantity of acetone (30 ml), dimethylsulphate (0.1mole) was added dropwise with continuous stirring over a period of half an hour. The mixture was there after stirred at room temp. for an additional hour, and then after refluxed for three hours. The acetone was removed by distillation. The heavy oil residue was poured in ice cold water and filtered. The filtrate was basified using Na_2CO_3 (10% aq. Na_2CO_3). The solid separated was filtered, washed with cold water, dried and recrystallised.

Procedure for Synthesis of designated series: 5-Cyano-6-methylmercapto-2,4-di[(substituted aryl)amino]pyrimidines (8)

A solution of 3-amino-2-cyano-3-methylthioacrylonitrile (4) (0.1 mole) and S-methyldiaryl isothioureas (7), (0.1 mole) in DMF was refluxed for 6-7 hrs with intermittent addition of pinch of K_2CO_3. The mixture was allowed to cool and poured in 4000 ml ice-cooled water. The solid separated was filtered washed with cold water, dried and recrystallised with chloroform-n-hexane.

III B. Pharmacological screening

III B.1 Anti-inflammatory activity *(in vivo)* carrageenan induced rat paw edema method[43]:

Method: The method of Winter et al was employed. Albino wistar rats of either sex (150-200 gm) were divided into various groups of four animal each. Animals were deprived of food for 12 hrs. prior to experiment and only water was given ad libitum. First group was used as control, and received 1 ml of 1% w/v sodium CMC suspension in water, the second group received sodium CMC suspension of celecoxib (50 mg./kg) orally and the third group received sodium CMC suspension of rofecoxib at a dose of 50 mg/kg, p.o. The remaining gar oups received sodium CMC suspension of test compounds at a dose of 50 mg./kg., p.o. One hour after the administration of the compounds, carrageenan suspension (0.1 ml of 1 % w/v suspension in 0.9% saline solution) was Injected into the sub plantar region of left hind paw of the animals. Immediately, the paw volume was measured using plethismometer (initial paw volume). Thereafter, the paw volume was measured after 3 hours (final paw volume).

Edema volume of control (Vc) and edema volume of treated (Vt) were used to calculate percentage (%) inhibition and edema volume (%) by using following formula.

% Inhibition = [1- (Vt/Vc)] x 100

% Edema volume =100 x Edema volume/Initial volume

III B.2 Ulcerogenic Potential: Development of hemorrhages and ulcer after treatment with drug.[44]

Ulcerogenic Potential is considered to be main gastrointestinal side effect of any analgesic and anti-inflammatory agent. Therefore, compounds were evaluated for their ulcerogenic potential.

METHOD: The Albino wistar rats of either sex, weighing 160-180 gm, were divided into various groups of three animals each. The rats were deprived of food for the 36 hrs before the

administration of the drug. First group was kept as control group. Second and third groups received standard drug celecoxib in the form of sodium CMC suspension at a dose of 100 mg/kg and 300mg/kg. Fourth group received standard drug aspirin in the form of sodium CMC Suspension at a dose of 100mg/kg. The rest of the groups were given test compounds in the form of sodium CMC suspension at a dose of 50mg/kg, 100mg/kg, 200mg/kg and 300 mg/kg. After six hours, the animals were sacrificed and stomach was taken out. The stomach was opened along the greater curvature and examined for hemorrhages and ulcers with the help of hand lens and compared with that of Celecoxib and aspirin treated groups. Ulcer index was calculated using following formula.

Ulcer Index = 10/x Where, x= (Total mucosal area/ Total ulcerated area)

Total mucosal area= μD^2 /8 Where D=Diameter of stomach.

III B.3 Inhibition of RBC aggregation (COX-1 inhibition)

Principle: COX-I enzyme derived serum TBX_2 is responsible for the aggregation of whole human blood. Selective COX-2 inhibitors like, celecoxib, rofecoxib etc. do not inhibit the COX-1 derived serum TBX_2 even at high doses. So, they do not inhibit lipopolysaccharide stimulated aggregation of RBC. They are having anti-inflammatory activity only through the inhibition of COX-2 derived PGE, synthesis.[45,46]

Method: Inhibition of gelatin induced red cell aggregation in rat/ human blood.[47]

Adult rats of either sex were anesthetized & blood was collected into a centrifuge tube containing heparin to give 20U/ml final concentration. From the blood collected from separate animals in about 15-20 minutes, 3.0 ml samples were pipetted into wassermann tubes. The blood was centrifuged for 20 minutes at 2000 rev./min. for rat blood and for 15 minutes at 3500 rev./min. for human blood. After which the plasma, leukocytes and platelets were removed and discarded. To the packed red cell, 2.0 ml of solution prepared from the substance to be tested was added. In each series two test, two standard and two control tubes were used. The cells were cautiously suspended by the inversion of the tube. After 5 minutes standing at 20 °C, 2.0 ml of 2% gelatin was added to the tubes and mixed by ten inversions. The measure of aggregation was estimated by the erythrocyte sedimentation rate (ESR). Height of supernant liquid was measured and % inhibition of aggregation was calculated by following formula.

% Inhibition = 100 x (1- Ht/Hc)

where, Hc=height of supernant liquid of control

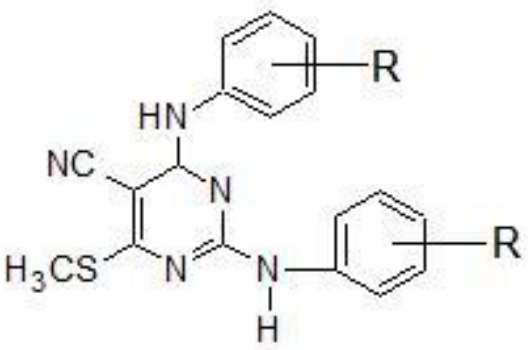
Ht=height of supernant liquid of test compounds

IV RESULTS AND DISCUSSION

IV A, Chemistry

Synthesis of designed derivatives was accomplished as per scheme in Figure 2.

IV A 1) Physical characteristic: All designed compounds were light yellow, amorphic solid compound, melting point was more than 200^0C. The title compounds are freely soluble in dimethylformamide, dimethylsulfoxide, chloroform, and sparingly soluble in rtanol, methanol, benzene and insoluble in n — hexane, petroleum ether, dil. NaOH and dil. HCl solution. They were recrystallised form chloroform-n-hexane mixture.



8 (a-e)

Table 1: Physical characteristic of target compounds.

No	R	Mot. Formula	Mol. Wt.	Recryst. Solvent*	M.P. (°C)	Rf**	Yield (%)
8a	H	$C_{18}H_{17}N_5S$	333.29	C:H	210-215	0.65	92
8b	p-CH_3	$C_{20}H_{17}N_5S$	361.33	C:H	240-245	0.70	90
8c	p-OCH_3	$C_{20}H_{21}N_5O_2S$	393.31	C:H	345-350	0.56	85
8d	p-F	$C_{18}H_{15}F_2N_5S$	369.27	C:H	232-235	0.69	79
8e	p-Cl	$C_{18}H_{15}Cl_2N_5S$	403.28	C:H	260-264	0.78	87

* C = Chloroform, H = n-Hexane **TLC [Benzene : Methanol (4.5:0.5)]

IV A 2) Spectral characteristic

The structures of the target compounds were confirmed by the spectral analysis.

INFRARED SPECTRA

The I.R. spectrum of all the compounds shows the characteristic stretching peaks of the secondary amino, The presence of two sharp peak at 3440-3320 cm^{-1} shows the presence of two secondary amino function. The CN stretching is observed at 2200 cm^{-1}.

U.V. SPECTRA

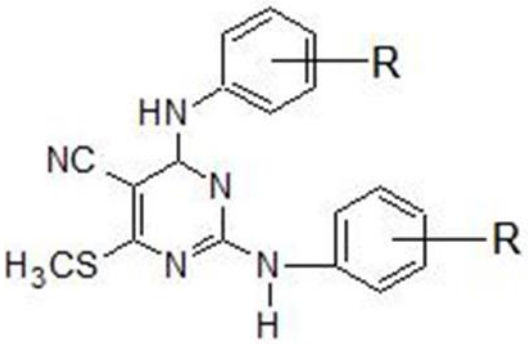
The U.V. spectra (in methanol) of all the compounds show two peaks. One in range of 283-295 nm and other in 360-375 nm. All the compounds have shown identical pattern of U.V. spectra, which confirms that all the compounds belong to same series.

MASS SPECTRA

All compounds were also characterized by mass spectral analysis. All the analyzed compounds gave M^+ peak at the molecular weight. Chlorinated Compound containing halides Cl on aryl ring shows M^{+2} peaks also.

NMR SPECTRA

The ^1H NMR Spectra of the compounds were studied in $CDCl_3$ the compound exhibited the following shifts. The aryl protons resonate around δ 7.1-7.7 as complex multiplets corresponding to 8/10 protons. The secondary amino proton resonates sharply at δ 5.1 as singlet corresponding to a single proton. The three protons of methylmercaptan shows sharp peak at region of δ 2.3.



(8a-8e)

Table 2: Spectral characteristic analysis of target compounds.

No	R	U.V. λmax (nm)	I.R. Spectra (cm^{-1})	Mass Peak (m/e)	1H NMR shift (δ ppm)
8a	H	283, 362	3240 (NH), 2200 (CN)	333 (M^+) 352.4 (M^{+1})	2.3 (s, 3H, -SC<u>H</u>$_3$) 5.6 (s,2H, -N<u>H</u>-C_6H_4), 7.2-7.6 (m, 10H, -C_6<u>H</u>$_4$-)
8b	p-CH_3	283.3, 360	3240 (NH), 2200 (CN)	361 (M^+), 362 (M^{+1})	1.8 (s, 6H, -C<u>H</u>$_3$) 2.4 (s, 3H, -SC<u>H</u>$_3$) 5.7 (s,2H, -N<u>H</u>-C_6H_4), 7.2-7.7 (m, 8H, -C_6<u>H</u>$_4$-),
8c	p-OCH_3	283, 316	3200 (NH), 2200 (CN)	393(M^+), 394.4 (M^{+1})	2.4 (s, 3H, -SC<u>H</u>$_3$) 3.4-3.7 (s, 6H, -OC<u>H</u>$_3$) 5.8 (s, 2H, -N<u>H</u>-C_6H_4),

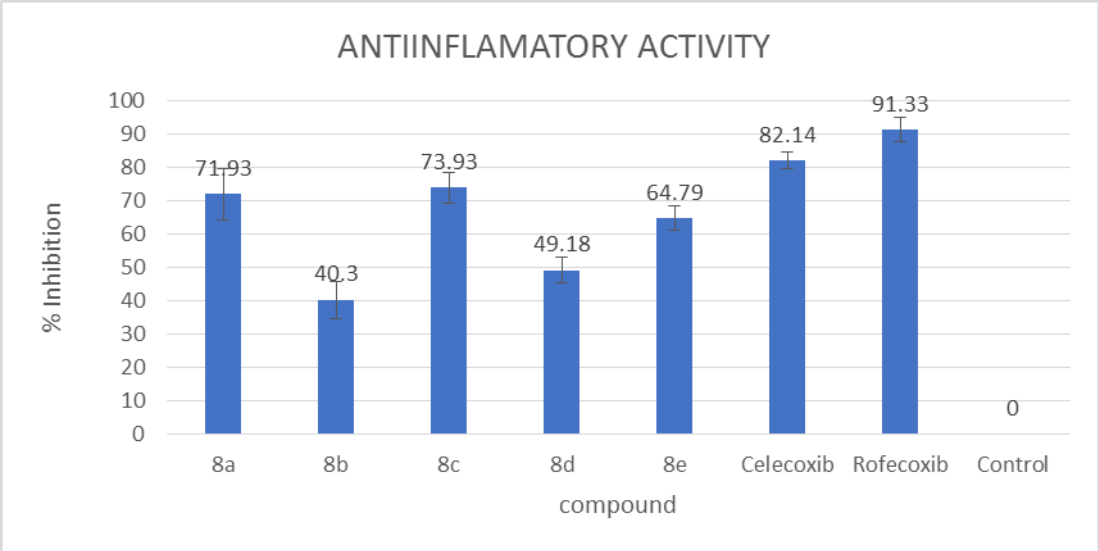
					7.2-7.7 (m, 8H, -$C_6\underline{H}_4$-),
8d	p-F	280.5, 322.5	3280 (NH), 2200 (CN)	369 (M^+), 370 (M^{+1})	2.3 (s, 3H, -S$C\underline{H}_3$) 5.4 (s,2H, -N$\underline{H}$-C_6H_4), 7.2-7.7 (m, 8H, -$C_6\underline{H}_4$-)
8e	p-Cl	281, 325.5	3280 (NH), 2200 (CN),	402(M^+), 404.3 (M^{+2})	2.3 (s, 3H, -S$C\underline{H}_3$) 5.5 (s,2H, -N$\underline{H}$-C_6H_4), 7.1-7.7 (m, 8H, -$C_6\underline{H}_4$-)

IV B. Pharmacological screening

IV B.1 Anti-inflammatory activity (in vivo) carrageenan induced rat paw edema method.

Table 3: Anti-inflammatory activity of target compounds (8a-8e).

Compound No	Edema volume [after 3 hours] [Mean ± s.e.m.]	% Edema volume [Mean ± s.e.m.]	% Inhibition [Mean ± s.e.m.]
8a	0.27 ± 0.75	17.94 ± 6.18	71.93 ± 7.65
8b	0.585 ± 0.055	34.36 ± 5.08	40.3 ± 5.61
8c	0.25 ± 0.045	14.38 ± 1.66	73.93 ± 4.59
8d	0.38 ± 0.075	26.87 ± 2.83	49.18 ± 3.67
8e	0.345 ± 0.035	22.16 ± 1.58	64.79 ± 3.57
Celecoxib	0.175 ± 0.025	11.55 ± 2.73	82.14 ± 2.55
Rofecoxib	0.085 ± 0.035	5.35 ± 1.78	91.33 ± 3.57s
Control	0.98 ± 0.07	95.54 ± 11.12s	



All the synthesized compounds were screened for anti-inflammatory activity by carrageenan induced rat paw edema method (in vivo), using celecoxib and rofecoxib as standard drugs. Both standard and test compounds were screened at dose of 50 mg/kg p.o. All the compounds have exhibited significant anti-inflammatory activity. In a series of 2,4-di[(substituted aryl)amino]pyrimidines, compound 8c, 5-cyano-6-methylmercapto-2,4-di[(4'-methoxyphenyl

)amino]pyrimidine was found to be most potent with % inhibition of 73.93 % and compound 8b, 5-cyano-6-methylmercapto-2,4-di[(4'-methylphenyl)amino]pyrimidine was found to be least potent with % inhibition of 40.30 %.

IV B.2 Ulcerogenic Potential: Development of hemorrhages and ulcer after treatment with drug Ulcerogenic potential of the most potent compound from the series 8c was screened for ulcerogenic potential in comparison to standard drugs celecoxib and Aspirin.

Table 4: Comparison of ulcerogenic potential.

Compound	Ulcer Index (Under experimental condition)			
	50 mg/kg	100 mg/kg	200 mg/kg	300 mg/kg
Celecoxib (Std)	-	-	-	0.020 ± 0.002
Aspirin (Std)	-	0.844 ± 0.0102		-
8c	0.055 ± 0.002	0.0263 ± 0.025	0.497 ± 0.015	0.568 ± 0.026

Compounds 8c have exhibited very low ulcer index at its therapeutic dose i.e. 50 mg/kg. But in the test compound ulcer index was found to increase with increase in dose. Compared to celecoxib the ulcer index was found to be high at six-time higher dose then the therapeutic dose i.e, 300 mg/kg. Ulcer index of test compound was found to be very less than the standard drug aspirin at the Same dose level, 100 mg/kg.s.

IV B.3 Inhibition of RBC aggregation (COX-1 inhibition)

Table 5: Inhibition of gelatin-induced R.B.C. aggregation.

	In Rat Blood after 1 hour		In Human Blood after 1 hour	
	Ht. of supernant (mm)	% Inhibition	Ht. of supernant (mm)	% Inhibition
Control	47	-	27.5	-
Celecoxib	42	10.63	26	5.46
8c	40	14.89	25	9.09
Aspirin	35	51	13	52.72

RBC aggregation is observed due to COX-1 enzyme, Aspirin has exhibited higher % inhibition with 52.72 %, while celecoxib has exhibited lower % inhibition with 10.63 % (in rat blood) and 5.46% (in human blood). The test compound 8c has exhibited % inhibition of 14.89 % in rat blood and 9.09 % in human blood. Thus the test compound 8b have exhibited very low % inhibition comparable to the standard drug, celecoxib. This suggests that, compounds are having very less affinity for COX-1 enzyme.

From the above result we can propose that the anti-inflammatory activity of test compounds may be due to inhibition of COX-2 enzyme.

SUMMERY AND CONCLUSION

series of 5-Cyano-6-methylmercapto-2,4-di[(substituted aryl)amino]pyrimidines were synthesized. All the compounds were characterized by TLC, UV, IR Mass and ^{1}H NMR spectral analysis. All the synthesized compounds have been evaluated for anti-inflammatory activity by rat paw edema method. (invivo) All the compounds have exhibited significant anti-inflammatory activity with inhibition ranging from 40.3 to 74%. Of all the compounds screened, compound (8c) was found equipotent with the standard drug celecoxib at the same dose level i.e. 50 mg/kg, p.o. Most potent compounds of both the 8c was checked for ulcerogenic potential. The test compounds have exhibited very low ulcer index compared to the standard drug aspirin at its therapeutic dose i.e. 50 mg/kg. Compounds was screened for inhibition of gelatine induced RBC aggregation in rat and human blood. The compounds have exhibited very low inhibition comparable to standard drug celecoxib. This suggests that, the compounds c is having very less affinity for COX-1 enzyme and they may act through selective COX-II inhibition.

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