SYNTHESIS, CHARACTERIZATION, AND BIOLOGICAL EVALUATION OF PYRAZOLYL AMINOPYRIMIDINE DERIVATIVES

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ABSTRACT : A new series of pyrazolyl aminopyrimidine derivatives 5a-5d had been synthesized and tested for antimicrobial agent against different bacteria and fungi and anti-inflammatory activity in the Carrageenan-induced acute inflammation in rats. The structures of new sulfanilamide derivatives were characterized by NMR spectroscopy and mass spectrometry. The synthesized compounds were tested for their in vitro antimicrobial activity using the broth diffusion method against Gram-positive bacteria S. Aureus, S. Pyogenus the Gram-negative bacteria E. Coli, P. Aeruginosa, the fungal strain C.Albicans, A. Niger, A. Clavatus. It has been observed that the maximum anti-inflammatory activity was recorded by compound 5a having 65.38% percentage protection of edema followed by compound 5b (60.25 %), 5c (50 %) &45 (39.74%) anti-inflammatory potential. Compound5a showed more percentage protection of edema than that of the standard drug Diclofenac sodium.

Keywords: Antibacterial, Antifungal, Griseofulvin, Anti-inflammatory activity.

I.INTRODUCTION

Inflammatory diseases can affect the quality of life of many patients; however, the current medicinal drugs are not always effective and may cause serious adverse effects[1]. Nonetheless, infectious diseases are among the leading causes of death globally[2], and antimicrobial resistance has been commonly reported worldwide[2,3]. These obstacles necessitate the search for new therapies with potential antioxidant, anti-inflammatory, and antimicrobial activities. Despite massive efforts by various global academic and industrial research laboratories over the years, developing anti-inflammatory and antimicrobial agents that are potent, safe, and selective remains quite challenging. The pyrimidine ring is responsible for the properties of many natural and synthetic biomolecules. The pyrimidine ring is a key structural moiety of life-supporting substances, such as vitamins, coenzymes, and uric acid, as well as of drugs, such as Veronal, Sulfadiazine, Fluorouracil, Glivec, and Rosuvastatin. Since the aminopyrimidine ring is a fragment of nucleotide bases in DNA and RNA, which are the most important components of living cells, the significance of these compounds in nature can scarcely be overestimated.

The pyrimidine structural motif is a fundamental part of nucleic acids and is associated with numerous biological activities[4]. Substituted aminopyrimidine nuclei are common in the marketed drugs such as anti-atherosclerotic Aronixil®, anti-histaminic Thonzylamine® (I), antianxielytic Buspirone® (II), anti-psoriatic Enazadrem® (III), and other medicinally relevant compounds[5].

The discovery of a series of aminopyrimidine derivatives inhibiting the protein kinases is one of the most important advances in the field of synthetic chemistry of amino pyrimidines.1a,3 Some other biological activities of these molecules include antitubercular,4 adenosine receptor antagonists[6], analgesic[7,] and anti-inflammatory[6].

Previous reports have suggested that beginning with modification of compounds known to have pharmacological effects or conjugating two structures with promising biological effects into an interesting motif may be useful for developing new effective therapeutic agents. Therefore, this work aimed to design and synthesize new pyrazolyl aminopyrimidine derivatives[8], in order to evaluate their anti-inflammatory and antimicrobial activities against medically important bacterial and fungal strains. In this study, the aim isto obtain compounds with more powerful desired effects.

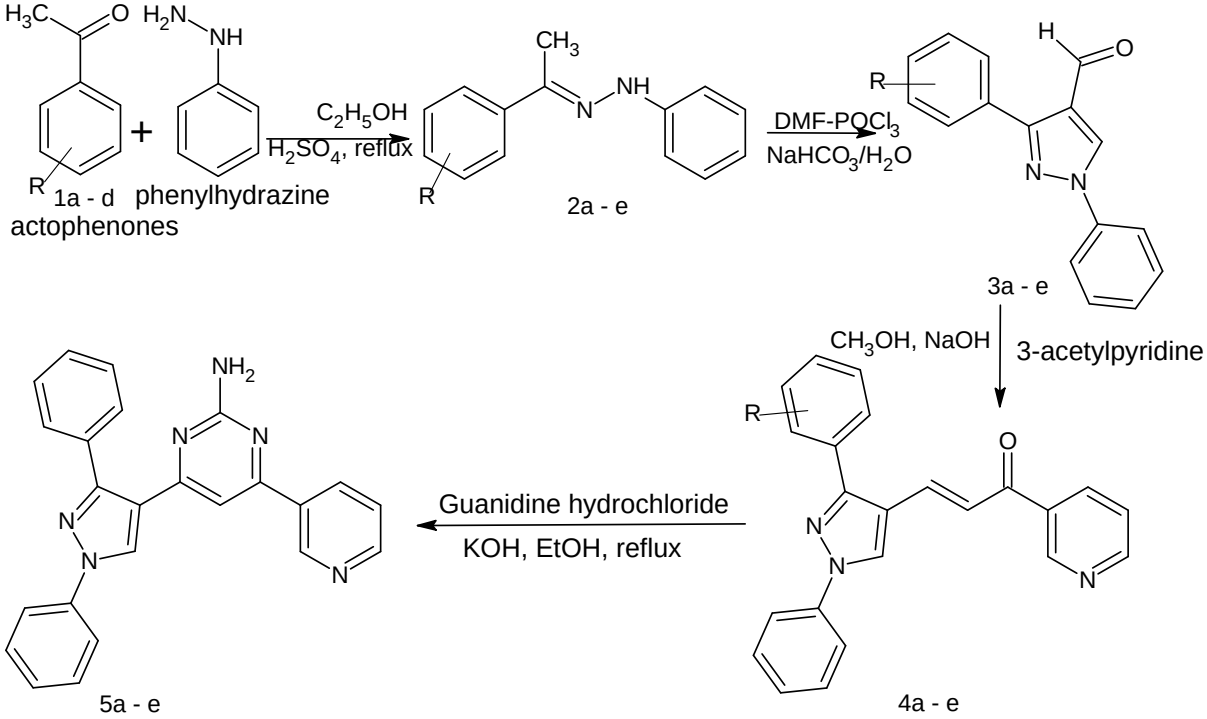
II. MATERIALS AND METHODS

2.1 Experimental

All chemicals and reagents used are of analytical grade. Melting points were determined on an electrothermal apparatus using open capillaries and are uncorrected. Thin-layer chromatography was accomplished on 0.2-mm

precoated plates of silica gel G60 F254 (Merck). Visualization was made with UV light (254 and 365nm) or with iodine vapor.Perkin Elmer 1600 series Fourier Transformer-Infrared Spectrophotometer in KBr-Pellet method. ^{1}H NMR spectra were recorded on a **Bruker DPX-400 MHz** spectrometer. Chemical shifts are expressed in δ ppm downfield from TMS as an internal standard.

2.2 Current working scheme



a: R= H, b: R= 4-F, c: R= 4-Cl, d: R= 3-Br, R =3- NO_2

2.3 Synthesis Procedure

2.3.1 General procedure for the synthesis of* pyrazolic chalcones*(3a-e)

To a solution of acetophenones (**1a-e)**(10 mmol) and phenylhydrazine (10 mmol) in EtOH (10 mL) was added a drop of concentrated H_2SO_4 and the resultant solution was refluxed for 2 h. On cooling, precipitates (**2a-e)** were filtered, washed with cold EtOH, and dried, provided sufficiently pure (by TLC) hydrazone**,** which was immediately used for the next step. $POCl_3$ (15 mmol) was added dropwise to dry DMF (15 mmol) in the round bottom flask at 0 °C and the resultant mixture was stirred for 30 minutes until the formation of the Vilsmeier-Haack reagent appeared. The corresponding solution of phenylhydrazones (**2a-e)**(5 mmol) in the minimum amount of dry DMF was added dropwise to the Vilsmeier-Haack reagent, which was warmed at r.t. and heated at 70- 80 °C for 5 h. The cool reaction mixture was poured into crushed ice and neutralized with a cool saturated K2CO3 solution. The precipitates were filtered, strongly washed with water, and crystallized from ethanol, affording pure **3a-e.**

2.3.2 General procedure for the synthesis of pyrazolic chalcones 4a-e

A mixture of 3-aryl-1-phenylpyrazol-4-carboxaldehydes **3a-e** (10 mmol) and 3-acetyl pyridine (10 mmol) was stirred in methanolic sodium hydroxide solution for 24 h at room temperature. Upon completion, the precipitates formed were filtered off, washed with water, and dried, affording compounds **4a-e**. The compounds were crystallized from ethanol

2.3.3 General procedure for the synthesis of compounds 5a-e

A mixture of (E)-3-(3(aryl)-1-phenyl-1H-pyrazol-4-yl)-1-(pyridin-3-yl) prop-2-en-1- ones **4a-e (**5 mmol) and guanidine hydrochloride (5 mmol) in ethanolic KOH (3%, 20 ml) was refluxed for 10 h. On completion, the reaction mixture was allowed to stand overnight. The precipitates were filtered off, washed with water, and dried, affording compounds **5a-e**. The compounds were re-crystallized in ethanol.

4-(1,3-Diphenyl-1H-pyrazol-4-yl)-6-(pyridine-3-yl) pyrimidine-2-amine (5a)

IR (neat, νmax cm^{-1}): 3312 (NH_2stretching), 3149(NH_2 group), 1655 (C=C), 1573, 1544, 1503(Ar C=C stretching)

^{1}H NMR (300 MHz, CDCl3): δ 8.87 (s, 1H), 8.60-8.64 (m, 1H), 8.59 (s, 1H), 7.28-8.08 (m, 12 H), 6.92 (s, 1H), 5.18 (s, 2H);

^{13}C NMR (125 MHz, DMSO-d6): δ 164.36, 162.30, 161.54, 151.57, 151.34, 148.33, 139.59, 134.45, 133.36, 133.23, 130.54, 130.13, 129.36, 128.82, 128.67, 127.35, 124.21, 121.53, 119.08, 104.93;

Anal. Calcd. for $C_{24}H_{18}N_6$: C, 73.83; H, 4.65; N, 21.52. Found: C,73.75; H, 4.59; N, 21.48; MS (ESI) $[M + H]^+$ Calcd. for $C_{24}H_{18}N_6$: 391.44, Found:391.4 $[M + H]^+$.

4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(pyridine-3-yl) pyrimidin-2- amine (5b)

IR (neat, νmax cm-1): 3320(NH_2stretching), 3160(NH_2 group), 1655 (C=C), 1577, 1547, 1510(Ar C=C stretching);

^{1}H NMR (300 MHz, CDCl3): δ 8.95 (s, 1H), 8.65-8.66 (m, 1H), 8.56 (s, 1H), 7.13-8.11 (m, 11 H), 6.95 (s, 1H), 5.13 (s, 2H);

^{13}C NMR (125 MHz, DMSO-d6): δ 164.26, 163.63, 162.42, 161.68, 161.45, 151.60, 150.37, 148.42, 139.54, 134.51, 133.23, 131.62, 131.55, 130.87, 130.14, 129.80, 129.78, 127.41, 124.23, 121.34, 119.07, 115.53, 115.36, 104.81

Anal. Calcd. for $C_{24}H_{17}FN_6$: C, 70.58, 4.20; N, 20.58. Found: C, 70.61; H, 4.16; N, 20.54; **MS (ESI) $[M + H]^+$** Calcd. for $C_{24}H_{18}FN_6$: 409.43. Found: 409.6 $[M + H]^+$.

4-(3-(4-Chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(pyridine-3-yl) pyrimidin-2- amine (5c)

IR (neat, νmax cm-1): 3324, (NH_2stretching), 3164(NH_2 group),1652(C=C), 1585, 1547, 1506(Ar C=C stretching);

^{1}H NMR (300 MHz, CDCl3): δ 8.99 (s, 1H), 8.7 (d, J =3.3 Hz, 1H), 8.54 (s, 1H), 8.10 (d, J = 7.5 Hz, 1H), 7.82 (d, J = 7.8 Hz, 2H), 7.66 (d, J = 8.1 Hz, 2H), 7.35-7.52 (m, 6H), 6.96 (s, 1H), 5.13 (s, 2H);

^{13}C NMR (125 MHz, DMSO-d6): δ 164.25, 162.49, 161.40, 151.61, 150.04, 148.46, 139.50, 134.54, 133.43, 133.22, 132.19, 131.19, 131.11, 130.15, 128.58, 127.48, 124.23, 121.47, 119.11, 104.89

Anal. Calcd. for $C_{24}H_{17}ClN_6$: C, 67.84; H, 4.03; N, 19.78. Found: C, 67.86; H, 4.04; N, 19.81;

MS (ESI) $[M + H]^+$ Calcd. for C24H18ClN6: 425.88, Found: 425.5 $[M + H]^+$.

4-(3-(3-Bromophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(pyridine-3-yl)pyrimidin-2- amine(5d)

IR (neat, νmax cm^{-1}): 3335(NH_2stretching), 3204(NH_2 group), 1648(C=C), 1585, 1566, 1536(Ar C=C stretching)

^{1}H NMR (300 MHz, CDCl3): δ 8.97 (s, 1H), 8.66 (d, J =4.8 Hz, 1H), 8.57 (s, 1H), 8.13 (d, J = 7.8 Hz, 1H), 7.95 (s, 1H), 7.82 (d, J = 7.5 Hz, 2H), 7.27-7.64 (m, 7H), 6.98 (s, 1H), 5.13 (s, 2H);

^{13}C NMR (125 MHz, DMSO-d6): δ 164.26, 162.54, 161.28, 151.63, 149.54, 148.44, 139.46, 135.61, 134.54, 133.19, 131.58, 131.46, 131.10, 130.75, 130.16, 128.47, 127.56, 124.23, 121.78, 121.55, 119.18, 104.96

Anal. Calcd. for $C_{24}H_{17}BrN_6$: C, 61.42, H, 3.65; N, 17.91. Found: C, 61.39; H, 3.64; N, 17.89;

MS (ESI) [M]+ Calcd. for C24H17BrN6: 469.34, Found: 469.5 [M]+.

III. BIOLOGICAL EVALUATION

3.1 Antimicrobial activity

In this study, Broth Dilution Method[7] was employed to evaluate the antibacterial activity. It is one of the non-automated *in vitro* bacterial susceptibility tests. This classical method yields a quantitative result for the number of antimicrobial agents that are needed to inhibit the growth of specific microorganisms. It is carried out in tubes.

- Macrodillution method in tubes.
- Microdilution format using plastic trays.

The *in vitro* antibacterial activity evaluation was conducted using Escherichia coli (MTCC 443) and Pseudomonas aeruginosa (MTCC 441) from gram-negative group of bacteria while Staphylococcus aureus (MTCC 96) and Streptococcus pyogenus (MTCC 442) from gram-positive group of bacteria. The *in vitro* antifungal activity of all compounds and standard drugs was evaluated against three fungi viz. Candida albicans (MTCC 227) Aspergillus niger (MTCC 282) and Aspergillus clavatus (MTCC 1323). The strains were procured from the Institute of Microbial Technology, Chandigarh. The size of the inoculum for test strain was adjusted to 108 CFU/mL (Colony Forming Unit per milliliter) by comparing the turbidity (turbidimetric method). Mueller Hinton Broth was used as fortifying medium for bacterial strains to cultivate and dilute the compound suspension for the microbial strains. Sabouraud Dextrose Broth was used as the nutrition of fungal strains. Ampicillin was used as standard antibacterial drug, whereas Greseofulvin was used as standard antifungal drugs. DMSO was used as a diluent/vehicle to get the proper concentration of synthesized compounds and standard drugs were used to test upon standard microbial strains. Serial dilutions were prepared in primary and secondary screening.

All compounds and standard drugs were diluted to obtain 2000 µg/Ml concentration as a stock solution. In primary screening 1000, 500, and 250 µg/mL concentrations of the synthesized compounds were used. The active compounds found in this primary screening were further diluted to obtain 200, 100, 62.5, 50, 25, 12.5, and 6.25 µg/mL concentrations for secondary screening to test in the second set of dilution against all microbial strains. The control tube containing no antibiotic was immediately subcultured (before inoculation) by spreading a loopful evenly over a quarter of a plate of medium suitable for the growth of the test organism. The tubes were then put

for incubation at 37°C for 24 h for bacteria and 48 h for fungi.

The highest dilution (lowest concentration) preventing the appearance of turbidity was considered as minimal inhibitory concentration (MIC, µg/mL) i.e., the amount of growth from the control tube before incubation (representing the original inoculum) was compared. A set of tubes containing only seeded broth and the solvent controls were maintained under identical conditions so as to make sure that the solvent had no influence on strain growth. The result of this was much affected by the size of the inoculum. The test mixture should contain 108 CFU/mL organisms.

3.2 Anti-inflammatory Activity

The anti-inflammatory activity was assessed as suggested by Winter et al[8] by using carrageenan as an edematogenic agent on adult albino rats of either sex weighing between 125- 150g. The selected albino rats were housed in groups of six each in acrylic cages under laboratory conditions. The test compounds 4a, 4b, 4c, and 4d were administered intraperitoneally in the form of suspension with tween-40 and normal saline at a dose of 50mg/kg b.w. The Diclofenac sodium 10mg/kg was used as a reference standard and administered in a similar manner. The control group received the solvent (tween-40 + normal saline) at 2ml/kg b.w. in a similar manner. All the test samples were administered 30mins before injection of carrageenan (0.1ml of 1% w/v in normal saline) into the sub-plantar region of left hind paw of each rat. The contra-lateral paw was injected with an equal volume of saline. The increase in paw swelling (volume) was determined by a plethysmometer by calculating the difference between the volumes of the mercury displaced by the two paws in ml. The increase in paw volume was measured at 1, 2 & 3 hrs after the administration of the test compounds. The percentage protection of edema was calculated at the end of 3 hrs as per the following formula.

$$\%\ of\ protection = \frac{P_C - P_T}{P_C} \times 100$$

Where P_C = Increase in paw volume at time‘t’ of solvent control & P_T = Increase in paw volume at time‘t’ of test.

IV. RESULT AND DISCUSSION

Table 1: Physicochemical character of synthesized compound

Sl. No.	Compound	Molecular formula	Molecular weight	% of yield	Melting point (^{0}C)	Nature
1	5a	$C_{24}H_{18}N_6$	391.44	74	238-240	Light yellow solid
2	5b	$C_{24}H_{18}FN_6$	*409.43*	76	258-260	Yellow solid
3	5c	$C_{24}H_{18}ClN_6$	*425.88*	69	266-268	Light yellow solid
4	5d	$C_{24}H_{17}BrN_6$	*479.34*	83	240-242	White solid
5	5e	$C_{24}H_{17}BrN_6$	*471.34*	79	242-244	Light yellow
6	5f	$C_{24}H_{17}N_7O_2$	436.44	78	266-268	Pale yellow solid
7	5g	$C_{24}H_{17}N_7O_2$	436.44	81	274-276	Yellow solid

Table 2: Elemental analysis of synthesized compound

Compound	Molecular formula	Calculated (%)			Found (%)		
		C	H	N	C	H	N
5a	$C_{24}H_{18}N_6$	73.83	4.65	21.52	73.75	4.59	21.48
5b	$C_{24}H_{18}FN_6$	70.58	4.20	20.58	70.61	4.16	20.54
5c	$C_{24}H_{18}ClN_6$	67.84	4.03	19.78	67.86	4.04	19.81
5d	$C_{24}H_{17}BrN_6$	61.42,	3.65	17.91	61.39	3.64	17.89
5e	$C_{24}H_{17}BrN_6$	61.42	3.65	17.91	61.39	3.64	17.87
5f	$C_{24}H_{17}N_7O_2$	66.20	3.94	22.52	66.16	3.95	22.51
5g	$C_{24}H_{17}N_7O_2$	66.20	3.94	22.52	66.18	3.92	22.56

4.1. Biological Evaluation

4.2 Antimicrobial activity

In antibacterial potency compounds 5a and 5b were found to be equipotent to Ampicillin while compound 5c was found as more potent than Ampicillin against *E. coli*. Compound 4b found to be more potent than ampicillin against P. Aeruginosa. Compound 5d was found to be equivalent to ampicillin and compound 5c and 5d found to be more potent than ampicillin against gram positive bacteria S. Aureus. Compound 5c found to be equivalent to ampicillin against gram positive bacteria S. Pyogenus.

In antifungal activity compounds 5c and 5d found to be equivalent to Greseofulvin against C. Albicans and all four compounds were found to be less potent than Greseofulvin against A. Niger and A. Clavatus.

Table3: Antibacterial activity screening

Compound	Minimum Inhibition Concentration (MIC, µg/mL)			
	Gram-negative		Gram-positive	
	E. Coli	P. Aeruginosa	S. Aureus	S. Pyogenus
5a	100	200	250	200
5b	100	62.5	500	500
5c	50	125	100	100
5d	200	250	100	250
5e	100	62.5	500	500
Ampicillin	100	100	250	100

Table 4: Antifungal activity screening

Compound	Minimum Inhibition Concentration (MIC, µg/mL)		
	C.Albicans	A. Niger	A. Clavatus
5a	1000	1000	1000
5b	1000	250	500
5c	**500**	500	1000
5d	**500**	500	1000
5e	**1000**	250	500
Greseofulvin	500	100	100

4.3 Anti-inflammatory activity

The anti-inflammatory activity of present synthesized compounds was evaluated by carrageenan induced acute inflammatory method in rats on comparison with standard diclofenac sodium. The result revealed that the all-synthesized compounds shown anti-inflammatory activity (*p<0.05, **p<0.01, ***p<0.001) when compare to control and positive control group. These showed the percentage protection of edema ranges between 39.74 and 65.38 at the end of 3 hrs; where as the standard drug Diclofenac sodium registered 64.10% (Table – 2). It has been observed that the maximum anti-inflammatory activity was recorded by the compound **5a** having 65.38% percentage protection of edema followed by compound **5b** (60.25 %), 5**c** (50 %) &**5d** (39.74%) anti-inflammatory potential. The compound **5a** showed more percentage protection of edema than that of standard drug Diclofenac sodium. It has been found that the extent of increase in paw volume reduces significantly up to 3 hrs when compared with solvent control.

Table 5: Anti-inflammatory activity of pyrazolyl aminopyrimidine (5a, 5b, 5c, 5d, and 5e)

Sl. No.	Compound	Increase in paw vol (ml)			% Protection at 3h
		1h	2h	3h	
1	**Solvent**	0.48±0.02	0.73±0.03	0.78±0.01	---
2	**5a**	0.24±0.03***	0.29±0.01***	0.27±0.03***	65.38
3	**5b**	0.36±0.03**	0.49±0.06**	0.47±0.02***	39.74
4	**5c**	0.27±0.03***	0.34±0.05***	0.31±0.05***	60.25
5	**5d**	0.29±0.02***	0.40±0.01***	0.39±0.05***	50.00
6	**5e**	0.31±0.0.03**	0.42±0.05**	0.41±0.004**	41.72
6	**Diclofenac**	0.38±0.02**	0.33±0.04***	0.28±0.03***	64.10

*Values expressed as Mean □ SEM, n=6, *p<0.05, **p<0.01, ***p<0.001, Dose of the test compound: 50 mg/kg.*

V. CONCLUSION

The present study concluded the beneficial effect of synthesized novel pyrazolyl aminopyrimidine derivatives as an antimicrobial agent against different bacteria and fungi and in the Carrageenan induced acute inflammation in rats. This study confirms the rational basis for its use in synthesized novel pyrazolyl aminopyrimidine and its derivatives for the treatment of microbial infection and inflammation in patients. Further pharmacological investigations are under way to characterize active novel pyrazolyl aminopyrimidine and to establish exact mechanism of antimicrobial and anti-inflammation action, which may have fewer side effects. This work, we believe, will be useful for further antimicrobial and inflammation research works.

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