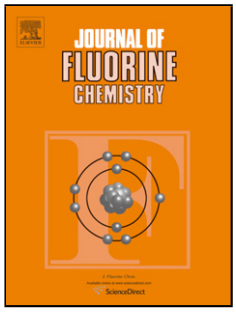

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Synthesis and biological evaluation of 7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines as anti-inflammatory and antimicrobial agents

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Abstract

A series of 2-*H*/methyl-3-phenyl-5-alkyl/aryl/heteroaryl-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines (**4a-l**) were synthesized by refluxing 3(5)-amino-4-phenyl-5(3)-*H*/methyl-1*H*-pyrazoles (**1-2**) with trifluoromethyl-*β*-diketones (**3a-f**) in ethanol for 6 hrs. The structure of the compounds was assigned on the basis of ^{1}H, ^{13}C , ^{19}F NMR and IR spectral data. The intermediate, 5-methyl-4-phenyl-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyrimidin-5,7-diol (**5b**), involved in the reaction was also isolated and characterized in one case by performing the reaction in DCM at -15 °C. A total of nine compounds **4a-f**, **4h-i**, **4k** were tested for their anti-inflammatory activity by Carrageenan-induced rat paw edema assay. Compound **4e** exhibited the comparable anti-inflammatory activity (83.4 %) to the standard drug Indomethacin (84.2 %).To rationalize the anti-inflammatoy activity, docking experiments were performed to study the ability of these compounds to bind into the active site of COX-2 enzyme. All the twelve compounds synthesized (**4a-l**) were screened for their antimicrobial activity *in vitro* against two

Gram +ve, two Gram -ve bacteria and two fungi. Preliminary results reveal that some of the synthesized compounds revealed promising antimicrobial activity against Gram +ve bacteria and pathogenic fungi used in this study.

Keywords

2-*H*/methyl-3-phenyl-5-alkyl/aryl/heteroaryl-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines; Trifluoromethyl-*β*-diketone; Anti-inflammatory activity; Antifungal activity; Antibacterial activity.

1. Introduction

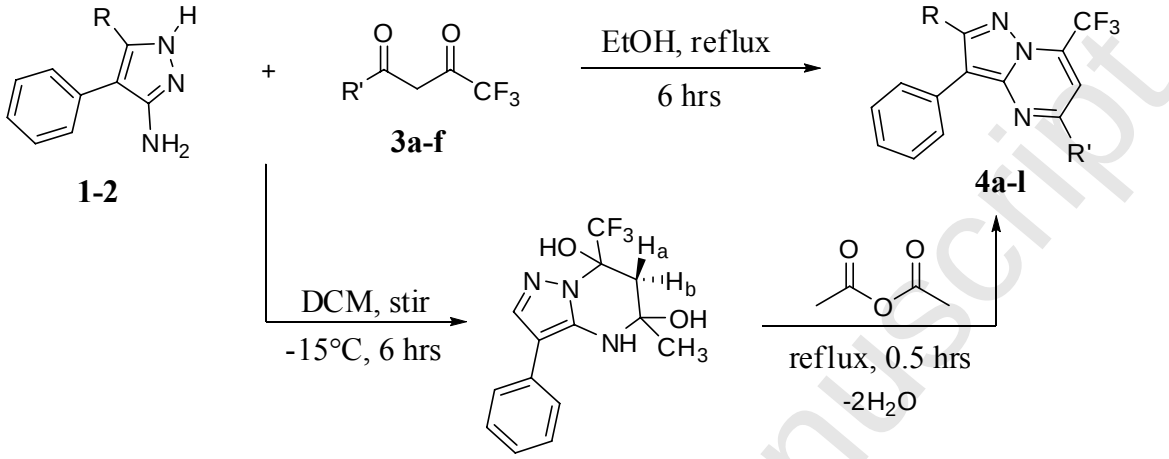
Pyrazolo[1,5-*a*]pyrimidines and their derivatives constitute a class of heterocyclic compounds currently employed in the field of medicinal chemistry for demonstrating antimicrobial [1-5], antifungal [6], antitumour [7,8], anticancer [9] and anti-trichromonal [10] activities. Different pyrazolo[1,5-*a*]pyrimidine containing compounds possess antischistosomal [11], hypnotic [12] and anti-inflammatory [13,14] activity and some are known to be used as pharmaceutical as well as agrochemical products. Zaleplon [15-17] and Indiplon [18] are some of the well known hypnotic drugs which belong to this class of compounds. Furthermore, pyrazolo[1,5-*a*]pyrimidine derivatives act as potent inhibitors of various enzymes such as adenokinase [19], CHK 1 [20,21], C-Src kinase [22], human cyclin-dependent kinase 2 [23], DPP-IV [24], B-Raf^{V600E} kinase [25] and estrogen receptors ligands [26]. In addition to this, it has been observed that the addition of trifluoromethyl group, due to its unique stereoelectronic properties, increases

the lipophilicity when present in the biologically active molecules [27]. Hence, the introduction of trifluoromethyl group into bioactive molecules becomes an important strategy in perfecting pharmaceuticals.

Several methods have been described in the literature for the synthesis of pyrazolo[1,5-*a*]pyrimidines. Most of them involve the reaction between 5-amino-1*H*-pyrazoles with 1,3-bielectrophilic reagents, such as *β*-dicarbonyl, *α,β*-unsaturated carbonyl, alkoxymethylene-*β*-dicarbonyl and *β*-enaminone compounds [28-31]. Reaction of various unsymmetrical 1,3-bis-electrophiles with 5-amino-4-substituted-1*H*-pyrazoles usually affords a mixture of two regioisomers, however, in some of the cases regiospecific/regioselective synthesis of pyrazolo[1,5-*a*]pyrimidines has been reported [32-34]. Recently a chemo and regioselective synthesis of some new pyrazol-1'-ylpyrazolo[1,5-*a*]pyrimidines has been achieved by us involving reaction between 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride and several β-diketones using water as a solvent [3]. Prompted by these investigations and in continuation of our efforts to synthesize trifluoromethylated bioactive molecules [35-39], we considered to synthesize a series of 2-*H*/methyl-3-phenyl-5-alkyl/aryl/heteroaryl-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines with an aim to find more potent anti-inflammatory and antimicrobial agents.

2. Results and discussion

The synthetic pathway to the title compounds is summarized in **Scheme-1**.



	R
1	H
2	CH_3

3	R'
a	CF_3
b	CH_3
c	C_6H_5
d	4-$CH_3C_6H_4$
e	4-BrC_6H_4
f	2-thienyl

4	R	R'
a	H	CF_3
b	H	CH_3
c	H	C_6H_5
d	H	4-$CH_3C_6H_4$
e	H	4-BrC_6H_4
f	H	2-thienyl
g	CH_3	CF_3
h	CH_3	CH_3
i	CH_3	C_6H_5
j	CH_3	4-$CH_3C_6H_4$
k	CH_3	4-BrC_6H_4
l	CH_3	2-thienyl

Scheme-1 Synthetic route to the synthesis of 7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines (**4a-l**)

The starting compounds, 3(5)-amino-4-phenyl-1*H*-pyrazole (**1**) & 3(5)-amino-4-phenyl-5(3)-methyl-1*H*-pyrazole (**2**) were obtained by the condensation of *α*-phenylformylacetonitrile and *α*-phenylacetylacetonitrile with hydrazine hydrate, respectively [48]. The reaction of **1-2** with equimolar amount of trifluoromethyl-*β*-diketones (**3a-f**) in refluxing ethanol afforded the target compounds, 2-*H*/methyl-3-phenyl-5-alkyl/aryl/heteroaryl-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines **(4a-l**) regioselectively in good yield. The reaction between binucleophilic centres in **1-2** and bielectrophilic centres in **3a-f**, in principle, may yield two regioisomers, however, in the present study due to differential reactivities of nucleophilic and electrophilic sites, only a single isomer was isolated.

The compounds were characterized by a combined application of ^{1}H, ^{13}C and ^{19}F NMR spectroscopy.

The ^{1}H NMR spectra of **4a-f** exhibited a singlet of one proton intensity at about δ 8.64 due to the pyrazolopyrimidine 2-H while in **4g-l** this singlet was replaced by a sharp singlet of three proton intensity at ~ δ 2.65 due to methyl protons at C-2. For **4a-l** another singlet of one proton intensity was observed situated at ~ δ 7.55 corresponding to the proton present at position-6 of the pyrazolo[1,5-a]pyrimidine ring**.** Compounds **4b** and **4h** displayed a singlet of three proton intensity at δ 2.76 and δ 2.68 ppm, respectively which was assigned to methyl protons at position-5. Had the methyl group been on the position-7 of the pyazolopyimidine ring it would have appeared as a doublet. This argument is based on our previous observation where ^{1}H NMR and 2-Dimensional NMR spectrum of 2-(3',5'-dimethylpyrazol-1'-yl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidine [40] showed a singlet of three proton intensity at δ 2.54 corresponding to the C-5 methyl and a doublet of three proton intensity at δ 2.70 having coupling constant ^{4}J= 1.0 Hz for the C-7 methyl protons due to coupling with the proton at position-6 of pyrazolopyrimidine. This

coupling split the signal for the proton at position-6 into a quartet at δ 6.55 (^4J= 1.0 Hz) (CH_3-7, H-6). (**Fig-1**)

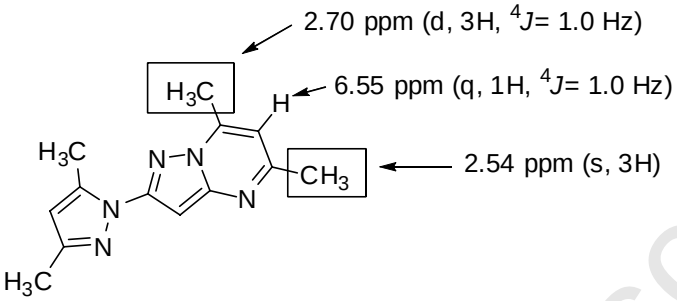
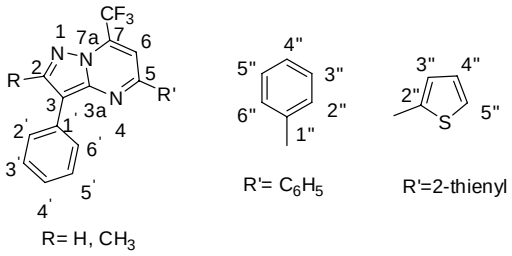


Fig-1 ^{1}H NMR data of 2-(3',5'-dimethylpyrazol-1'-yl)-5,7-dimethylpyrazolo[1,5-a]pyrimidine [40]

The structure of regioisomers **4a-f** can further be established on the basis of ^{13}C NMR. The methyl group in compounds **4b** and **4h** appear as a sharp signal at δ 25.26 and 25.12 ppm respectively, which is characteristic for a methyl group at carbon-5 of the pyrazolopyrimidine ring [32,33]. Moreover, in all the compounds, while C-5 appears as a singlet at a ~ 150 ppm, C-6 exhibits a quartet at ~ 102 ppm (s ^3J=4.0 Hz (C-6, CF_3)) and C-7 exhibits a quartet at ~ 133 ppm (^2J=37.0 Hz (C-7, CF_3)) due to coupling with the CF_3 carbon. This data is concordant with our earlier reports [41]. The signal for the CF_3 carbon appears as a doublet at about ~ 118 ppm (^2J= 273.0 Hz) in agreement with the literature value [32,41].

Further, in the ^{19}F NMR spectra of **4a-l**, signals in the range δ -68.79 to -69.55 ppm, values typical to the CF_3 at position-7 suggested the isomer as 2-H/methyl-3-phenyl-5-alkyl/aryl/heteroaryl-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidine in agreement with literature values [41] (**Table-1)**.

Table 1 ^{13}C and ^{19}F NMR data of **4a**, **4d-e**, **4g**, **4j-l** (in ppm)



Compounds	4a	4d	4e	4g	4j	4k	4l
C-2	157.27	154.25	153.89	156.08	154.23	153.57	154.06
C-3	110.00	110.70	112.43	113.35	110.68	111.20	110.46
C-3a	142.59	141.50	144.24	144.97	146.91	146.79	146.43
C-5	144.35[a]	154.84	145.46	145.52[a]	154.81	154.64	150.15
C-6	106.25[b]	102.84[c]	103.26[c]	101.64[b]	102.77[c]	102.54[c]	102.23[c]
C-7[a]	132.52	133.56	132.45	135.00	133.78	132.32	133.64
Aryl carbons							
C-1'	130.19	131.78	134.96	130.11	133.41	131.50	130.87
C-2',6'	125.54	129.09	127.03	127.62	128.53	128.61	126.68
C-3',5'	125.74	126.66	128.90	129.14	129.07	128.69	128.95
C-4'	127.75	127.18	126.08	128.77	126.65	129.14	126.82
							Thienyl carbons
C-1"	-	136.11	134.84	-	133.54	135.15	142.47 (C-2")
C-2",6"	-	129.82	131.09	-	127.15	126.88	128.52 (C-3")
C-3",5"	-	128.54	128.75	-	129.80	129.10	128.61 (C-4")
C-4"	-	133.98	126.66	-	131.79	125.76	131.60 (C-5")
Other carbons							
2-CH_3	-	-	-	14.54	14.58	14.55	14.69
5-CH_3	-	-	-	-	-	-	-
5-CF_3[d]	114.35	-	-	117.25	-	-	-
7-CF_3[e]	119.80	118.32	118.14	118.43	118.35	118.75	118.19
C-5"-CH_3	-	21.48	-	-	21.46	-	-
^{19}F Data							
7-CF_3	-69.25	-68.92	-68.94	-69.20	-68.86	-68.88	-68.79
5-CF_3	-68.21	-	-	-68.18	-	-	-

aQuartet, 2*J*CF = 37.2 Hz
bMultiplet
cQuartet, 2*J*CF = 4.0 Hz
dQuartet, 1*J*CF = 274.7 Hz
eQuartet, 1*J*CF = 274.7 Hz
The assignments are on the basis of data of compound 4c and 4f [41].

To gain an insight of the reaction mechanism, attempts were made to isolate an intermediate of the reaction. The intermediate, 5-methyl-3-phenyl-7-trifluoromethyl-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyrimidin-5,7-diol (**5b**), could successfully be isolated in one case by performing the reaction in DCM by stirring 3(5)-amino-4-phenyl-1*H*-pyrazole (**1**) and 1,1,1-trifluoromethylpent-2,4-dione (**3b**) at -15 °C for 6 hrs. Two nitrogens, endo (part of the pyrazole ring) and exo (amino group) of **1**, in principle, may react with two carbonyls of 1,1,1-trifluoromethylpent-2,4-dione (**3b**), however, the intermediacy of **5** indicates that the amino group of **1** reacts with methyl carbonyl of **3b** and the carbonyl carbon near to CF_3 is attacked by endo N. (**Scheme-1**)

The intermediate **5b** was characterized by NMR & IR spectroscopy. The ^{1}H NMR spectrum of compound **5b** showed a set of signals at δ 3.03-3.07 & 3.15-3.19 showing two doublets of AB system (gem-coupling) belonging to diastereotopic methylene protons at position-6 with J = 16.0 Hz. Broad singlets at δ 5.56, 7.29 and 7.98 were assigned to –NH, C_7-OH and C_5-OH protons respectively. The IR spectrum showed a broad absorption band at 3124 cm^{-1} and a sharp band at 3479 cm^{-1}, characteristic for the –OH and –NH groups. The product was identified as 5-methyl-3-phenyl-7-trifluoromethyl-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyrimidin-5,7-diol (**5b**) which upon dehydration by refluxing with acetic anhydride for 0.5 hr gave **4b**. This was confirmed by co-TLC and mixed m.pt with the standard sample prepared by reaction between **1** and **3b** (**Scheme-1**).

3. Biological results and discussion

3.1 Anti-inflammatory evaluation and docking studies

The anti-inflammatory activity of compounds **4a-f, 4h-i** and **4k** was studied using Carrageenan induced paw edema method [42]. The protocol of animal experiments has been approved by the Institutional Animal Ethics Committee (IAEC). Each test compound was dosed orally (50 mg/kg body weight) 30 min prior to induction of inflammation by carageenan injection. Indomethacin was utilized as a reference anti-inflammatory drug at a dose of 10 mg/kg. The anti-inflammatory activity was then calculated 60-240 min after induction and presented in **Table-2** as the mean paw volume (ml) in addition to the percentage anti-inflammatory activity (AI%).

A careful analysis of **Table-2** and **Fig-2** reveals that most of the tested compounds showed good anti-inflammatory activity after the 2nd hour of drug treatment comparable to the standard drug Indomethacin. After 1 hour of drug treatment, compound **4a** was fairly effective with an activity of 77.4 % in comparison with Indomethacin (94.3). Compounds **4a**, **4d**, **4h-i** and **4k** showed activity in the range 58.6- 77.6 % while compound **3e**, showing an activity of 83.4 % was comparable to the standard drug Indomethacin (84.2 %). Some of the compounds **(4a-c),** showing activity in the

range 58.8-71.7 % were more effective after 3 hours. Only one of the compounds, **4a**, was found to be effective after 4 hrs.

Though no general trend can be assigned to the compounds showing good activity in terms of substituents present at position-2 and 5 of the pyrazolopyrimidine ring, most of the compounds exhibited good anti-inflammatory activity. Comparative analysis indicates that compound **4a** was

found to be most potent showing very high activity after 1st, 2nd, 3rd as well as 4th hour of drug treatment while compound **4e** showed excellent activity after 2 hrs as compared to Indomethacin.

Table-2 Anti-inflammatory activity of compounds **4a-f**, **4h-i** and **4k** through Carrageenan-induced paw edema test

Compound	1 hr	2 hr	3 hr	4 hr
Tween 80 (5 %)	0.71 ± 0.08	1.21 ±.10	1.36 ± .15	1.25 ±0.09
Indomethacin (10 mg/kg)	0.04 ± 0.02**(94.3)	0.19 ±0.03**(84.2)	0.26±0.02**(80.8)	0.17 ±0.03**(86.4)
4a	0.16 ±0.04**(77.4)	0.27 ±0.09**(77.6)	0.33±0.05**(75.7)	0.48 ±0.11**(61.6)
4b	0.34 ±0.06(52.1)	0.78 ±0.13(35.5)	0.52±0.20**(61.7)	0.73 ±0.11(41.6)
4c	0.55 ±0.20(22.5)	0.68 ±0.16(43.8)	0.56±0.29**(58.8)	0.70 ±0.12(44.0)
4d	0.30 ± 0.03(57.7)	0.28 ±0.06**(76.8)	0.86 ±0.05(36.6)	1.04 ±0.13(16.8)
4e	0.67 ±0.18(5.6)	0.20 ±0.07**(83.4)	0.69±0.11*(49.2)	0.86 ±0.11(31.2)
4f	0.53 ± 0.09(23.3)	0.68 ± 0.21(43.8)	0.89 ±0.19(34.5)	1.17 ±0.23(6.4)
4h	0.44 ± 0.04(38.0)	0.46±0.11**(61.9)	0.76 ±0.14(44.1)	0.59 ±0.21*(52.8)
4i	0.36 ± 0.09(49.2)	0.50 ±0.22**(58.6)	0.73 ±0.11(46.3)	1.04 ±0.13(16.8)
4k	0.51 ± 0.11(28.1)	0.40 ±0.10**(66.9)	1.03 ±0.21(24.2)	0.76 ±0.08(39.2)

All values are expressed as mean ±SEM of five rats in each group.

Statistically significant **p<0.01,*p>0.05 compared to control.

Values in parenthesis represent % inhibition.

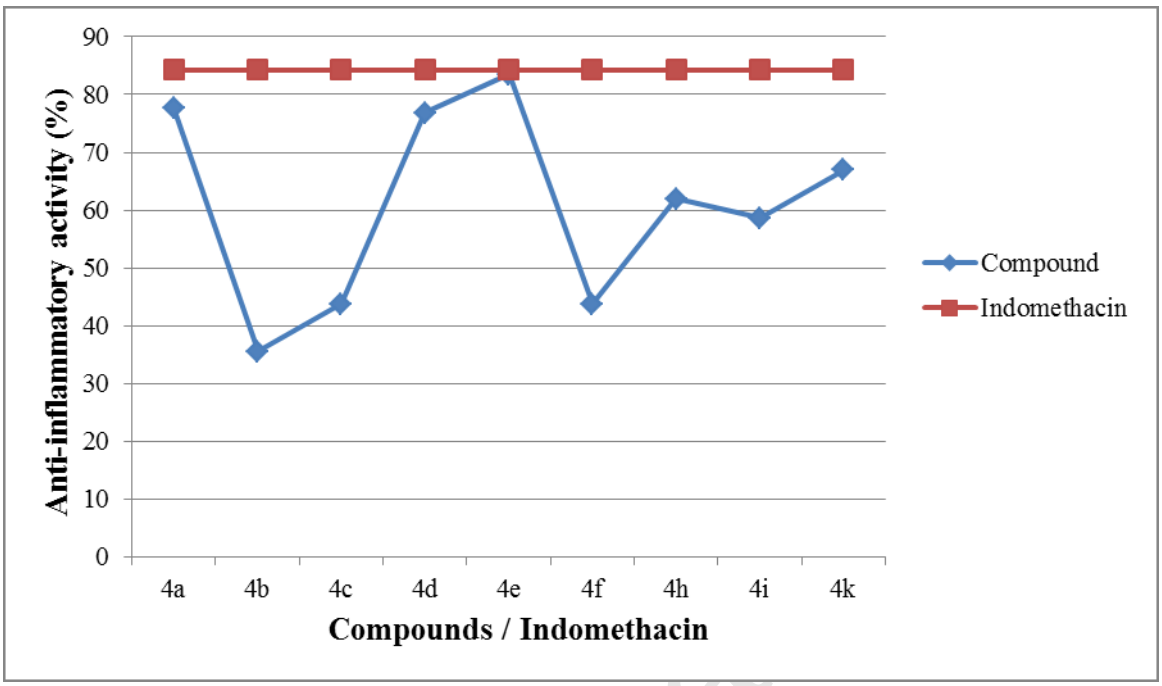


Fig-2 Percentage anti-inflammatory activity of compounds **4a-f, 4h-i** and **4k** / reference after 2 hrs of drug treatment

Significant anti-inflammatory activity of novel synthesised compounds prompted us to perform molecular docking studies of compounds **4a-f**, **4h**, **4i** and **4k** to understand the ligand-protein interactions and Cyclooxygenase-2 (COX-2) selectivity in detail. Automated docking studies were carried out using Molegro Virtual Docker 2010.4.1 [43], the scoring functions and hydrogen bonds formed with the surrounding amino acids are used to predict their binding modes and their binding affinities at the active site of COX-2 enzyme.

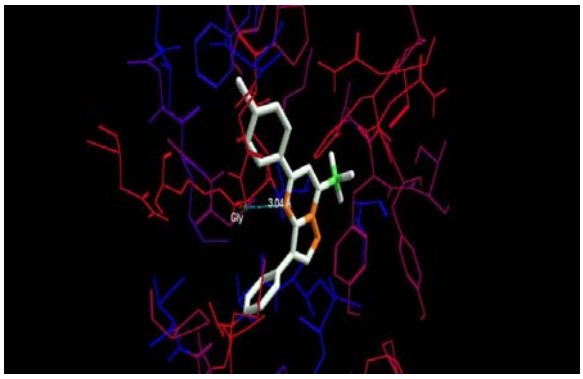
The standard compound Indomethacin showed one hydrogen bond interaction of oxygen of C=O group with amino acid Lys 56 having hydrogen bond length 2.87 Å. In most of the compounds (**4**), N-4 of pyrazolopyrimidine ring showed interaction with amino acid Thr 60 (2.92 Å - 3.19 Å) , Lys 56 (3.44 Å) and Arg 61 (3.50 Å) except compound **4c** and **4d** which showed interaction with amino acid Thr 60 (2.73 Å - 3.43 Å) *via* N-1 and N-7a. Compounds **4e**, **4f**, **4i** and **4k**

exhibited relatively similar binding affinity for COX-2 having docking score 102.82, 100.77, 106.26 and 108.82 respectively, which is comparable to the reference drug Indomethacin (the original ligand) as presented in **Table-3** and **Fig-3.** (For complete docking interactions see Supplementary data). Though dock score may be used as an index to explain the better activity exhibited by **4a, 4d, 4e** and **4k**, however, no correlation can be made between anti-inflammatory activity and dock score of these four compounds. Further studies need to be carried out to find the specific target of these compounds.

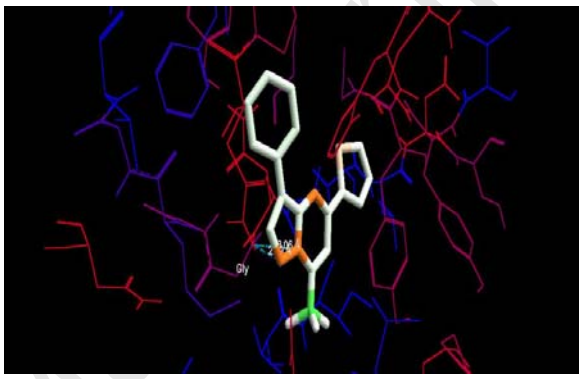
Table-3 Dock score and bond interactions of reference drug Indomethacin and synthesized compounds **4a-f**, **4h**, **4i** and **4k** with amino acids of COX-2 enzyme

Compound	Dock Score (-)	No. of interactions	Distance (Å)	Amino acids involved	Atoms of ligand pyrazolopyrimidine
4a	89.08	3	3.09	Thr 60	N-4
			3.28	Arg 61	N-1
			3.46	Arg 61	N-7a
4b	82.80	2	3.26	Thr 60	N-1
			3.44	Lys 56	N-4
4c	92.61	2	2.73	Thr 60	N-1
			3.43	Thr 60	N-7a
4d	98.59	2	2.97	Thr 60	N-1
			3.13	Thr 60	N-7a
4e	102.82	3	3.27	Thr 60	N-7a
			3.33	Arg 61	N-1
			3.50	Arg 61	N-4
4f	100.77	1	3.01	Thr 60	N-4
4h	87.68	1	3.05	Thr 60	N-4
4i	106.26	3	2.92	Thr 60	N-4

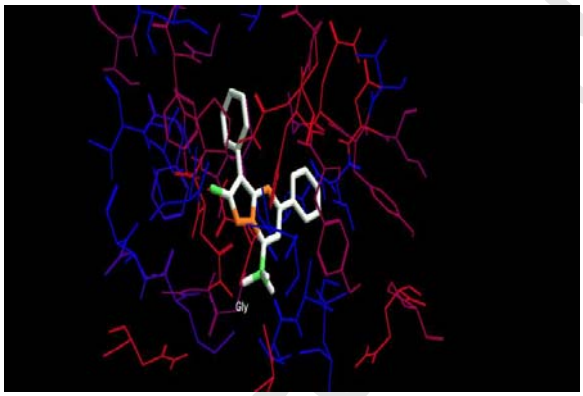
			3.36	Arg 61	N-1
			3.38	Arg 61	N-7a
4k	108.82	2	3.19	Thr 60	N-4
			3.35	Arg 61	N-1
Indomethacin	110.92	1	2.87	Lys 56	Oxygen of =CO



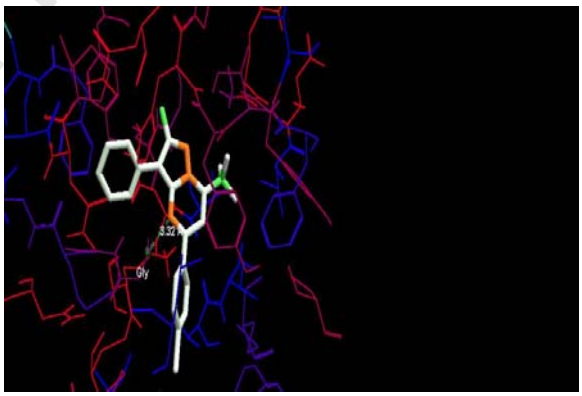
4e



4f



4i



4k

Fig 3 Zoomed images showing bond interactions of compounds **4e**, **4f**, **4i** and **4k** with amino acids of COX-2 enzyme

3.2 Antimicrobial evaluation

Twelve chemically synthesized compounds (**4a-l**) were assayed for their antibacterial activity *in vitro* against *Staphylococcus aureus* (MTCC 96) and *Bacillus subtilis* (MTCC 121) as examples of two Gram +ve bacteria and *Escherichia coli* (MTCC 1652) and *Pseudomonas aeruginosa* (MTCC 741) as examples of two Gram -ve bacteria. Compounds **4a-l** were also screened for their antifungal activity against two fungi *Aspergillus niger* and *Aspergillus flavus*.

The Agar well diffusion method [44] was used for the determination of antibacterial activity while antifungal activity was evaluated by poison food technique [45]. DMSO was used as a negative control whereas Ciprofloxacin was used as positive control in antibacterial activity while Fluconazole was used as standard drug for antifungal activity. Minimum Inhibitory Concentaration (MIC) measurements were performed using a micro dilution tube method [46,47].

3.2.1 Antibacterial activity

Results revealed that in general, all the tested compounds possessed moderate antibacterial activity against Gram +ve bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). However, none of them was found to be effective against any of the Gram -ve bacteria (*E. coli* and *P. aeruginosa*).

On the basis of diameter of growth of inhibition zone shown against Gram +ve bacteria, compound **4g** was found to be the most effective against *S. aureus* with zone of inhibition of 17.6 mm and two compounds namely **4g** and **4h**, against *B. subtilis*, with zone of inhibition ranging between 19.3 mm and 18.6 mm comparable to the standard drug Ciprofloxacin. Moderate antibacterial activity was observed by compounds **4h**, **4j** and **4l** with zone of inhibition > 15.0 mm and compounds **4b**, **4d**, **4i** and **4k** with zone of inhibition ≥ 15.0 mm (**Table-3**). In

the whole series, the MIC of chemical compounds ranged between 64 and 256 μg/ml against Gram +ve bacteria. Compound **4g** was found to be best as it exhibited the lowest MIC of 64 μg/ml against *S. aureus* and compounds **4g** and **4h** showed an MIC of 64 μg/ml against *B. subtilis* (**Table-3**).

The general trend in **Table-4** reveals that compounds **4a-l** exhibited fairly good activity against *B. subtilis* than *S. aureus* as compared to the standard drug Ciprofloxacin. Moreover, the substituent at position-2 of the pyrazolopyrimidine ring also affects the results. Replacement of –H group at position-2 by $–CH_3$ group (**4g-l**) enhances the antibacterial activity against both the Gram +ve bacteria *B. subtilis* and *S. aureus*. Further, compounds **4b** and **4g-h** having $–CH_3$ and $–CF_3$ group at position-5 showed better activity as compared to rest of the compounds having phenyl and substituted phenyl groups. Ineffective nature of the tested compounds against Gram -ve bacteria *E. coli* and *P. aeruginosa* may be attributed to the outer harder, lipopolysaccharide containing membrane which makes them more resistant against antibiotics. As can be seen from **Table-4**, MIC was lowest for compounds **4g** and **4h**. Thus, in general, it can be concluded that compounds containing simple substituents like $–CH_3$ and $–CF_3$ at position-5 exhibit better antibacterial activity than compounds having aryl/heteroaryl substituents.

Table -4 Antibacterial activity of chemical compounds **4a-l** through agar well diffusion method

Compound No.	Diameter of growth of inhibition zone (mm)[a]				Minimum inhibitory concentration (MIC) (µg/mL)	
	Staphylococcus aureus	***Bacillus subtilis***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Bacillus subtilis***
4a	12.3	13.6	**-**	**-**	>256	256
4b	14.6	15.3	-	-	256	128
4c	13.6	12.6	-	-	256	>256
4d	12.0	15.6	-	-	>256	128
4e	12.6	13.6	-	-	>256	256
4f	13.6	14.0	-	-	256	256
4g	**17.6**	**19.3**	-	-	128	**64**
4h	15.6	**18.6**	**-**	**-**	**64**	**64**
4i	12.3	15.3	-	-	>256	128
4j	15.3	14.3	-	-	128	256
4k	13.6	15.0	**-**	**-**	**256**	128
4l	15.3	12.6	-	-	128	>256
Ciprofloxacin	26.6	24.0	25.0	22.0	5	5

- No activity

a Values, including diameter of the well (8mm), are mean of three replicates

3.2.2 Antifungal activity

As can be observed from **Table-5**, of the twelve chemical compounds (**4a-l**) screened for their antifungal activity against *Aspergillus niger* and *Aspergillus flavus* fungal strains, two compounds **4d** and **4j** showed more than 56 % inhibition of mycelial growth against *Aspergillus niger* whereas compounds **4d**, **4h**, **4i** and **4k** showed more than 55 % inhibition against *A. flavus* as compared to the standard drug Fluconazole (81.8 % inhibition). Compound **4j** showed highest inhibition of fungal mycelium (61.1%) against *A. flavus***.** Data reported in **Table-5** revealed that compound **4j** is most active as compared to other compounds and standard drug**.**

Table-5 Antifungal activity *in vitro* of synthetic chemical compounds **4a-l** through poisoned food method [45]

Compound No.	Mycelial growth inhibition (%)	
	Aspergillus niger	***Aspergillus flavus***
4a	52.2	53.3
4b	50	51.1
4c	51.1	50
4d	**58.8**	**55.5**
4e	53.3	50
4f	51.1	52.2
4g	50	51.1
4h	52.2	**56.6**
4i	53.3	**58.8**
4j	**56.6**	**61.1**
4k	51.1	55.5
4l	50	48.8
Fluconazole	81.1	77.7

6. Conclusion

In conclusion, we have synthesized a series of 2-H/methyl-3-phenyl-5-substituted-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines (**4a-l**) regioselectively and evaluated them for their antimicrobial and anti-inflammatory activitites. Most of the tested compounds (**4a-l**) were moderately active as Gram +ve antibacterial and the antifungal agents. Compounds **4g** and **4h** were most effective in antibacterial activity showing an inhibition zone of 19.3 and 18.6 mm respectively against *B. subtilis* as compared to the standard drug Indomethacin (24.0 mm). Antifungal activity was best shown by compounds **4d** and **4h-i** while **4j** was most effective against *A. flavus.* Anti-inflammatory activity results of the tested compounds showed that compounds **4a**, **4d**, **4h-i** and **4k** showed activity in the range 58.6- 77.68 % while compound **4e**,

showing an activity of 83.47 % was comparable to the standard drug Indomethacin (84.29 %) after 2 hrs of drug treatment.

7. Experimental

Melting points were determined in open capillaries and are uncorrected. IR spectra were recorded on a Buck Scientific IR M-500 spectrophotometer in KBr pellets (ν max in cm^{-1}), ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker instrument at 300 / 400 MHz and 75 MHz, respectively; chemical shifts are expressed in δ-scale downfield from TMS as an internal standard. ^{19}F NMR spectra were run on DRX 300 and DPX 400 at 282 and 376 MHz, respectively, using deuteriochloroform as a solvent. The internal standard for ^{19}F spectra was fluorotrichloromethane, setting the $CFCl_3$ signal at δ 0.0. The reactions were monitored by the TLC carried out on pre-coated silica gel glass plates. Mass spectra were measured in EI mode on a Kratos MS-50 spectrometer at University of California, San Francisco, USA. Elemental analyses were performed at Sophisticated Analytical Instrument Facility, Central Drug Research Institute, Lucknow, India.

The starting materials 3(5)-amino-4-phenyl-1*H*-pyrazole **1** & 3(5)-amino-4-phenyl-5(3)-methyl-1*H*-pyrazole **2** were prepared according to literature procedure [48]. Fluorinated-*β*-diketone **3a** was purchased from Sigma-Aldich and other **3b-f** were prepared according to literature procedure [49,50].

7.1 Synthesis of 3-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidine (4a)

To a warm solution of 3(5)-amino-4-phenyl-1*H*-pyrazole **1** (1.0 g, 6.2 mmol) in ethanol (20 ml) was added 1,1,1,5,5,5-hexafluoropentan-2,4-dione **2a** (1.3 g, 6.2 mmol) and the mixture was

refluxed for 6 hrs. The reaction was monitored by TLC carried out on pre-coated silica gel glass plates. The pale yellow solid obtained on cooling was recrystallised from ethanol.

All other compounds, **4b-l,** were synthesized according to procedure mentioned for **4a** using **1-2** with fluorinated-*β*-diketones **3a-f**.

The characterization data for these compounds is given below:

7.1.1 3-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidine (4a)

Mp 132-135 ^{o}C; **Yield** 88.5%; **IR** (KBr, cm^{-1}) 3032, 2361, 1582, 1450, 1265, 1211, 1134; **^{1}H NMR** (300 MHz, $CDCl_3$) δ: 7.38-7.43 (m, 1H, Ph^{a}- 4'H), 7.50 (s, 1H, C_6-H), 7.53-7.56 (m, 2H, Ph^{a}- 3'H, 5'H), 8.06-8.08 (m, 2H, Ph^{a}- 2'H, 6'H), 8.74 (s, 1H, C_2-H). **MS (EI)** *m/z*: 332.05 $[M+1]^{+}$; **Elemental analysis** calcd. for $C_{14}H_7F_6N_3$: C, 50.77; H, 2.13; N, 12.69. Found: C, 50.73; H, 2.06; N, 12.76.

7.1.2 5-Methyl-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4b)

Mp 106-108 °C (Lit [32] mp 116 °C).

7.1.3 3,5-Diphenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4c)

Mp 174-175 °C (Lit [41] mp 176-177 °C).

7.1.4 5-(4''-Methylphenyl)-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4d)

Mp 180-182 ^{o}C; **Yield** 89%; **IR** (KBr, cm^{-1}**) :** 2361, 1605, 1574, 1404; **^{1}H NMR** (300 MHz, $CDCl_3$) δ: 2.49 (s, 3H, Ph^{b}-4''-CH_3), 7.35-7.55 (m, 5H, Ph^{a}), 7.67 (s, 1H, C_6-H), 8.12-8.18 (m,

4H, Ph^b- 2"H, 3"H, 5"H, 6"H), 8.57 (s, 1H, C_2-H). **MS (EI)** *m/z*: 354.11 $[M+1]^+$; **Elemental analysis** calcd. for $C_{20}H_{14}F_3N_3$: C, 67.98; H, 3.99; N, 11.89 Found: C, 67.80; H, 3.89; N, 11.92.

7.1.5 5-(4"-Bromophenyl)-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4e)

Mp 174-176 ^oC; **Yield** 83%; **IR** (KBr, cm^{-1}**)** 3063, 2361, 1582, 1396, 1335, 1265, 1196; **^{1}H NMR** (300 MHz, $CDCl_3$) δ: 7.31-7.35 (m, 1H, Ph^a-4' H), 7.47-7.50 (m, 2H, Ph^a-3'H, 5'H), 7.57 (s, 1H, C_6-H), 7.67-7.70 (m, 2H, Ph^a-2'H, 6'H), 8.00 (m, 2H, Ph^b-3"H, 5"H), 8.06-8.09 (m, 2H, Ph^b-2"H, 6"H), 8.54 (s, 1H, C_2-H). **MS (EI)** *m/z*: 418.01/420.01 (1:1) $[M+1]^+/[M+1+2]^+$; **Elemental analysis** calcd. for $C_{19}H_{11}BrF_3N_3$: C, 54.57; H, 2.65; N, 10.05. Found: C, 54.53; H, 2.70; N, 10.18.

7.1.6 3-Phenyl-5-(2"-thienyl)-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4f)

Mp 141-142 °C (Lit [41] mp 142-143 °C).

7.1.7 2-Methyl-3-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidine (4g)

Mp 110-112 ^oC; **Yield** 86.5%; **IR** (KBr, cm^{-1}**)** 2361, 1450, 1512, 1450, 1265, 1180; **^{1}H NMR** (300 MHz, $CDCl_3$) δ: 2.85 (s, 3H, C_2- CH_3), 7.42 (s, 1H, C_6-H), 7.52- 7.57 (m, 3H, Ph^a- 3'H, 4'H, 5'H), 7.71-7.74 (m, 2H, Ph^a- 2'H, 6'H). **MS (EI)** *m/z*: 346.07 $[M+1]^+$; **Elemental analysis** calcd. for $C_{15}H_9F_6N_3$: C, 52.18; H, 2.63; N, 12.17. Found: C, 52.27; H, 2.67; N, 12.03.

7.1.8 2,5-Dimethyl-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4h)

Mp 122-124 °C (Lit [32] mp 126-127 °C).

7.1.9 2-Methyl-3,5-diphenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4i)

Mp 128-130 °C (Lit [32] mp 133-135 °C).

7.1.10 2-Methyl-5-(4"-methylphenyl)-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4j)

Mp 138-140 ^{o}C; **Yield** 88.4%; **IR** (KBr, cm^{-1}**) :** 3433, 2361, 1628, 1566, 1412, 1335, 1211, 1149; **^{1}H NMR** (300 MHz, $CDCl_3$) δ: 2.46 (s, 3H, Ph^b- 4"H), 2.74 (s, 3H, C_2- CH_3), 7.33- 7.41 (m, 3H, Ph^a- 3'H, 4'H, 5'H), 7.52-7.57 (m, 2H, Ph^a- 2'H, 6'H), 7.59 (s, 1H, C_6-H), 7.82-7.84 (d, 2H, *J*= 6.0 Hz, Ph^b- 3"H, 5"H), 8.05-8.07 (d, 2H, *J*= 6.0 Hz, Ph^b- 2"H, 6"H). **MS (EI)** *m/z*: 368.13 $[M+1]^+$; **Elemental analysis** calcd. for $C_{21}H_{16}F_3N_3$: C, 68.66; H, 4.39; N, 11.44. Found: C, 68.70; H, 4.49; N, 11.32.

7.1.11 5-(4"-Bromophenyl)-2-methyl-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4k)

Mp 180-185 ^{o}C; **Yield** 87%; **IR** (KBr, cm^{-1}**) :** 3472, 3425, 1628, 1566, 1528, 1404, 1335, 1211, 1149; **^{1}H NMR** (300 MHz, $CDCl_3$) δ: 2.74 (s, 3H, C_2-CH_3), 7.38-7.43 (m, 1H, Ph^a- 4'H), 7.52 (s, 1H, C_6-H), 7.55-7.57 (m, 2H, Ph^a- 3'H, 5'H), 7.66- 7.69 (d, 2H, *J*= 8.7 Hz, Ph^b- 3"H, 5"H), 7.78-7.81 (m, 2H, Ph^a- 2'H, 6'H), 8.02- 8.05 (d, 2H, *J*= 8.7 Hz, Ph^b- 2"H, 6"H). **MS (EI)** *m/z*: 432.02/434.02 (1:1) $[M+1]^+$/ $[M+1+2]^+$; **Elemental analysis** calcd. for $C_{20}H_{13}BrF_3N_3$: C, 55.57; H, 3.03; N, 9.72. Found: C, 55.53; H, 3.13; N, 9.76.

7.1.12 2-Methyl-3-phenyl-5-(2"-thienyl)-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4l)

Mp 120-125 ^{o}C; **Yield** 83%; **IR** (KBr, cm^{-1}**)** 3464, 3078, 1628, 1528, 1427, 1211, 1157; **^{1}H NMR** (300 MHz, $CDCl_3$) δ: 2.73 (s, 3H, C_2- CH_3), 7.17-7.20 (m, 1H, Ph^a- 4'H), 7.34-7.38 (m,

2H, Ph[a]- 3'H, 5'H), 7.44 (m, 1H, C_6-H), 7.51-7.56 (m, 2H, Ph[a]-2'H, 6'H), 7.73-7.83 (m, Th-3"H, 4"H, 5"H). **MS (EI)** *m/z*: 360.07 $[M+1]^+$; **Elemental analysis** calcd. for $C_{18}H_{12}F_3N_3S$: C, 60.16; H, 3.37; N, 11.69. Found: C, 60.18; H, 3.27; N, 11.73.

7.2 Synthesis of 5-Methyl-3-phenyl-7-trifluoromethyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrimidin-5,7-diol (5b)

To a stirred solution of 3(5)-amino-4-phenyl-1*H*-pyrazole **1** (1.0 g, 6.2 mmol) in DCM (15 ml) was added 1,1,1-trifluoromethylpent-2,4-dione (**3b**) (0.7 g, 6.2 mmol) at -15 °C. The reaction mixture was stirred for 0.5 hr. The reaction was monitored by TLC carried out on pre-coated silica gel glass plates. The solid mass separated on stirring was filtered off and washed with cold dichloromethane.

7.2.1 5-Methyl-3-phenyl-7-trifluoromethyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrimidin-5,7-diol (5b)

Mp 112-115 °C; **Yield** 56%; **IR** (KBr, cm^{-1}) 3479 (-NH), 3124 (-OH), 1628, 1428, 1373; **^{1}H NMR** (400 MHz, $CDCl_3$) δ: 2.34 (s, 3H, C_5- CH_3), 3.03-3.07 (d, 1H, *J*= 16.0 Hz, C_6-H[a]), 3.15-3.19 (d, 1H, *J*= 16.0 Hz, C_6-H[b]), 5.56 (bs, 1H, -NH), 7.26-7.49 (m, 5H, Ph[a]), 7.29 (bs, 1H, C_5-OH), 7.98 (bs, 1H, C_7-OH)

7.3 Conversion of 5-Methyl-3-phenyl-7-trifluoromethyl-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrimidin-5,7-diol (5b) to 5-Methyl-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (4b)

5-Methyl-3-phenyl-7-trifluoromethyl-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyrimidin-5,7-diol **5b** (0.5 g, 1.6 mmol) was refluxed in acetic anhydride (10 ml) for 0.5 hr and the reaction was

monitored by TLC carried out on pre-coated silica gel glass plates. The reaction mixture was cooled and poured in ice. Orange coloured solid so obtained was filtered, dried in air and recrystallised in chloroform. It was characterized as 5-Methyl-3-phenyl-7-trifluoromethylpyrazolo[1,5-a]pyrimidine (**4b**).

Ph^a represents phenyl ring at position-3 of the pyrazolopyrimidine ring

Ph^b represents phenyl ring at position-5 of the pyrazolopyrimidine ring

8. Pharmacological assay

8.1 Anti-inflammatory assay

The anti-inflammatory activity was evaluated by using the Carragenan-induced paw edema test [42]. Male Wister albino rats weighing 200-250 g were used throughout the study. They were kept in the animal house under standard conditions of light and temperature with free access to food and water. Food was withdrawn 12 h before and during experimental hours. The animals were randomly divided into groups each consisting of six rats. One group of six rats was kept as control and received tween 80 (95:5). Another group received the standard drug Indomethacin at a dose of 10 mg/kg body weight, i.p. Other groups of rats were administered the test compounds at a dose of 50 mg/kg body weight orally. A mark was made on the left hind paw just beyond the tidiotarsal articulation, so that every time the paw was dipped up to fixed mark and constant paw volume was ensured. Paw volumes were measured using a plethsymometer (model 7140, Ugo Basile, Italy). Thirty minutes after administration of test and standard drugs, 0.1 ml of 1% w/v of carageenan suspension in normal saline was injected into subplanter region of the left hind paw

of all the animals. The initial paw volume was measured within 30 sec of the injection and remeasured again 1 hour, 2 hours, 3 hours and 4 hours after administration of Carrageenan. The anti-inflammatory effect of ethanolic extract was calculated by the following equation:

Anti-inflammatory activity (%) = (Vc - V_t/ Vc) x 100

Where V_t represents the paw volume in drug treated animals and V_c represents the paw volume of control group of animals.

8.2 Antimicrobial assay

8.2.1 Antibacterial assay

The antibacterial activity of newly synthesized compounds was evaluated by the agar well diffusion method [44]. All the microbial cultures were adjusted to 0.5 McFarland standard, which is visually comparable to a microbial suspension of approximately 1.5×108 cfu/mL [46,51]. 20 mL of Mueller Hinton agar medium was poured into each Petri plate and the agar plates were swabbed with 100 mL inocula of each test bacterium and kept for 15 min for adsorption. Using sterile cork borer of 8 mm diameter, wells were bored into seeded agar plates and these were loaded with a 100 mL volume with concentration of 4.0 mg/mL of each compound reconstituted in dimethylsulphoxide (DMSO). All the plates were incubated at 37 °C for 24 h. Antibacterial activity of 12 compounds was evaluated by measuring the zone of growth inhibition against the test bacteria with zone reader (Hiantibiotic zone scale). DMSO was used as a negative control whereas ciprofloxacin was used as a positive control. The experiments were performed in triplicates. The antibacterial activity of the compounds was compared with ciprofloxacin as standard. Minimum Inhibitory Concentration (MIC) of newly synthesized compounds against tested bacteria was determined using macrodilution tube method as

recommended by NCCLS [46,47]. MIC is the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after overnight incubation. In this method, various test concentrations of newly synthesized compounds were prepared from 128 to 0.25 mg/mL in sterile tubes No. 1-10. 100 mL sterile Mueller Hinton Broth (MHB) was poured in each sterile tube followed by addition of 200 mL test compound in tube 1. Two fold serial dilutions were carried out from tube 1 to tube 10 and excess broth (100 mL) was discarded from the last tube No. 10. To each tube, 100 mL of standard inoculums (1.5×108 cfu/mL) was added. Ciprofloxacin was used as control Turbidity was observed after incubating the inoculated tubes at 37 °C for 24 h.

8.2.2 Antifungal assay

The antifungal activity of newly synthesized compounds was evaluated by the poisoned food method [45]. The molds were grown on Saburaud Dextrose Agar (SDA) at 25 °C for 7 days and used as inocula. 15 mL of molten SDA (45 °C) was poisoned by the addition of 100 mL volume of each compound having concentration of 4.0 mg/mL, reconstituted in DMSO, poured into a sterile Petri plate and allowed to solidify at room temperature. The solidified poisoned agar plates were inoculated at the centre with fungal plugs (8 mm diameter), obtained from the actively growing colony and incubated at 25 °C for 7 days. DMSO was used as a negative control whereas fluconazole was used as a positive control. The experiments were performed in triplicates. Diameter of the fungal colonies was measured and expressed as percent mycelial inhibition determined by applying the following formula:

Inhibition of mycelial growth % = (dc - dt)/dc × 100

where dc = average diameter of fungal colony in negative control plates; dt = average diameter of fungal colony in experimental plates.

8.3 Docking methodology

Docking study was carried out for the target compounds using Molegro Virtual Docker version 2010. MolDock scoring function is used by MVD program is defined by:

$E_{score} = E_{inter} + E_{intra}$

where, E_{score} = MolDock score.

E_{inter} = ligand - Protein interaction

E_{intra} = internal energy of the ligand:

The molecules/ligands were built using Marvin Sketch 5.11.0. The 2D structure was then converted into 3D which was saved as MDL MolFile. Crystal structure of COX-2 (PDB code: 1CX2) was obtained from the Protein Data Bank in order to prepare protein for docking studies. Compounds were docked into the active sites using Molegro Virtual Docker 2010.4.1 software using the standard protocol. [43]

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Schemes and Figures Caption

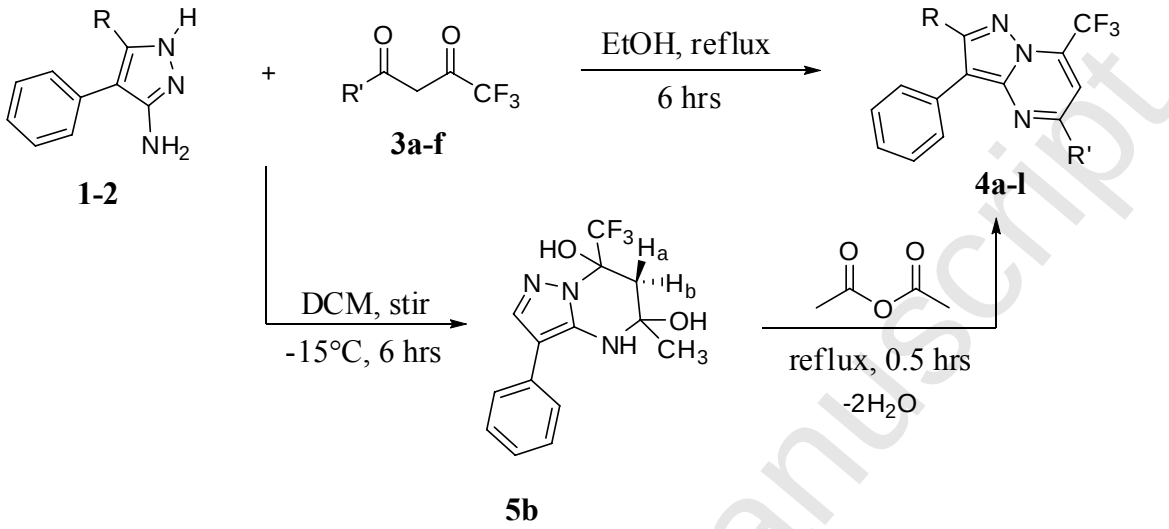
Scheme-1 Synthetic route to the synthesis of 7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines (**4a-l**)

Fig-1 ^{1}H NMR data of 2-(3',5'-dimethylpyrazol-1'-yl)-5,7-dimethylpyrazolo[1,5-a]pyrimidine [40]

Fig-2 Percentage anti-inflammatory activity of compounds **4a-f, 4h-i** and **4k** / reference after 2 hrs of drug treatment

Fig 3 Zoomed images showing bond interactions of compounds **4e**, **4f**, **4i** and **4k** with amino acids of COX-2 enzyme

Schemes and Figures



	R
1	H
2	CH_3

3	**R'**
a	CF_3
b	CH_3
c	C_6H_5
d	4-$CH_3C_6H_4$
e	4-BrC_6H_4
f	2-thienyl

4	**R**	**R'**
a	H	CF_3
b	H	CH_3
c	H	C_6H_5
d	H	4-$CH_3C_6H_4$
e	H	4-BrC_6H_4
f	H	2-thienyl
g	CH_3	CF_3
h	CH_3	CH_3
i	CH_3	C_6H_5
j	CH_3	4-$CH_3C_6H_4$
k	CH_3	4-BrC_6H_4
l	CH_3	2-thienyl

Scheme-1 Synthetic route to the synthesis of 7-trifluoromethylpyrazolo[1,5-*a*]pyrimidines (**4a-l**)

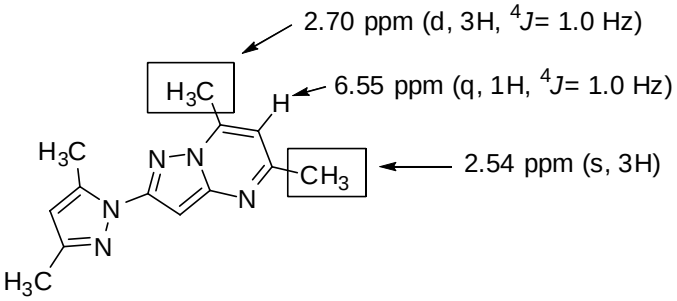


Fig-1 ^{1}H NMR data of 2-(3',5'-dimethylpyrazol-1'-yl)-5,7-dimethylpyrazolo[1,5-a]pyrimidine [40]

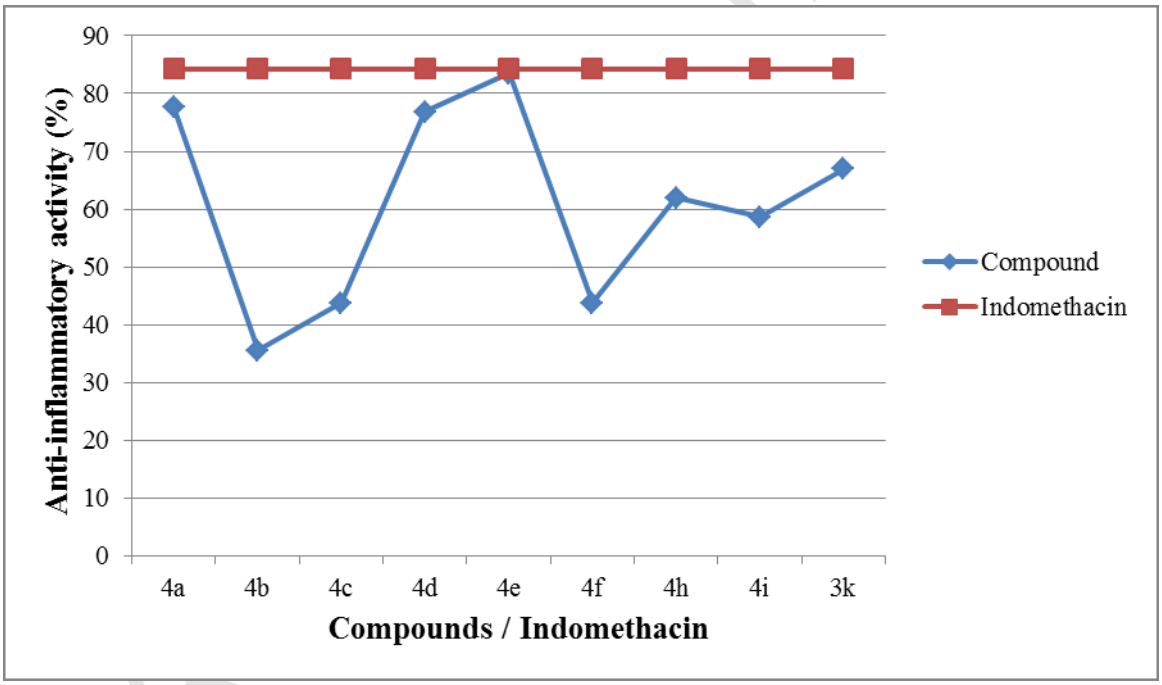
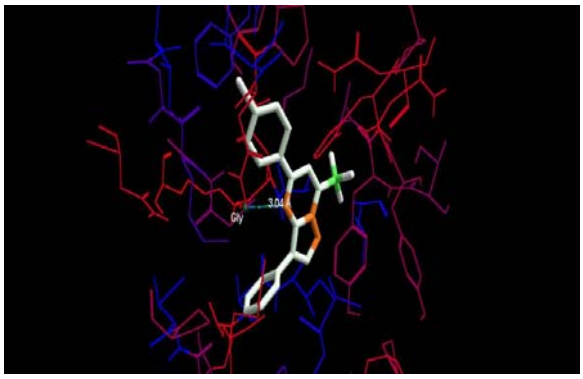
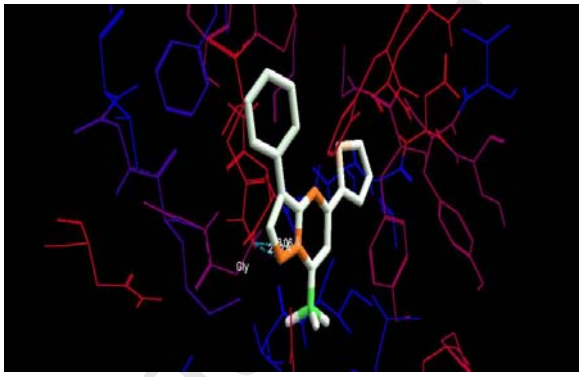


Fig-2 Percentage anti-inflammatory activity of compounds **4a-f, 4h-i** and **4k** / reference after 2 hrs of drug treatment



4e



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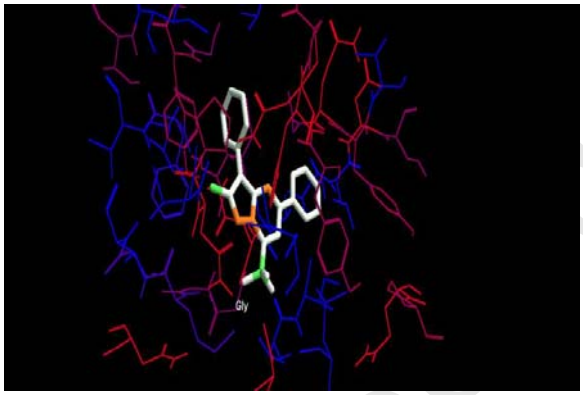

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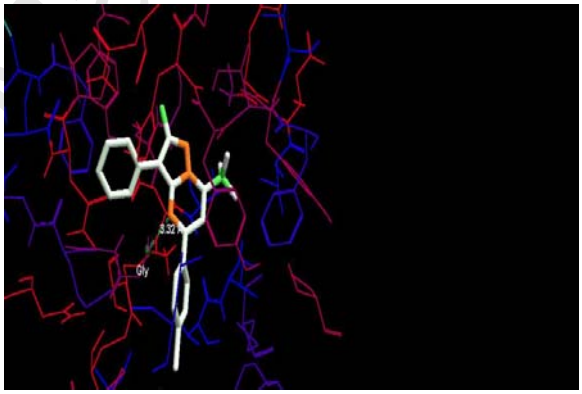
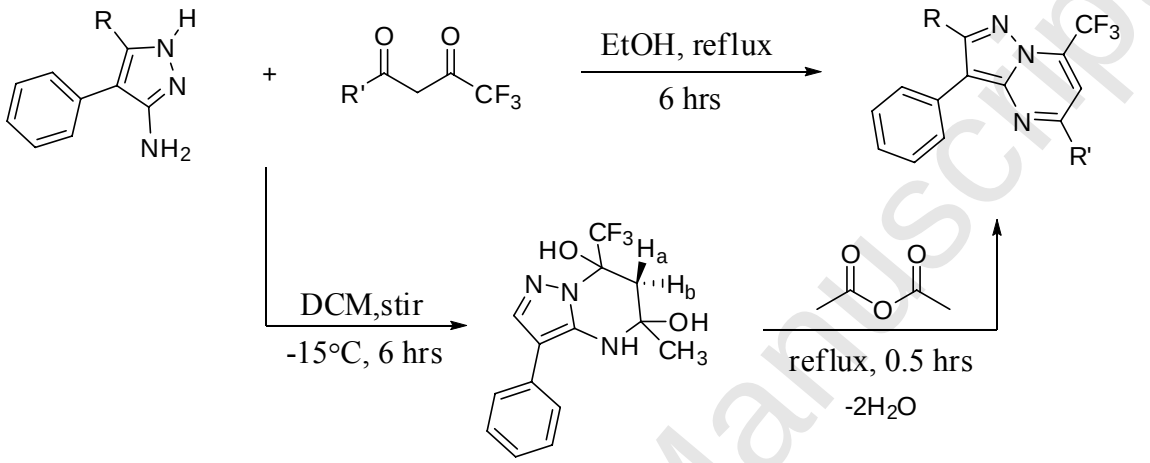


4k

Fig 3 Zoomed images showing bond interactions of compounds **4e**, **4f**, **4i** and **4k** with amino acids of COX-2 enzyme

Graphical Abstract:



Research Highlights

- Efficient regiospecific synthesis of 7-trifluoromethylpyrazolo[1,5-a]pyrimidines.
- Structures established on the basis of ^{1}H, ^{13}C , ^{19}F NMR spectra and IR data.
- Isolation and characterisation of an intermediate in DCM at -15 °C.
- Compound **4e** exhibited anti-inflammatory activity comparable to the standard drug.
- Promising antimicrobial activity against Gram +ve bacteria and pathogenic fungi.