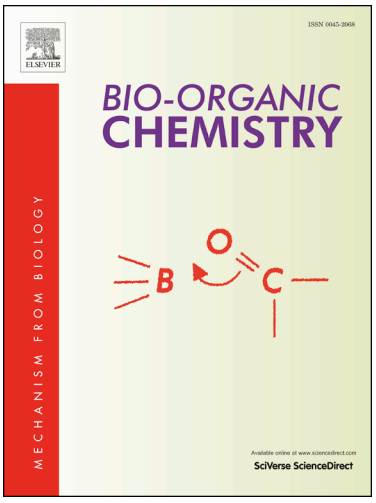

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Design, synthesis and evaluation of some pyrazolo[3,4-d]pyrimidines as anti-inflammatory agents

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Abstract:

New pyrazolo[3,4-d]pyrimidines substituted with various functionalities or attached to a substituted pyrazole ring through different linkages were synthesized. The synthesized compounds were evaluated for their anti-inflammatory activity using *in vitro* COX-1/COX-2 inhibition assay and *in vivo* formalin induced paw edema and cotton pellet-induced granuloma assays. Results revealed that compounds **17b** and **18** possessed COX-1/COX-2 selectivity indices higher than diclofenac sodium and celecoxib. However, compounds **16a,b** exhibited selectivity indices higher than diclofenac sodium and nearly equivalent to celecoxib, whereas, **9b** displayed selectivity index comparable to diclofenac sodium. *In vivo* anti-inflammatory data showed that compounds **9b, 16a, 18** displayed anti-inflammatory activity higher than both references in the formalin induced paw edema model. On the other hand, the pyrazolyl derivatives **9b, 16b** and **17b** displayed anti-inflammatory activity about 2-2.5 fold that of diclofenac sodium and nearly 8-10.5 fold that of celecoxib in the cotton pellet-induced granuloma assay. The ulcerogenic effect of the active compounds was also investigated and results revealed that compounds **16a, 17a,b** and **18** showed good gastrointestinal safety profile. Based on this, compounds **16a** and **18** were considered as safe and effective leads in managing acute inflammation, while, **17b** was prominent in controlling chronic inflammation.

Keywords:

Pyrazolo[3,4-d]pyrimidines, Pyrazoles, Anti-inflammatory activity, Ulcerogenic effect, COX-1/COX-2 selectivity index

1. Introduction:

Non-steroidal anti-inflammatory drugs (NSAIDs) are usually prescribed for treatment of acute and chronic pain, inflammation and fever. Their clinical efficacy is greatly related to their suppression of prostaglandin biosynthesis from arachidonic acid by inhibiting COX enzyme which exists in three distinct isoforms: COX-1, COX-2, and COX-3.[1-5] The constitutive COX-1 isoform provides cytoprotection in the gastrointestinal (GI) tract and controls renal function in the kidneys, whereas the inducible COX-2 is upregulated by proinflammatory mediators such as endotoxins, mitogens, or cytokines including tumor necrosis factor a (TNF-a) and interleukins (ILs) such as IL-6 and IL-1.[6, 7] Furthermore, COX-3 which is a variant of COX-1 present in central nervous system and has been proposed to be another target for anti-inflammatory agents.[8] GI injury is one of the major hallmarks of NSAIDs and is contraindicated in patients with history of GI bleeding or distress.[9] Moreover, use of additional gastroprotective therapy in conjunction with NSAIDs increases the risk of polypharmacy and drug-induced organ injury in elderly and infants.[10] Accordingly, research was directed towards the development of selective COX-2 inhibitors such as celecoxib that should retain the therapeutic potency of NSAIDs with less gastrointestinal adverse effects. However, significant cardiovascular side effects associated with these drugs led to reconsideration of their clinical use. Therefore, development of novel compounds having anti-inflammatory activity with an improved gastrointestinal safety profile is a necessity.

Pyrazolo[3,4-d]pyrimidines either linked to or fused with other heterocyclic ring systems constitute an important scaffold in several pharmacologically active compounds including anti-inflammatory,[11-16] and analgesic agents.[17-20] Literature survey revealed that DPP; (N^4-benzyl-1-(tert-butyl)-N^6,N^6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidine-4,6-diamine) (**A, Figure 1**) possess anti-inflammatory and analgesic activities in addition to higher selectivity for COX-2 over COX-1.[21] Moreover, some pyrazolo[3,4-d]pyrimidines comprising benzamide or substituted benzamide moieties at position 4 or 5 showed remarkable anti-inflammatory activity and COX-2 selectivity (**B, C, Figure 1**).[22, 23]

On the other hand, pyrazoles represent a key scaffold in heterocyclic chemistry and occupy a significant position in medicinal chemistry because of their wide range of bioactivities. They were found to display anti-inflammatory[24-26] and analgesic activities[27, 28] in addition to COX-2 inhibitory activity.[29, 30] Moreover, the pyrazole unit is one of the core structures in a number of selective COX-2 inhibitors such as celecoxib **(D, Figure 1)** and SC-558 **(E, Figure 1)**.[31-33] Additionally, much research has been focused N-aryl pyrazole derivatives as dual anti-inflammatory and antimicrobial agents.[34-36] On the other hand, great attention has been devoted to pyrazoline derivatives as a potent class of anti-inflammatory, analgesic, and antipyretic agents[25, 37, 38] since the development of antipyrine, the first pyrazoline derivative used in the management of pain, inflammation, and fever.

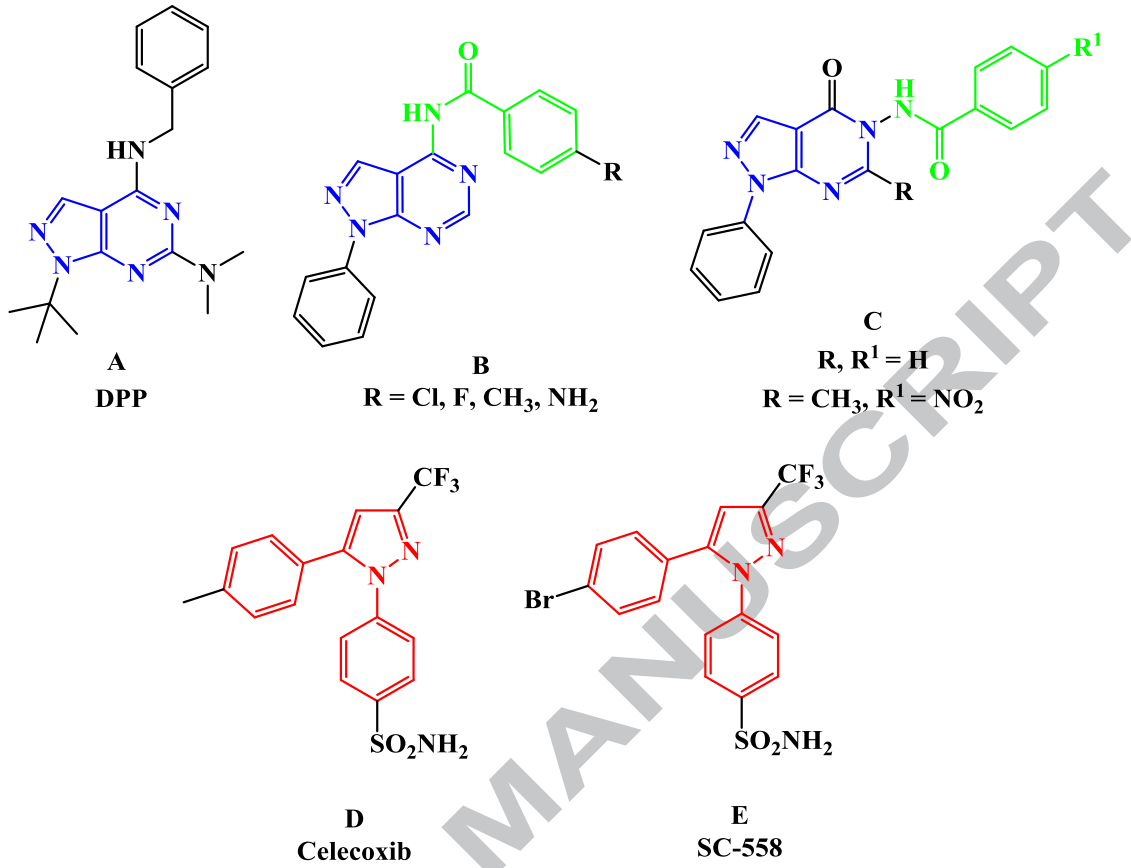


Figure 1. Chemical structures of compounds A-E

Motivated by these findings we deemed it interesting to synthesize some new pyrazolo [3,4-d]pyrimidines to be investigated for their anti-inflammatory activity. The synthesized compounds were designed so as to comprise the pyrazolo[3,4-d]pyrimidine core substituted with various functionalities or attached to a substituted pyrazole ring at position 5 or 6 through different linkages (**Figure 2**). Such substituents were selected so as to confer different electronic, lipophilic and steric environment to the molecules which would influence the targeted biological activity. The target compounds were evaluated for their *in vitro* COX-1/COX-2 inhibition assay and their *in vivo* anti-inflammatory activity in two inflammatory models and ulcerogenic liability in addition to histopathological examination to confirm the degree of inflammatory reaction in the gastric layers of treated rats' stomachs.

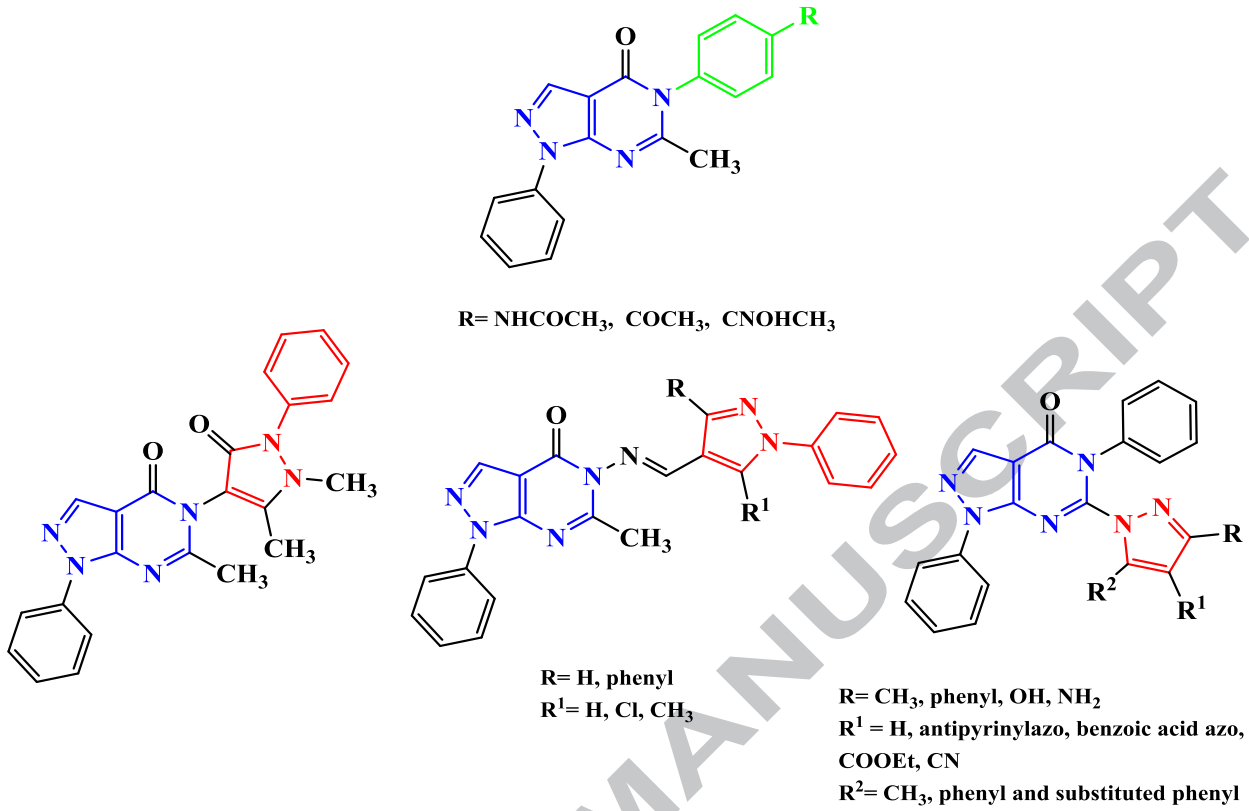


Figure 2. Design of new COX-2 inhibitors

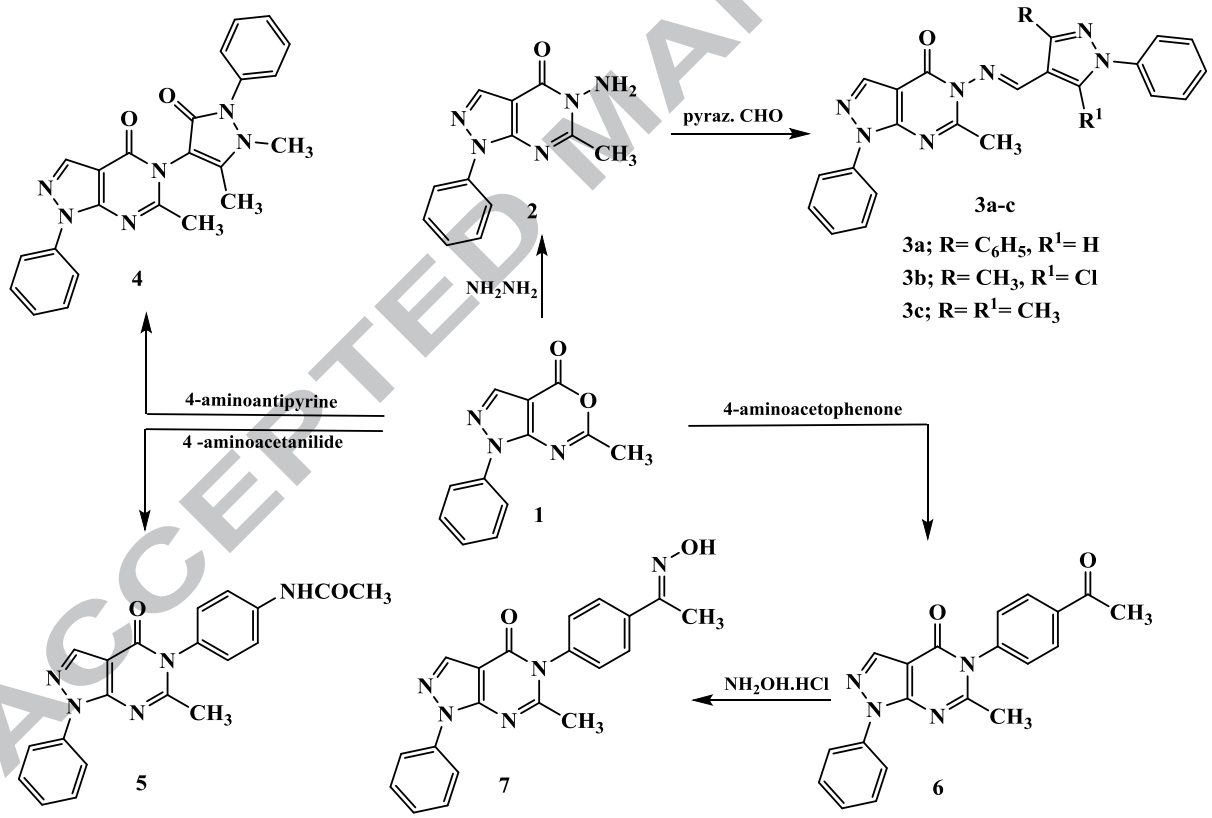
2. Results and Discussion

2.1. Chemistry

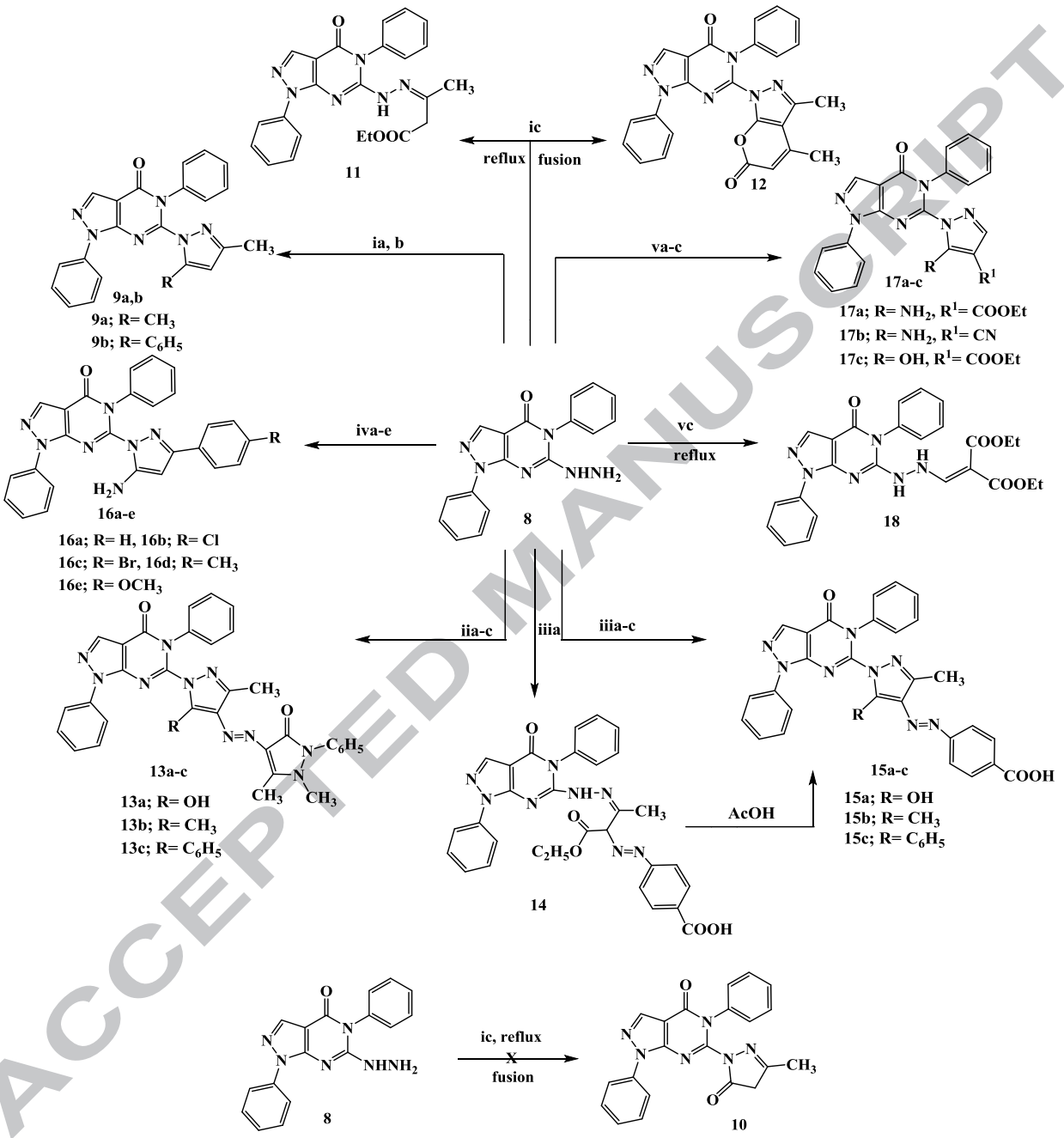
The synthetic strategies adopted for the synthesis of the intermediate and final compounds are depicted in Schemes 1 and 2. **In Scheme 1**, reaction of the oxazine **1** [20] with hydrazine hydrate following previously published reaction conditions[39] gave rise to the 5-amino pyrazolopyrimidine **2** which upon condensation with different pyrazole aldehydes[40-42] furnished the corresponding azomethines **3a-c**. Moreover, fusion of the oxazine **1** with 4-aminoantipyrine or 4-aminoacetanilide at 180 ^{o}C resulted in formation of the antipyrinyl pyrazolopyrimidine **4** and the acetamide derivative **5** respectively. On the other hand, reflux of **1** with 4-aminoacetophenone afforded the target 5-acetylphenylpyrazolopyrimidine derivative **6**. Finally, reaction of the latter compound with hydroxylamine hydrochloride in ethanol containing sodium acetate furnished the oxime derivative **7**.

In Scheme 2, cyclocondensation of the hydrazine derivative **8**[43, 44] with acetyl acetone or benzoyl acetone in ethanol afforded the corresponding pyrazolyl derivatives **9a, b**. However, heating the hydrazine **8** with ethyl acetoacetate in ethanol in an attempt to synthesize the pyrazolinyl derivative **10**[45] gave the open chain ester **11** whereas, fusion with ethyl acetoacetate gave unexpectedly the corresponding pyranopyrazole **12**. The possible mechanism is clearly illustrated in **Figure 3**. On the other hand, reaction of **8** with antipyrylazoacetoacetate,[46] antipyryl azoacetylaetone[46] and antipyrylazobenzoylacetone[47] in boiling ethanol yielded the corresponding antipyrylazopyrazolyl derivatives **13a-c**. Moreover, refluxing the hydrazine **8** with

ethyl acetoacetate coupled with azobenzoic acid[48] in ethanol/ acetic acid mixture gave the open chain ester **14** whereas, upon refluxing **8** with acetyl acetone or benzoyl acetone coupled with azobenzoic acid[48] under the same reaction conditions, the corresponding pyrazolyl derivatives **15b, c** were obtained. However, cyclization of the open chain ester **14** to the corresponding pyrazole derivative **15a** was readily achieved by refluxing in glacial acetic acid. On the other hand, Cyclocondensation of hydrazine derivative **8** with phenacyl cyanides in ethanol/glacial acetic acid mixture afforded the corresponding aminopyrazoles **16a-e**. Similarly, reaction of hydrazine derivative **8** with (ethoxymethylidene) derivatives in ethanol/glacial acetic acid mixture gave rise to the corresponding pyrazoles **17a,b**. This procedure was successful when ethyl 2-cyano-3-ethoxypropenoate **(va)** and (ethoxymethylidene)propanedinitrile **(vb)** were used. However, applying the same method with diethyl (ethoxymethylidene)propanedioate **(vc)** resulted in the formation of open chain counterpart **18** rather than pyrazole derivative **17c.** However, fusion of the hydrazine derivative **8** with diethyl 2-(ethoxymethylidene)propanedioate **(vc)** at 180 °C succeeded in giving the requisite pyrazole **17c**.



Scheme 1



Scheme 2

Reagents :

ia = $CH_3COCH_2COCH_3$ ib = $C_6H_5COCH_2COCH_3$ ic = $CH_3COCH_2COOC_2H_5$ iia = antipyrylazoacetoacetate

iib = antipyrylazoacetylacetone iic = antipyrylazobenzoylacetone iii = 4-substituted azobenzoic acid iva-e = (4) R-$C_6H_5COCH_2CN$

R = H, Br, Cl, CH_3, OCH_3.

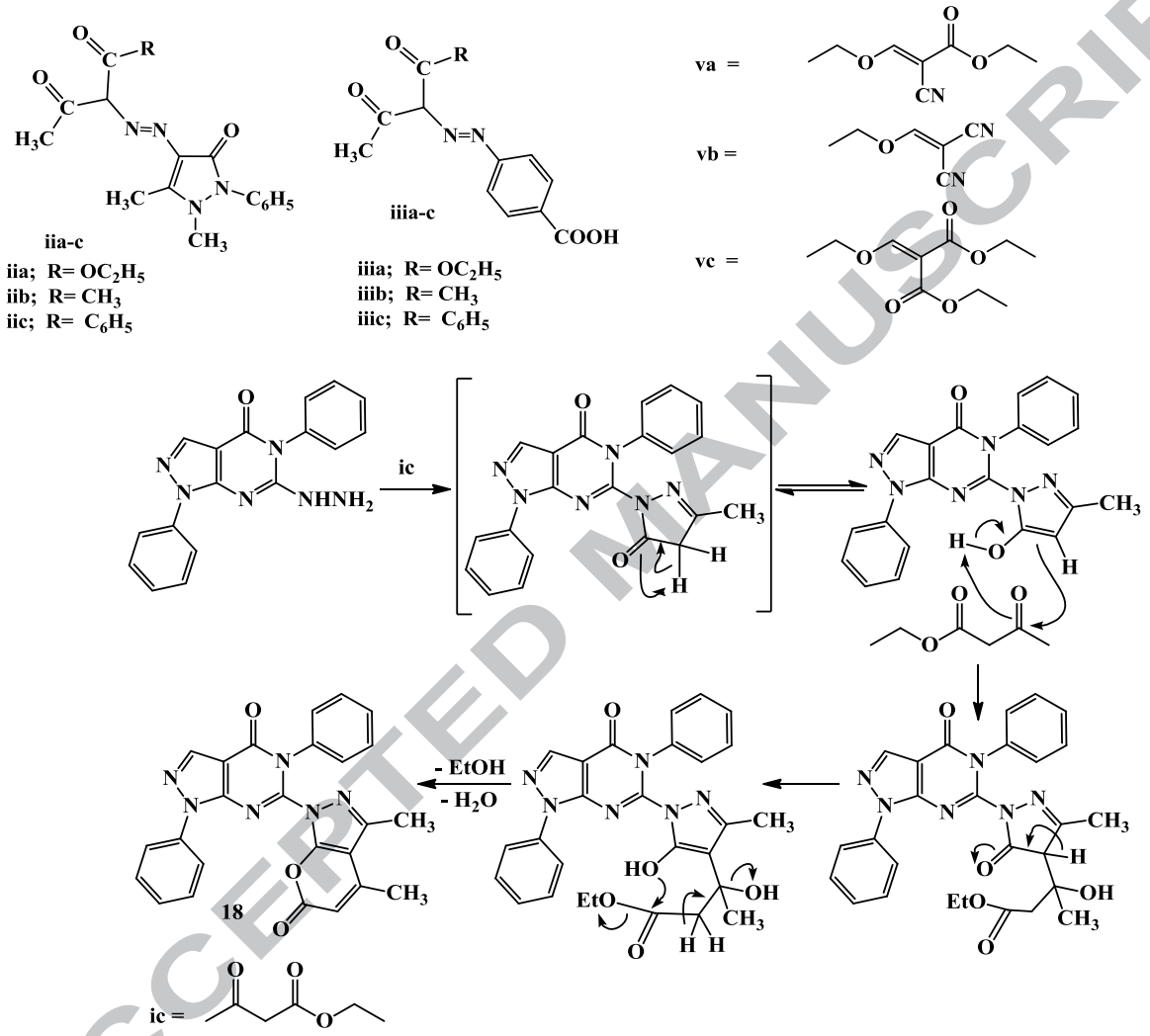


Figure 3. The possible mechanism for pyranopyrazolyl formation

2.2. Biological evaluation

2.2.1. *In vitro* cyclooxygenase (COX) inhibition assay

All the synthesized compounds were tested for their *in vitro* inhibition of COX-1 and COX-2 isoenzymes using Cayman colorimetric COX (ovine) inhibitor screening assay kit. The Colorimetric COX Inhibitor Screening Assay utilizes the peroxidase component of

cyclooxygenase. The peroxidase activity was assayed colorimetrically by monitoring the appearance of oxidized *N,N,N',N'*-tetramethyl-1,4-phenylenediamine (TMPD) which is produced during the reduction of PGG2 to PGH2, at 590 nm. The concentration produced 50 % inhibition of COX-1 and COX-2 isoenzymes (IC_{50} values) and the selectivity indices (SI = IC_{50} COX-1/ IC_{50} COX-2) of the test compounds were determined and results are recorded in **Table 1**.

In general, all the tested compounds showed relatively higher selectivity towards COX-2 than COX-1. Compounds (**3a, 4, 6, 9a, 9b, 12, 13a, 13c, 15a, 15c, 16a, 16b, 16d, 16e, 17a, 17b** and **18**) showed COX-1 IC_{50} values (IC_{50} = 2.74-6.59 μM) lower than celecoxib and diclofenac sodium (IC_{50} = 5.64 and 6.74 μM, respectively). On the other hand, compounds **16a, 16b, 17b** and **18** exhibited high COX-2 inhibitory activities (IC_{50} = 0.22-0.69 μM) which were lower than celecoxib (IC_{50} = 0.78 μM). Further investigation revealed that compound **17b** possesses selectivity index (SI = 12.45) higher than both references diclofenac sodium (SI = 6.12) and celecoxib (SI = 7.23). However, compounds **16a,b** exhibited selectivity indices (SI= 7.20 and 6.86 μM respectively) higher than diclofenac sodium and nearly equivalent to celecoxib, whereas, **9b** and **17a** displayed selectivity indices (SI= 5.44 and 5.78 μM respectively) comparable to diclofenac sodium. Interestingly, the open chain derivative **18**, exhibited selectivity index (SI= 8.52) higher than both references.

Table 1: *In vitro* COX-1 and COX-2 enzymes inhibitory activities, IC_{50} values and selectivity indices (SI) of the tested compounds:

Comp. No.	IC_{50} (μM)[a] COX-1	IC_{50} (μM) COX-2	SI[b] (COX-1/COX-2)
3a	5.91	1.36	4.34
3b	9.11	2.67	3.41
3c	7.89	1.81	4.35
4	3.64	0.85	4.20
5	8.24	2.34	3.50
6	4.21	0.97	4.30
7	6.74	1.52	4.43
9a	6.21	1.33	4.67
9b	4.74	0.87	**5.44**
12	4.56	1.17	3.89
13a	5.97	1.34	4.45
13b	7.85	1.97	3.98
13c	6.59	1.54	4.28
15a	3.98	0.89	4.47
15b	7.64	2.11	3.62
15c	6.52	1.74	3.70
16a	3.89	0.54	**7.20**
16b	4.74	0.69	**6.86**
16c	8.74	2.54	3.44
16d	6.52	1.89	3.45
16e	5.24	1.23	4.26

17a	5.61	0.97	**5.78**
17b	2.74	0.22	**12.45**
18	5.88	0.69	**8.52**
Celecoxib	5.46	0.78	7.23
Diclofenac Sodium	6.74	1.10	6.12

[a]Values are means of three determinations acquired using an ovine COX-1/COX-2 assay kit (catalog no. 760111, Cayman Chemicals, MI, USA) and the deviation from the mean is ±10 % of the mean value

[b] *In vitro* COX-2 selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).

Based on the *in vitro* results, compounds showing selectivity indices higher or nearly equivalent to celecoxib were selected for further evaluation of their *in vivo* anti-inflammatory and analgesic activities.

2.2.2. *In vivo* anti-inflammatory activity

2.2.2.1 Formalin-induced paw edema bioassay

In this acute inflammatory model, each test compound (**9b, 16a, 16b, 17a, 17b** and **18**) was dosed orally at a dose of (5 mg/kg body weight) for seven days prior to induction of inflammation by formalin injection. [49, 50] Celecoxib and diclofenac sodium were utilized as reference drugs at a dose of (5 mg/kg, po). The anti-inflammatory activity was then calculated 4 h after induction of inflammation and presented in **Table 2** as the mean paw volume (cm^3) ± SD and the percentage anti-inflammatory activity (AI %).

Table 2: *In vivo* anti-inflammatory activities of selected compounds in formalin-induced rat paw edema bioassay (acute inflammation model)

	Volume of edema (cm^3) Mean ± SD		% Inhibition (% AI)
Comp. No.	**0**	**4 h**	
9b	0.5±0.1	0.6[a] ±0.0	57.1
16a	5.0±0.0	0.5[a] ±0.0	92.9[b,c]
16b	0.5±0.1	0.6[a] ±0.0	42.8
17a	0.5±0.0	0.6[a] ±0.1	42.9
17b	0.4±0.1	0.6±0.1	23.8[b]
18	0.5±0.0	0.6[a] ±0.0	57.1
Celecoxib	0.5±0.0	0.6±0.1	46.4
Diclofenac sodium	0.5±0.0	0.6[a] ±0.0	50.0
Control	0.5[b] ±0.0	0.7±0.1	-

[a] statistically significant difference in comparison with control group
[b] statistically significant difference in comparison with diclofenac sodium treated group
[c] statistically significant difference in comparison with celecoxib treated group

A comparative study of the anti-inflammatory activity of the test compounds relative to the reference drug indicated that three compounds (**9b, 16a** and **18)** showed outstanding anti-inflammatory activity (% AI = 57.1- 92.2) that was superior to both diclofenac sodium and celecoxib (% AI = 50, 46.4 respectively) (**Figure 4**). Interestingly, compound **16a** displayed the highest potency as it showed anti-inflammatory activity (% AI = 92.9) twice that of celecoxib and diclofenac sodium (% AI = 46.4 and 50 % respectively). Meanwhile, the pyrazolyl derivatives **16b** and **17a** displayed anti-inflammatory activity (% AI = 42.8, and 42.9 respectively) equivalent to celecoxib and slightly lower than diclofenac sodium.

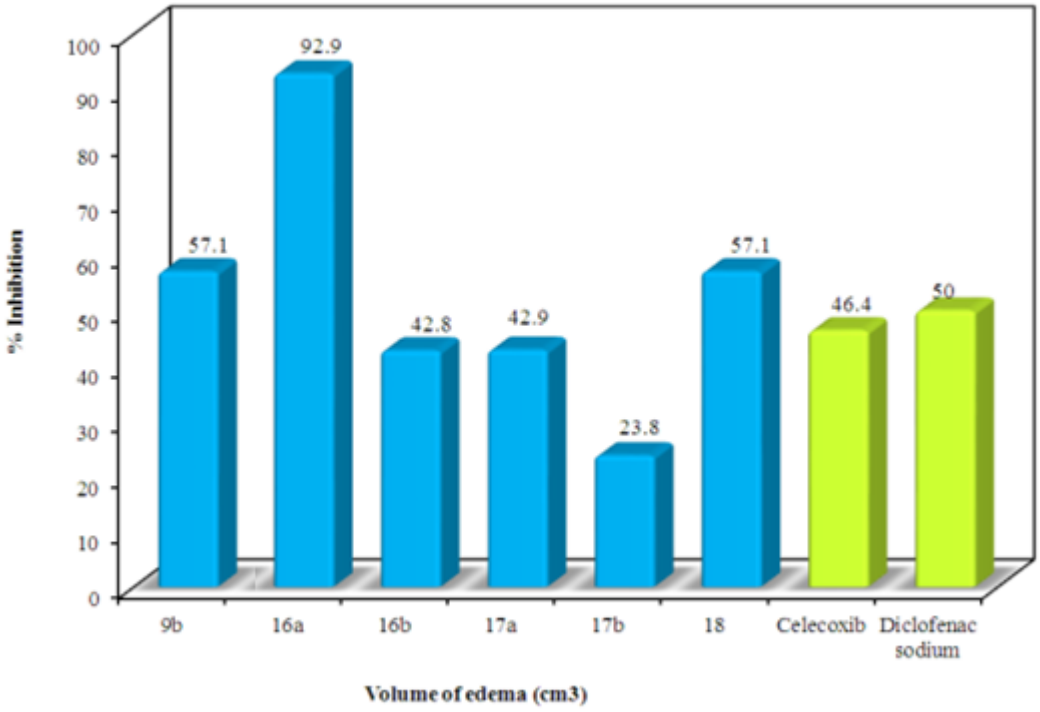


Figure 4: Anti-inflammatory activity of selected compounds in the formalin-induced rat paw edema test (% inhibition)

2.2.2.2 Cotton pellet-induced granuloma assay

In order to investigate the test compounds' efficacy against the later proliferative phase of inflammation caused by tissue degeneration and fibrosis, the cotton pellet induced granuloma assay was used.[51] The results listed in Table 3 represent the mean changes in weight of dry cotton in (mg) ± SD in animals pretreated with the reference drugs and test compounds (**9b, 16a, 16b, 17a, 17b** and **18**) after 7 days from the insertion of the cotton pellet and induction of inflammation, together with the percent inhibition of granuloma by the test compounds (% anti-inflammatory activity).

The screening results revealed that the pyrazolyl derivatives **9b, 16b** and **17b** displayed anti-inflammatory activity (% inhibition of granuloma = 71.2, 90.0 and 85.3 respectively) about 2-2.5 fold that of diclofenac sodium and nearly 8-10.5 fold that of celecoxib. On the other hand, compound **16a** displayed anti-inflammatory activity (% inhibition of granuloma = 30.8)

comparable to diclofenac sodium. The other compounds showed weak to moderate activity compared to the reference diclofenac sodium. (**Figure 5**)

Table 3: *In vivo* anti-inflammatory activities of selected compounds in Cotton pellet induced granuloma test (Chronic inflammation model)

Comp. No.	Dry weight of granuloma (mg) Mean ± SD	% Granuloma inhibition
9b	13.4[ac]±10.6	71.2
16a	32.2[ac]±5.4	30.8
16b	4.63[abc]±2.43	90.0
17a	50.3±79.9	8.1
17b	6.86[abc]±3.46	85.3
18	38.1[c]±10.5	18.1
Celecoxib	50.5±11.7	8.6
Diclofenac sodium	29.7[a]±13.3	36.1
Control	46.5±6.2	-

[a] statistically significant difference in comparison with control group
[b] statistically significant difference in comparison with diclofenac sodium treated group
[c] statistically significant difference in comparison with celecoxib treated group

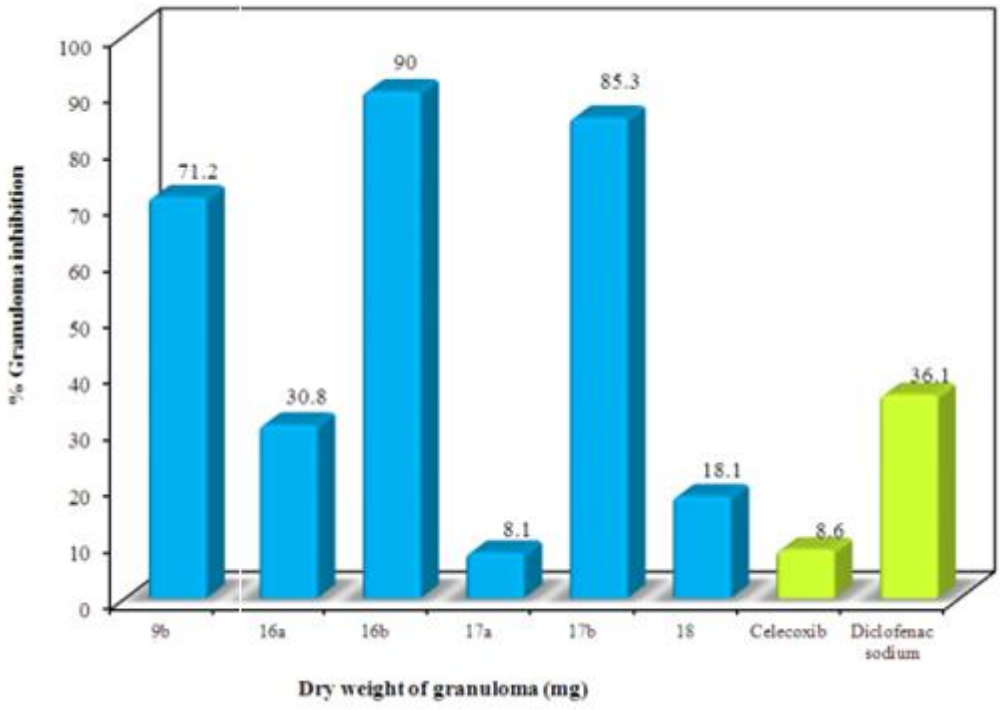


Figure 5: Anti-inflammatory activity of selected compounds in the Cotton pellet-induced granuloma test (% inhibition)

2.2.3. Structure activity relationship (SAR)

A deep insight in the structures of the tested compounds revealed that the 5-amino-3-phenylpyrazolyl derivative **16a** displayed nearly two fold the anti-inflammatory activity of diclofenac sodium in the acute model although, it was slightly less active than the reference drug in the chronic model. On the other hand, compound **16b** which possesses the lipophilic 4-bromophenyl group at position 3 of the pyrazole moiety showed a marked decrease in the anti-inflammatory activity in the acute model, however, the activity in the chronic model was markedly improved to exceed that of diclofenac sodium. In this respect, compound **16b** exhibited the highest activity among the tested compounds in the chronic model (% granuloma inhibition = 90.0) compared to diclofenac sodium (% granuloma inhibition = 36.1). Modification of structure **(16)** by replacing the lipophilic group (phenyl and p-bromophenyl at position 3) with another lipophilic group as cyano while retaining the amino group as in compound **17b** maintained the anti-inflammatory activity in the chronic model but the activity in the acute model was abolished. On the contrary, introduction of ester group (compound **17a**) at the same position reduced the anti-inflammatory activity in both models. In compound **16b**, replacement of the aromatic moiety at position 3 by methyl group and the 5-amino group by phenyl (compound **9b**) decreased the anti-inflammatory activity in the acute model, however, the activity in the chronic model was highly increased to be twice that of diclofenac sodium.

2.2.4. Gastric ulcerogenic activity

The tested compounds that exhibited *in vitro* COX-2 selectivity indices higher or nearly equivalent to reference drugs were further evaluated for their ulcerogenic potential in rats.[52, 53]

Gross observation of the isolated rats' stomachs showed a normal stomach texture for the tested compounds **16a, 17a, 17b, 18** as well as the reference celecoxib. While for compounds **9b** and **16b** variable degrees of hyperemia were observed (**Figure 6**).

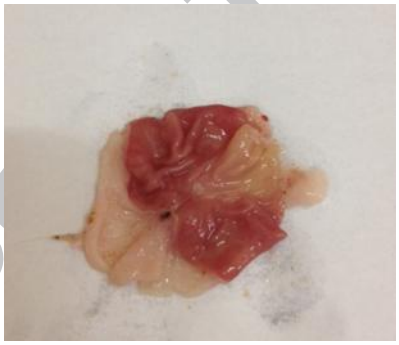


Figure 6a

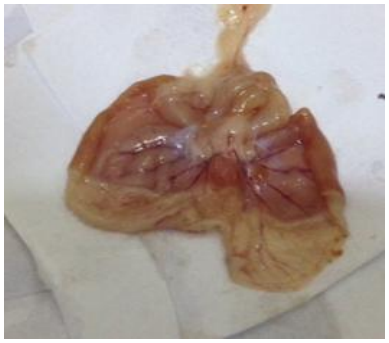


Figure 6b

Figure 6: Gross appearance of gastric mucosa

6a- Normal stomach texture for the tested compounds **(16a, 17a, 17b, 18)** and celecoxib

6b- Hyperemia observed in case of compounds **(9b, 16b)** and diclofenac sodium

Further histopathological examination was performed to confirm the degree of inflammatory reaction in the gastric layers of the treated rats' stomachs. Histopathological examination revealed that compounds **16a, 17a, 17b, 18** showed superior gastrointestinal safety profile (free, normal gastric and esophageal mucosa) as well as celecoxib and diclofenac sodium (**Figure 7a**). On the other hand, histopathological examination indicated that the compound **9b, 16b** revealed signs of gastro-esophageal inflammation (**Figure 7b**).

Figure 7: Histology of rat gastric mucosa (H&E stain)

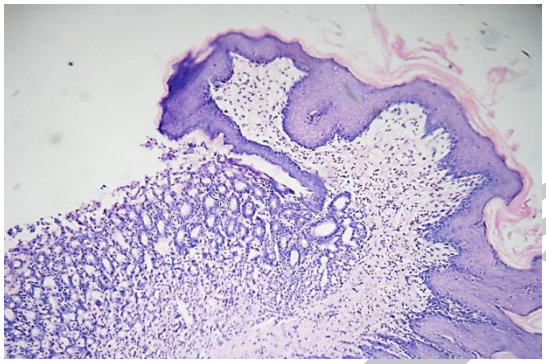


Figure 7a: Free, normal gastro-esophageal junction, (H&Ex100)

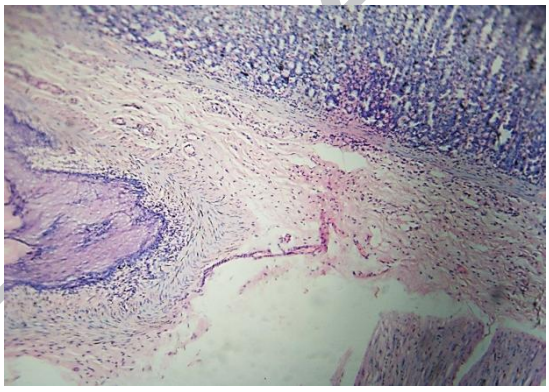


Figure 7b: Gastro-esophageal inflammation, (H&Ex100)

3. Conclusion

The aim of the present study was to synthesize and investigate the anti-inflammatory activity of new pyrazolo[3,4-d]pyrimidines substituted with various functionalities or attached to a substituted pyrazole ring through different linkages with the hope of discovering new lead structures devoid of the GI side effects associated with conventional NSAIDs. The obtained results from COX-1/COX-2 inhibition assay clearly revealed that compounds **17b** and **18** possessed selectivity indices higher than diclofenac sodium and celecoxib. While, compounds **16a,b** exhibited selectivity indices higher than diclofenac sodium and nearly equivalent to celecoxib, whereas, **9b** displayed selectivity index comparable to diclofenac sodium. *In vivo* anti-inflammatory data showed that compounds **9b, 16a, 18** displayed anti-inflammatory activity higher than both references in the formalin induced paw edema model, whereas, the pyrazolyl

derivatives **9b, 16b** and **17b** displayed anti-inflammatory activity about 2-2.5 fold that of diclofenac sodium and nearly 8-10.5 fold that of celecoxib in the cotton pellet-induced granuloma assay. Histopathological examination revealed that compounds **16a, 17a,b** and **18** showed good gastrointestinal safety profile. Collectively, the promising anti-inflammatory activity of **16a** and **18** in acute model and **17b** in chronic model as well as their reduced ulcerogenic potential make them good lead candidates for further optimization and development of potent and safe anti-inflammatory agents.

4. Experimental

4.1. Chemistry

All reagents and solvents were purchased from commercial suppliers and were dried and purified when necessary by standard techniques.

Melting points were determined in open glass capillaries using Griffin melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on Perkine-Elmer RXIFT-IR spectrophotometer using KBr discs. ^{1}H-NMR spectra were scanned on were run on Jeol spectrometer (500 MHz) at the Microanalytical Unit, Faculty of Science, Alexandria University, on Bruker high performance digital FT-NMR spectrometer avance III (400 MHz) at Faculty of Pharmacy, Cairo University and on Varian Mercury VX (300 MHz) spectrometer, Faculty of Science, Cairo University. ^{13}C-NMR proton decoupled spectra were run on Jeol spectrometer (125 MHz) at the Microanalytical Unit. Faculty of Science, Alexandria University and on Varian Mercury VX (75 MHz) spectrometer, Faculty of Science, Cairo University, using deuterated dimethylsulfoxide (DMSO-d_6) as a solvent. The data were reported as chemical shifts or δ values (ppm) relative to tetramethylsilane (TMS) as internal standard. Mass spectra were run on a gas chromatograph/mass spectrophotometer Shimadzu GCMS/QP-2010 plus (70 eV) at the faculty of Science, Al-Azhar University. Relative intensity % corresponding to the most characteristic fragments were recorded. Microanalyses were performed at the Microanalytical Unit, Faculty of Science, Cairo University, Egypt and the found values were within ±0.4% of the theoretical values. Follow up of the reactions and checking the purity of the compounds was made by thin layer chromatography (TLC) on silica gel-precoated aluminum sheets (Type 60 GF254; Merck; Germany) and the spots were detected by exposure to iodine vapour or UV lamp at λ 254 nm for few seconds.

4.1.1. 6-Methyl-1-phenylpyrazolo[3,4-d][1,3]oxazin-4(1*H*)-one (1)[20]; M.p. 94-95 °C as reported[54]

4.1.2. 5-Amino-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (2)[39]; M.p. 220-221 °C, reported 215-217 °C.[39]

4.1.3. General procedure for preparation of **5-{(Substituted pyrazol-4-yl)methylideneamino}-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-ones (3a-c)**

A mixture of the amino derivative **2** (0.24 g, 1 mmol) and the selected pyrazole aldehyde (1 mmol) in absolute ethanol (7 ml) was heated under reflux for 3 h. The separated product was filtered, washed with ethanol, dried and recrystallized from dioxane. Physical and spectral data for **3a-c** are listed below.

4.1.3.1. 5-{(1,3-Diphenyl-1*H*-pyrazol-4-yl)methylideneamino}-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (3a) Yield: 80 %; Mp: 226-227 °C. IR (KBr, cm^{-1}): 3113, 3046, 2926 (CH); 1706 (C=O); 1598 (C=N); 1563, 1539, 1504, 1455 (C=C). Anal. Calcd for $C_{28}H_{21}N_7O$ (471.51): C, 71.32; H, 4.49; N, 20.79. Found: C, 71.53; H, 4.57; N, 21.06.

4.1.3.2. 5-{(5-Chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylideneamino}-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (3b) Yield: 87 %; Mp: 232-234 °C. IR (KBr, cm^{-1}): 3063, 3042, 2963, 2921 (CH); 1714 (C=O); 1606 (C=N); 1565, 1531, 1505, 1462 (C=C). MS (m/z, %): 445.45 ($M^{+\bullet}$+2, 8); 443.72 ($M^{+\bullet}$, 24); 442 (13); 419 (9); 412 (15); 411 (11); 400 (11); 338 (22); 333 (16); 318 (10); 302 (26); 266 (16); 211 (21); 197 (11); 195 (13); 192 (54); 158 (21); 150 (20); 141 (100); 137 (14); 128 (32); 108 (19); 106 (68); 93 (18); 79 (81); 73 (41); 57 (16); 47 (11); 44 (50). Anal. Calcd for $C_{23}H_{18}ClN_7O$ (443.89): C, 62.23; H, 4.09; N, 22.09. Found: C, 62.41; H, 4.15; N, 22.34.

4.1.3.3. 5-{(3,5-Dimethyl-1-phenyl-1*H*-pyrazol-4-yl)methylideneamino}-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (3c) Yield: 86 %; Mp: 216-217 °C. IR (KBr, cm^{-1}): 3105, 3037, 2964, 2920 (CH); 1709 (C=O); 1607 (C=N); 1563, 1535, 1501, 1460 (C=C). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.47, 2.50, 2.56 (3s, each 3H, 3 CH_3); 7.42-7.61 (m, 8H, phenyl-H); 8.11 (d, *J* = 7.8 Hz, 2H, phenyl $C_{2,6}$-H); 8.37 (s, 1H, pyrazolopyrimidine C_3-H); 8.77 (s, 1H, N=CH). Anal. Calcd for $C_{24}H_{21}N_7O$ (423.47): C, 68.07; H, 5.00; N, 23.15. Found: C, 68.25; H, 4.98; N, 23.34.

4.1.4. 5-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (4)

A mixture of the oxazine derivative **1** (0.23 g, 1 mmol), 4-aminoantipyrine (0.20 g, 1 mmol) and a catalytic amount of p-toluene sulphonic acid was heated in an oil bath at 180 °C for 2 h. The reaction mixture was left to cool, then triturated with ethanol, filtered, washed with diethyl ether, dried and crystallized from ethanol. Yield: 66 %; Mp: 232-234 °C. IR (KBr, cm^{-1}): 3023, 2985, 2923 (CH); 1708, 1678 (C=O); 1597 (C=N); 1568, 1544, 1512, 1494 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.20, 2.46 (2s, each 3H, 2 C-CH_3); 3.28 (s, 1H, N-CH_3); 7.38-7.61 (m, 8H, phenyl-H); 8.08 (d, *J* = 7.5 Hz, 2H, phenyl $C_{2,6}$-H); 8.36 (s, 1H, pyrazolopyrimidine C_3-H). MS (m/z, %): 413.20 ($M^{+\bullet}$+1, 32); 412.19 ($M^{+\bullet}$, 100); 397 (21); 291 (34); 210 (24); 143 (58); 77 (80); 56 (99). Anal. Calcd for $C_{23}H_{20}N_6O_2$ (412.44): C, 66.98; H, 4.89; N, 20.38. Found: C, 67.23; H, 4.94; N, 20.52.

4.1.5. N-{4-(6-methyl-4-oxo-1-phenyl-1*H*,5*H*-pyrazolo[3,4-d]pyrimidin-5-yl)phenyl}acetamide (5)

A mixture of oxazine derivative **1** (0.23 g, 1 mmol), 4-aminoacetanilide (0.15 g, 1 mmol) and a catalytic amount of p-toluene sulphonic acid was heated in an oil bath at 180 °C for 2 h. The reaction mixture was left to cool, triturated with ethanol. The separated solid was filtered, washed with diethyl ether, dried and crystallized from ethanol. Yield: 67 %; Mp. 265-266 °C. IR (KBr, cm^{-1}): 3419, 3293 (NH); 3064 (CH); 1693 (C=O); 1601 (C=N); 1548, 1504 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.09, 2.19 (2s, each 3H, 2 CH_3); 7.31-7.76 (m, 7H, phenyl-H);

8.09 (d, *J* = 7.5 Hz, 2H, phenyl $C_{2,6}$-H); 8.34 (s, 1H, pyrazolopyrimidine C_3-H); 10.15 (s, 1H, NH, D_2O exchangeable). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 24.57 (CH_3); 25.23 (CH_3); 105.97 (pyrazolopyrimidine C_{3a}); 120.25, 122.22, 132.54 (phenyl C_{2-6}); 127.66, 129.28, 129.85 (phenyl C_{2-6}); 136.87 (phenyl C_1); 138.78 (phenyl C_1); 140.32 (pyrazolopyrimidine C_3); 151.20 (pyrazolopyrimidine C_{7a}); 158.20 (pyrazolopyrimidine C_6); 159.90 (pyrazolopyrimidine C_4); 169.22 (NHC=O). Anal. Calcd for $C_{20}H_{17}N_5O_2$ (359.38): C, 66.84; H, 4.77; N, 19.49. Found: C, 67.01; H, 4.81; N, 19.62.

4.1.6. 5-(4-Acetylphenyl)-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (6)

A mixture of oxazine derivative **1** (2.27 g, 10 mmol) and 4-aminoacetophenone (1.35 gm, 10 mmol) in dry pyridine (30 ml) was heated under reflux for 6 h. The reaction mixture was poured into ice-cold water and the reaction mixture was neutralized with diluted hydrochloric acid. The separated product was filtered, washed with water, dried and crystallized from methanol. Yield: 55 %; Mp. 216-217 ^{o}C. IR (KBr, cm^{-1}): 3050, 3014 (CH); 1706, 1688 (C=O); 1581 (C=N); 1562, 1502 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.19, 2.66 (2s, each 3H, 2 CH_3); 7.43-7.64 (m, 5H, phenyl-H) 7.99 (d, *J* = 8.4 Hz, 2H, phenyl $C_{2,6}$-H); 8.11 (d, *J* = 8.4 Hz, 2H, phenyl $C_{3,5}$-H); 8.37 (s, 1H, pyrazolopyrimidine C_3-H). Anal. Calcd for $C_{20}H_{16}N_4O_2$ (344.37): C, 69.76; H, 4.68; N, 16.27. Found: C, 69.94; H, 4.73; N, 16.43.

4.1.7. 5-{4-(1-Hydroxyiminoethyl)phenyl}-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (7)

A mixture of the acetophenone derivative **6** (0.34 g, 1 mmol), hydroxylamine hydrochloride (0.07 g, 1 mmol) and sodium acetate (0.08 g, 1 mmol) in ethanol (10 ml) was heated under reflux for 4 h. The separated beage shiny crystals were filtered while hot, washed with ethanol, dried then recrystallized from dioxane. Yield: 78 %; Mp. 248-249 ^{o}C. IR (KBr, cm^{-1}): 3281 (OH); 3076, 2958, 2907(CH); 1685 (C=O); 1598 (C=N); 1561, 1543, 1501 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.19, 2.22 (2s, each 3H, 2 CH_3), 7.39-7.62 (m, 5H, phenyl-H); 7.83 (d, *J* = 8.7 Hz, 2H, phenyl $C_{2,6}$-H); 8.10 (d, *J* = 8.7 Hz, 2H, phenyl $C_{3,5}$-H); 8.36 (s, 1H, pyrazolopyrimidine C_3-H); 11.37 (s, 1H, OH, D_2O exchangeable). Anal. Calcd for $C_{20}H_{17}N_5O_2$ (359.38): C, 66.84; H, 4.77; N, 19.49. Found: C, 67.01; H, 4.81; N, 19.62.

4.1.8. 6-Hydrazinyl-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (8) [44] Mp. 268-270 ^{o}C as reported.[44]

4.1.9. General procedure for preparation of **6-(3-Methyl-5-substituted-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-ones (9a,b)**

A mixture of the hydrazine derivative **8** (0.32 g, 1mmol) and acetylacetone **(ia)** or benzoylacetone **(ib)** (1 mmol) in absolute ethanol (10 ml) was heated under reflux for 3-6 h. The reaction mixture was concentrated under reduced pressure, left overnight at room temperature and the separated crystals were filtered, washed with diethyl ether, dried and recrystallized from ethanol. Yield, physical properties and elemental microanalyses of compounds **(9a,b)** are listed below.

4.1.9.1. 6-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (9a) Yield: 85 %; Mp: 184-186 ^{o}C. IR (KBr, cm^{-1}): 3082, 3036, 2968, 2925 (CH); 1710 (C=O); 1575(C=N); 1537, 1496, 1460 (C=C). ^{1}H-NMR (500 MHz, DMSO-d_6) δ 1.78, 2.37 (2s,

each 3H, 2 CH_3); 5.83 (s, 1H, pyrazole C_4-H); 7.22-7.59 (m, 8H, phenyl- H); 7.96 (d, *J* = 7.7 Hz, 2H, phenyl $C_{2,6}$-H); 8.50 (s, 1H, pyrazolopyrimidine C_3-H). Anal. Calcd for $C_{22}H_{18}N_6O$ (382.42): C, 69.10; H, 4.74; N, 21.98. Found: C, 69.24; H, 4.81; N, 22.21.

4.1.9.2. 6-(3-Methyl-5-phenyl-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (9b) Yield: 84 %; Mp: 196-198 ^oC. IR (KBr, cm^{-1}): 3033 (CH); 1715 (C=O); 1647 (C=N); 1577, 1540, 1495 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.02 (s, 3H, CH_3); 6.37 (s, 1H, pyrazole C_4-H); 7.15-7.50 (m, 15H, phenyl-H); 8.45 (s, 1H, pyrazolopyrimidine C_3-H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 13.00 (CH_3); 105.94 (pyrazole C_4); 107.95 (pyrazolopyrimidine C_{3a}); 121.48, 128.53, 129.18 (phenyl C_{2-6}); 127.31, 128.76, 129.10 (phenyl C_{2-6}); 128.17, 128.63, 128.86 (phenyl C_{2-6}); 135.15 (phenyl C_1); 136.88; (phenyl C_1); 137.34 (phenyl C_1); 146.41 (pyrazolopyrimidine C_3); 147.43 (pyrazolopyrimidine C_{7a}); 148.71 (pyrazole C_3); 150.84 (pyrazole C_5); 156.98 (pyrazolopyrimidine C_6); 162.00 (pyrazolopyrimidine C_4). MS (m/z, %): 445 ($M^{+\bullet}$+1, 13); 444 ($M^{+\bullet}$, 41); 443 (15); 288 (19); 287 (100); 77 (24). Anal. Calcd for $C_{27}H_{20}N_6O$ (444.49): C, 72.96; H, 4.54; N, 18.91. Found: C, 73.17; H, 4.61; N, 19.05.

4.1.10 Ethyl 3-{2-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl) hydrazinylidene}butanoate (11)

A mixture of the hydrazine derivative **8** (0.32 g, 1 mmol) and ethyl acetoacetate **(ic)** (0.13 g, 0.127 ml, 1 mmol) in absolute ethanol/glacial acetic acid (10 ml) (2:1) was heated under reflux for 6 h. The reaction mixture was left to cool to room temperature and poured into ice-cold water, the separated solid was filtered, washed with water, dried and crystallized from ethanol. Yield: 59 %; Mp. 152-153 ^oC. IR (KBr, cm^{-1}): 3382 (NH); 3104, 3063, 2978 (CH); 1737, 1709 (C=O); 1596 (C=N); 1567, 1547, 1494 (C=C); 1257, 1027 (C-O-C). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.19, 1.26 (2t, *J* = 7.0 Hz, each 3H, 2 CH_2-$\underline{CH_3}$, E and Z isomers); 2.11, 2.19 (2s, each 3H, 2 CH_3, E and Z isomers); 2.44, 2.61 (2s, each 2H, 2 CH_2, E and Z isomers); 4.19, 4.45 (2q, *J* = 7.0 Hz, each 2H, 2 $\underline{CH_2}$-CH_3, E and Z isomers); 7.16-8.36 (m, 22H, phenyl-H and NH, E and Z isomers); 8.46, 8.51 (2s, each 1H, pyrazolopyrimidine C_3-H, E and Z isomers). Anal. Calcd for $C_{23}H_{22}N_6O_3$ (430.46): C, 64.17; H, 5.15; N, 19.52. Found: C, 64.34; H, 5.21; N, 19.75.

4.1.11. 6-(3,4-Dimethyl-6-oxo-1*H*-pyrano[2,3-c]pyrazol-1-yl)1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (12)

A mixture of the hydrazine derivative **8** (0.32 g, 1 mmol) and ethyl acetoacetate **(ic)** (1 ml) was heated in an oil bath at 180 °C for 15 minutes. The reaction mixture was left to attain room temperature, triturated with ethanol, then the solid product was filtered, washed with ethanol, dried and crystallized from dioxane. Yield: 88 %; Mp. > 300 °C. IR (KBr, cm^{-1}): 3060, 2968, 2924 (CH); 1741, 1710 (C=O); 1616 (C=N); 1597, 1541, 1485 (C=C); 1230, 1048 (C-O-C). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.19, 2.34 (2s, each 3H, 2 CH_3); 5.98 (s, 1H, pyranopyrazole C_5-H), 7.34-7.60 (m, 8H, phenyl-H); 8.08 (d, *J* = 8.0 Hz, 2H, phenyl $C_{2,6}$-H); 8.56 (s, 1H, pyrazolopyrimidine C_3-H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 14.51 (CH_3); 19.53 (CH_3); 106.10 (pyrazolopyrimidine C_{3a}); 122.31 (pyranopyrazole C_{3a}); 122.62 (pyranopyrazole C_5); 128.17, 129.11, 129.45 (phenyl C_{2-6}); 128.99, 129.18, 129.98 (phenyl C_{2-6}); 135.57 (pyranopyrazole C_3); 137.42 (pyrazolopyrimidine C_3); 137.61 (phenyl C_1); 138.21 (phenyl C_1); 144.77 (pyranopyrazole C_4); 146.71 (pyrazolopyrimidine C_{7a}); 149.42 (pyrazolopyrimidine C_6); 154.54 (pyrazolopyrimidine C_4); 157.40 (pyranopyrazole C_6); 158.87 (pyranopyrazole C_{7a}). MS (m/z, %): 451 ($M^{+\bullet}$+1, 6); 450 ($M^{+\bullet}$, 19); 288 (19); 287 (100.00); 258 (5); 232 (4); 77 (24); 69 (4).

Anal. Calcd for $C_{25}H_{18}N_6O_3$ (450.45): C, 66.66; H, 4.03; N, 18.66. Found: C, 66.78; H, 4.11; N, 18.78.

4.1.12. General procedure for preparation of **6-{4-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yldiazenyl)-5-substituted-3-methyl-1*H*-pyrazol-1-yl}-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-ones (13a-c)**

An equimolar amount of the hydrazine derivative **8** and the selected antipyrylazo derivative **(iia-c)** in ethanol/glacial acetic acid mixture (15 ml) (2:1) was heated under reflux for 18 h. The separated solid product was filtered while hot, washed with ethanol, dried and recrystallized from dimethylformamide. Yield, physical properties and elemental microanalyses of compounds **(13a-c)** are listed below.

4.1.12.1. 6-{4-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yldiazenyl)-5-hydroxy-3-methyl-1*H*-pyrazol-1-yl}-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (13a) Yield: 89 %; Mp: 228-229 ^{o}C. IR (KBr, cm^{-1}): 3217 (OH); 3059, 2978, 2929 (CH); 1688 (C=O); 1594 (C=N); 1539, 1501, 1467 (C=C). ^{1}H-NMR (500 MHz, DMSO-d_6) δ 2.44, 2.65 (2s, each 3H, 2 CH_3); 3.26 (s, 3H, N-CH_3); 7.17-8.24 (m, 15H, phenyl-H); 8.54 (s, 1H, pyrazolopyrimidine C_3-H); 13.36 (s, 1H, OH, D_2O exchangeable). MS (m/z, %): 598.80 ($M^{+\bullet}$, 1); 361 (7); 319 (6) 229 (5); 207 (5); 202 (12); 193 (4); 131 (14); 117 (12); 116 (26); 105 (50); 104 (15); 103 (16); 102 (12); 91 (91); 90 (10); 89 (11); 78 (13); 77 (65); 76 (12); 65 (20); 55 (16); 43 (100). Anal. Calcd for $C_{32}H_{26}N_{10}O_3$ (598.61): C, 64.21; H, 4.38; N, 23.40. Found: C, 64.37; H, 4.43; N, 23.73.

4.1.12.2. 6-{4-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yldiazenyl)-3,5-dimethyl-1*H*-pyrazol-1-yl}-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one(13b) Yield: 87 %; Mp: 267-272 ^{o}C. IR (KBr, cm^{-1}): 3054, 2978, 2919 (CH); 1716, 1674 (C=O); 1570 (C=N); 1535, 1494, 1451 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.04, 2.45, 2.66 (3s, each 3H, 3 CH_3); 3.31 (s, 1H, N-CH_3); 7.33-8.04 (m, 15H, phenyl-H); 8.55 (s, 1H, pyrazolopyrimidine C_3-H). Anal. Calcd for $C_{33}H_{28}N_{10}O_2$ (596.64): C, 66.43; H, 4.73; N, 23.48. Found: C, 64.59; H, 4.82; N, 23.71.

4.1.12.3. 6-{4-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yldiazenyl)-3-methyl-5-phenyl-1*H*-pyrazol-1-yl}-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one(13c) Yield: 84 %; Mp: 279-280 ^{o}C. IR (KBr, cm^{-1}): 3062 (CH); 1698, 1680 (C=O); 1648 (C=N); 1516, 1460 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.22, 2.45 (2s, each 3H, 2 CH_3); 3.27 (s, 1H, N-CH_3); 7.20-8.04 (m, 20H, phenyl-H); 8.50 (s, 1H, pyrazolopyrimidine C_3-H). Anal. Calcd for $C_{38}H_{30}N_{10}O_2$ (658.71): C, 69.29; H, 4.59; N, 21.26. Found: C, 69.48; H, 4.63; N, 21.82.

4.1.13. 4-{1-Ethoxy-1-oxo-3-(2-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)hydrazinylidene)butan-2-yl}diazenylbenzoic acid (14)

A mixture of the hydrazine derivative **8** (0.32 g, 1 mmol) and 4-(1-ethoxy-1,3-dioxobutan-2-yl)diazenylbenzoic acid **(iiia)** (0.28 g, 1 mmol) in ethanol/glacial acetic acid mixture (15 ml) (2:1) was heated under reflux for 18 h. The formed red precipitate was filtered while hot, washed with ethanol, dried and recrystallized from dimethylformamide. Yield: 77 %; Mp. 228-229 ^{o}C. IR (KBr, cm^{-1}): 3421-3100 (OH); 3281 (NH); 3042, 2978, 2927 (C=C); 1734,

1704, 1653 (C=O); 1599 (C=N); 1530, 1451 (C=C); 1268, 1084 (C-O-C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.18, 1.28 (2t, *J* = 7.0 Hz, each 3H, 2 $CH_2\underline{CH_3}$, E and Z isomers); 1.78, 1.88 (2s, each 3H, 2 CH_3, E and Z isomers); 4.24, 4.36 (2q, *J* = 7.0 Hz, each 2H, 2 $\underline{CH_2}CH_3$, E and Z isomers); 6.84-8.51 (m, 30H, phenyl-H and pyrazolopyrimidine C_3-H, E and Z isomers); 10.66 (s, 1H, NH, D_2O exchangeable, one isomer); 12.55 (s, 2H, NH and OH, D_2O exchangeable, one isomer); 13.75 (s, 1H, OH, D_2O exchangeable, one isomer). Anal. Calcd for $C_{30}H_{26}N_8O_5$ (578.58): C, 62.28; H, 4.53; N, 19.37. Found: C, 62.49; H, 4.62; N, 19.59.

4.1.14. 4-{5-Hydroxy-3-methyl-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazol-4-yl}diazenylbenzoic acid (15a)

The intermediate **14** (0.58 g, 1 mmol) was heated under reflux in glacial acetic acid (4 ml) for 3 h. The reaction mixture was left to attain room temperature and the obtained yellow crystals were filtered, washed with ethanol, dried and recrystallized from dioxane. Yield: 70 %; Mp. > 300 ^oC. IR (KBr, cm^{-1}): 3398-3103 (OH); 3058, 2929, 2819 (CH); 1723, 1683 (C=O); 1601 (C=N); 1538, 1494, 1427 (C=C). ^{1}H-NMR (500 MHz, DMSO-d_6) δ 2.02 (s, 3H, CH_3); 7.28-8.05 (m, 14H, phenyl-H); 8.52 (s, 1H, pyrazolopyrimidine C_3-H); 12.89 (s, 2H, 2 OH, D_2O exchangeable). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 10.20 (CH_3); 106.82 (pyrazolopyrimidine C_{3a}); 116.86 (pyrazole C_4); 122.60, 126.55, 129.56 (phenyl C_{2-6}); 128.01, 128.09, 129.14 (phenyl C_{2-6}); 128.21, 129.96, 131.36 (phenyl C_{2-6}); 135.69 (phenyl C_1); 137.46 (phenyl C_1); 138.26 (phenyl C_1); 145.30 (pyrazole C_3); 146.47 (pyrazolopyrimidine C_3); 149.94 (pyrazolopyrimidine C_{7a}); 150.03 (pyrazolopyrimidine C_6); 156.46 (pyrazolopyrimidine C_4); 157.59 (pyrazole C_5); 167.22 (carboxylic C=O). Anal. Calcd for $C_{28}H_{20}N_8O_4$ (532.51): C, 63.15; H, 3.79; N, 21.04. Found: C, 63.24; H, 3.84; N, 21.31.

4.1.15. General procedure for preparation of **4-{3-Methyl-5-sustituted-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazol-4-yl}diazenylbenzoic acids (15b,c)**

An equimolar amount of the hydrazine derivative **8** and the selected 4-substituted azo benzoic acid derivative **(iiib,c)** in ethanol/glacial acetic acid mixture (15 ml) (2:1) was heated under reflux for 18 h. The reaction mixture was concentrated, left to cool then poured into ice-cold water, the separated solid was filtered, washed with water, dried and crystallized from dioxane/ethanol. Yield, physical properties and elemental microanalyses of compounds **(15b,c)** are listed below.

4.1.15.1. 4-{3,5-Dimethyl-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazol-4-yl}diazenylbenzoic acid (15b) Yield: 85 %; Mp: 237-238 ^oC. IR (KBr, cm^{-1}): 3392-3100 (OH); 3056, 2983, 2930, 2829 (CH); 1709, 1681 (C=O); 1633 (C=N); 1603, 1575 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.15, 2.81 (2s, each 3H, 2 CH_3); 7.36-8.16 (m, 14H, phenyl-H); 8.34 (s, 1H, pyrazolopyrimidine C_3-H); 13.66 (s, 1H, OH, D_2O exchangeable). Anal. Calcd for $C_{29}H_{22}N_8O_3$ (530.54): C, 65.65; H, 4.18; N, 21.12. Found: C, 65.81; H, 4.25; N, 21.37.

4.1.15.2. 4-{3-Methyl-5-phenyl-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazol-4-yl}diazenylbenzoic acid (15c) Yield: 78 %; Mp: 251-253 ^oC. IR (KBr, cm^{-1}): 3361-3218 (OH); 3030, 2885 (CH); 1693 (C=O); 1648 (C=N); 1608, 1578, 1510 (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.29 (s, 3H, CH_3); 7.23-8.07 (m, 19H, phenyl-H); 8.51 (s, 1H, pyrazolopyrimidine C_3-H); 11.32 (s, 1H, OH, D_2O exchangeable). ^{13}C-NMR (75 MHz,

DMSO-d_6) δ 14.67 (CH_3); 106.21 (pyrazolopyrimidine C_{3a}); 114.08 (pyrazole C_4); 121.56, 126.91, 130.03 (phenyl C_{2-6}); 121.85, 127.46, 128.87 (phenyl C_{2-6}); 128.44, 128.73, 129.18 (phenyl C_{2-6}); 128.99 , 130.47, 130.87 (phenyl C_{2-6}); 132.15 (phenyl C_1); 134.36 (phenyl C_1); 134.81 (phenyl C_1); 137.29 (phenyl C_1); 142.47 (pyrazole C_3); 146.78 (pyrazolopyrimidine C_3); 148.38 (pyrazolopyrimidine C_{7a}); 154.89 (pyrazole C_5); 156.78 (pyrazolopyrimidine C_6); 166.62 (pyrazolopyrimidine C_4); 166.90 (carboxylic C=O). Anal. Calcd for $C_{34}H_{24}N_8O_3$ (592.61): C, 68.91; H, 4.08; N, 18.91. Found: C, 69.08; H, 4.14; N, 19.12.

4.1.16. General procedure for preparation of **6-(5-Amino-3-aryl-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-ones (16a-e)**

An equimolar amount of the hydrazine derivative **8** and the selected phenacyl cyanide derivative **(iva-e)** in ethanol/glacial acetic acid mixture (2:1) (10 ml) was heated under reflux for 6 h. The reaction mixture was left to attain room temperature then the separated solid product was filtered, washed with diethyl ether, dried and recrystallized from dioxane. Yield, physical properties and elemental microanalyses of compounds **(16a-e)** are listed below.

4.1.16.1. 6-(5-Amino-3-phenyl-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (16a) Yield: 80 %; Mp: 226-227 ^oC. IR (KBr, cm^{-1}): 3452, 3339 (NH); 3095, 3058 (CH); 1719 (C=O); 1610 (C=N); 1546, 1479 (C=C). Anal. Calcd for $C_{26}H_{19}N_7O$ (445.48): C, 70.10; H, 4.30; N, 22.01. Found: C, 70.36; H, 4.38; N, 22.19.

4.1.16.2. 6-(5-Amino-3-(4-bromophenyl)-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (16b) Yield: 80 %; Mp: 285-286 ^oC. IR (KBr, cm^{-1}): 3457, 3316 (NH); 3111, 3063 (CH); 1700 (C=O); 1616 (C=N); 1572, 1542, 1484 (C=C). ^{1}H-NMR (500 MHz, DMSO-d_6) δ 5.52 (s, 1H, pyrazole C_4-H); 6.11 (s, 2H, NH_2, D_2O exchangeable); 7.23-7.59 (m, 12H, phenyl-H); 8.03 (d, *J* = 8.5 Hz, 2H, phenyl $C_{2,6}$-H); 8.51 (s, 1H, pyrazolopyrimidine C_3-H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 84.45 (pyrazole C_4); 106.81 (pyrazolopyrimidine C_{3a}); 121.74, 128.86, 131.85 (phenyl C_{2-6}); 122.52, 127.85, 129.95 (phenyl C_{2-6}); 128.11, 128.72, 128.99 (phenyl C_{2-6}); 132.41 (phenyl C_1); 136.56 (phenyl C_1); 137.42 (phenyl C_1); 138.38 (pyrazolopyrimidine C_3); 147.59 (pyrazolopyrimidine C_{7a}); 150.17 (pyrazole C_5); 151.25 (pyrazolopyrimidine C_6); 151.41 (pyrazole C_3); 158.03 (pyrazolopyrimidine C_4). MS (m/z, %): 526 ($M^{+\bullet}$+2, 48); 524 ($M^{+\bullet}$, 53); 315 (7); 313 (7); 287 (100); 261 (5); 258 (7); 232 (5); 205 (4); 143 (5); 102 (8); 77 (22). Anal. Calcd for $C_{26}H_{18}BrN_7O$ (524.37): C, 59.55; H, 3.46; N, 18.70. Found: C, 59.79; H, 3.49; N, 18.94.

4.1.16.3. 6-(5-Amino-3-(4-chlorophenyl)-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (16c) Yield: 70 %; Mp: > 300 ^oC. IR (KBr, cm^{-1}): 3458, 3315 (NH); 3110, 3065 (CH); 1702 (C=O); 1618 (C=N); 1573, 1543, 1486 (C=C). Anal. Calcd **for** $C_{26}H_{18}ClN_7O$ (479.92): C, 65.07; H, 3.78; N, 20.43. Found: C, 65.31; H, 3.75; N, 20.82.

4.1.16.4. 6-(5-Amino-3-(4-methylphenyl)-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (16d) Yield: 65 %; Mp: 237-238 ^oC. IR (KBr, cm^{-1}): 3457, 3305 (NH); 1705 (C=O); 1612 (C=N); 1540, 1478 (C=C). Anal. Calcd for $C_{27}H_{21}N_7O$ (459.50): C, 70.57; H, 4.61; N, 21.34. Found: C, 70.74; H, 4.67; N, 21.58.

4.1.16.5. 6-(5-Amino-3-(4-methoxyphenyl)-1*H*-pyrazol-1-yl)-1,5-diphenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (16e) Yield: 70 %; Mp: 267-268 ^{o}C. IR (KBr, cm^{-1}): 3447, 3320 (NH); 3112, 3068, 2951, 2925, 2827 (CH); 1703 (C=O); 1616 (C=N); 1580, 1544, 1482 (C=C); 1243, 1028 (C-O-C). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 3.74 (s, 3H, OCH_3); 5.47 (s, 1H, pyrazole C_4-H); 6.06 (s, 2H, NH_2, D_2O exchangeable); 6.85 (d, 2H, *J* = 8.8 Hz, methoxy phenyl $C_{3,5}$-H); 7.28-7.62 (m, 10H, phenyl-H); 8.07 (d, *J* = 7.7 Hz, 2H, phenyl $C_{2,6}$-H); 8.52 (s, 1H, pyrazolopyrimidine C_3-H). Anal. Calcd for $C_{27}H_{21}N_7O_2$ (475.50): C, 68.20; H, 4.45; N, 20.62. Found: C, 68.43; H, 4.51; N, 20.80.

4.1.17. General method for preparation of **Ethyl 5-amino-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazole-4-carboxylate (17a)** and **5-Amino-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazole-4-carbonitrile (17b)**

An equimolar amount of the hydrazine derivative **8** and ethyl 2-cyano-3-ethoxypropenoate **(va)** or (ethoxymethylidene)propanedinitrile **(vb)** in ethanol/glacial acetic acid mixture (4:1) (10 ml) was heated under reflux for 6-8 h. The reaction mixture was concentrated under reduced pressure and allowed to cool. The separated crystals were filtered, washed with water, dried and recrystallized from ethanol. Yield, physical properties and elemental microanalyses of compounds **(17a,b)** are listed below.

4.1.17.1. Ethyl 5-amino-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazole-4-carboxylate (17a) Yield: 65 %; Mp: 234-235 ^{o}C. IR (KBr, cm^{-1}): 3402, 3291 (NH); 2977, 2932 (CH); 1735, 1694 (C=O); 1633 (C=N); 1576, 1526, 1497 (C=C); 1253, 1058 (C-O-C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.21 (t, *J* = 7.0 Hz, 3H, CH_3); 4.12 (q, *J* = 7.0 Hz, 2H, CH_2); 6.97 (s, 2H, NH_2, D_2O exchangeable); 7.29-7.63 (m, 9H, phenyl-H and pyrazole C_3-H); 8.01 (d, *J* = 7.2 Hz, 2H, phenyl $C_{2,6}$-H); 8.55 (s, 1H, pyrazolopyrimidine C_3-H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 14.93 (CH_3); 59.55 (OCH_2); 92.90 (pyrazole C_4); 107.36 (pyrazolopyrimidine C_{3a}); 122.58, 128.25, 130.02 (phenyl C_{2-6}); 128.82, 129.16, 129.32 (phenyl C_{2-6}); 135.80 (phenyl C_1); 137.42 (phenyl C_1); 138.24 (pyrazole C_3); 146.79 (pyrazolopyrimidine C_{7a}); 150.03 (pyrazole C_5); 152.32 (pyrazolopyrimidine C_6); 157.89 (pyrazolopyrimidine C_4); 163.25 (ester C=O). Anal. Calcd for $C_{23}H_{19}N_7O_3$ (441.15): C, 62.58; H, 4.34; N, 22.21. Found: C, 62.80; H, 4.38; N, 22.48.

4.1.17.2. 5-Amino-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazole-4-carbonitrile (17b) Yield: 60 %; Mp: 284-285 ^{o}C. IR (KBr, cm^{-1}): 3317, 3246 (NH); 2971 (CH); 2224 (CN); 1704 (C=O); 1646 (C=N); 1592, 1524, (C=C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.27 (dd, *J* = 7.8, 1.8 Hz, 2H, phenyl-H); 7.39-7.64 (m, 9H, phenyl-H, NH_2 and pyrazole C_3-H); 8.00 (dd, *J* = 8.7, 1.2 Hz, 2H, phenyl-H); 8.56 (s, 1H, pyrazolopyrimidine C_3-H). Anal. Calcd for $C_{21}H_{14}N_8O$ (394.13): C, 63.95; H, 3.58; N, 28.41. Found: C, 64.14; H, 3.62; N, 28.67.

4.1.17.3. Ethyl 5-hydroxy-1-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)-1*H*-pyrazole-4-carboxylate (17c)

A mixture of the hydrazine derivative **8** (0.32 g, 1 mmol) and diethyl (ethoxymethylidene)propanedioate **(vc)** (0.43 g, 0.4 ml, 2 mmol) was heated in an oil bath at 180 °C for 15 min. The reaction mixture was allowed to attain room temperature, triturated with ethanol, then the solid product was filtered, washed with diethyl ether, dried and crystallized from ethanol. Yield: 52 %; Mp. > 300 ^{o}C. IR (KBr, cm^{-1}): 3391, 3363 (OH); 3102, 3066, 2982 (CH); 1719 (C=O); 1646 (C=N); 1568, 1541, 1519, 1497 (C=C); 1250, 1025 (C-O-C). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.22 (t, *J* = 6.9 Hz, 3H, CH_3); 4.15 (q, *J* = 6.9 Hz, 2H, CH_2); 7.10-8.15 (m, 11H, phenyl-H and pyrazole C_3-H); 8.33 (s, 1H, pyrazolopyrimidine C_3-H); 9.91 (s, 1H, OH, D_2O exchangeable). Anal. Calcd for $C_{23}H_{18}N_6O_4$ (442.43): C, 62.44; H, 4.10; N, 19.00. Found: C, 63.67; H, 4.16; N, 19.23.

4.1.18. Diethyl 2-{(2-(4-oxo-1,5-diphenyl-4,5-dihydro-1*H*-pyrazolo[3,4-d]pyrimidin-6-yl)hydrazin-1-yl)methylidene}propanedioate (18)

A mixture of the hydrazine derivative **8** (0.32 g, 1 mmol), diethyl 2-(ethoxymethylidene)propanedioate **(vc)** (0.21 g, 0.2 ml, 1 mmol) and anhydrous potassium carbonate (0.14 g, 1 mmol) in absolute ethanol (10 ml) was heated under reflux for 12 h. The reaction mixture was allowed to cool, acidified with (1 N) hydrochloric acid (until pH = 5) in an ice-water bath. The precipitated product was filtered, washed with water, dried and crystallized from ethanol; Yield 70 %; Mp. 215-217 ^{o}C. IR (KBr, cm^{-1}): 3272 (NH); 3070, 2976, 2902 (CH); 1718, 1698 (C=O); 1657 (C=N); 1612, 1551, 1492, 1419 (C=C); 1255, 1026 (C-O-C). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.17, 1.26 (2t, *J* = 8.0 Hz, each 3H, 2 $CH_2\underline{CH_3}$); 4.07, 4.22 (2q, *J* = 8.0 Hz, each 2H, 2 $\underline{CH_2}CH_3$); 7.29-7.67 (m, 8H, phenyl-H); 7.97 (d, *J* = 12 Hz, 2H, phenyl $C_{2,6}$-H); 8.20-8.26 (m, 2H, pyrazolopyrimidine C_3-H and =CH); 9.31 (s, 1H, NH, D_2O exchangeable); 10.27 (d, *J* = 12 Hz, 1H, NH, D_2O exchangeable). MS (m/z, %): 488.45 ($M^{+\bullet}$, 9); 309.22 26.12); 260.24 (22.36); 203.07 (24.24); 122.42 (20.02); 122.15 (25.53); 97.22 (23.52); 73.29 (30.20); 71.19 (22.97); 65.28 (100.00); 63.00 (24.96); 60.04 (24.27); 46.20 (29.95); 44.05 (94.71). Anal. Calcd for $C_{25}H_{24}N_6O_5$ (488.50): C, 61.47; H, 4.95; N, 17.20. Found: C, 61.72; H, 5.02; N, 17.49.

4.2. Biological evaluation

4.2.1. *In vitro* cyclooxygenase inhibition assay

The ability of the tested compounds to inhibit both COX-1 and COX-2 isozymes at three concentrations (25, 50 and 100 μM) was carried out using Cayman colorimetric COX (ovine) inhibitor screening assay kit (Catalog No. 760111)[55] supplied by Cayman chemicals, Ann Arbor, MI, USA. The Colorimetric COX Inhibitor Screening Assay utilizes the peroxidase component of cyclooxygenase. The peroxidase activity was assayed colorimetrically by monitoring the appearance of oxidized *N*,*N*,*N*',*N*'-tetramethyl-1,4-phenylenediamine (TMPD) which is produced during the reduction of PGG2 to PGH2, at 590 nm.[56] The preparation of reagent was carried out following the manufacturer instructions (Catalog No. 760111).[55] Briefly, aliquots contain 160 μl of assay buffer (0.1N Tris–HCl, pH 8.0), 10 μl of heme, 10 μl of enzyme (either ovine COX-1 or COX-2), and 10 μl of tested compounds were placed in a 96-well plate. Different concentrations of celecoxib, diclofenac sodium or tested compounds were

incubated with the enzymes for a period of 5 min at 25 ^{o}C. After the incubation period an addition of 20 µl of the colorimetric substrate (TMPD) and 20 µl of arachidonic acid was done then the plate was carefully shaken for a few seconds and then incubated for 5 min at 25 °C. The absorbance was measured at 590 nm using plate reader. The average absorbance of samples was determined; the absorbance of background wells was subtracted from absorbances of the 100% initial activity and the inhibitor wells; each inhibitor sample was subtracted from the 100% initial activity sample then divided by the 100% initial activity sample and multiplied by 100 to give the percent inhibition. Percent inhibition was graphed by the inhibitor concentration to determine the IC_{50} values.

4.2.2. *In vivo* anti-inflammatory activity

Adult female wistar rats weighing 150-250 g were used (procured from the Experimental Animal Centre in Alexandria University). All animals accessed to food and water *ad libitum* and were housed in 12 h dark/light cycle in a controlled condition at 23-25 ^{o}C. They were allowed to acclimatize for 1 week prior to experimentation. Procedures involving animals and their care were conducted in conformity with the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH publication No. 83-23, revised 1996) and following the ethical guidelines of Alexandria University on laboratory animals. In all tests, adequate considerations were adopted to reduce pain or discomfort of animals.

Celecoxib and diclofenac sodium (European Egyptian Pharmaceutical industries, Alexandria, Egypt), formalin 5 % made from formaldehyde 37 % and saline (Merck, Germany) were used as reference compounds. The novel compounds were synthesized based on the previously described methods.

Inflammatory models:

Compounds that showed *in vitro* selectivity indices higher or nearly equivalent to reference drugs towards COX 2 enzyme (**9b, 16a, 16b, 17a, 17b** and **18**) were further evaluated for their *in vivo* anti-inflammatory activity applying the formalin-induced paw edema screening protocol as an acute inflammation model[49, 50] and cotton pellet-induced granuloma protocol as a chronic inflammation model.[51] Celecoxib (5 mg/kg) and diclofenac sodium (5 mg/kg) were used as reference drugs. Animals were divided randomly into six groups of six rats each, treated with the different test compounds. Groups treated with celecoxib and diclofenac sodium served as reference and rats which were given the vehicle (DMSO) served as control. Same groups of rats were employed in both inflammation models and in ulcerogenic examination.

4.2.2.1. Formalin-induced paw edema test (acute inflammation model):

A solution of freshly prepared 5 % formalin was used as a phlogistic agent. A mark was made on the lateral malleolus of the rats' paws delineating the injection sites. The novel test compounds (5 mg/kg body weight), celecoxib (5 mg/kg body weight), diclofenac sodium (5 mg/kg body weight), were dissolved in DMSO and administered orally using gastric gavage once daily for 7 consecutive days, while DMSO was given to the control group. After the last dose, on the 8th day, the initial volume of paw was measured by means of digital calibrated Vernier caliper then 40 µl formalin were injected subcutaneously into the sub-plantar tissue of the right hind paw of all groups under light ether anesthesia.

The volume of the paw was measured in different treatment groups, 4 h following the formalin injection and the amount of increase in paw volume (edema volume) was calculated by subtracting the volumes before and 4 h after the injection of formalin.

Edema was expressed as an increase in the volume of the paw, and the percentage of edema inhibition (or percent protection against inflammation) for each rat and each group was calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{(Vt - Vo)\ \text{control} - (Vt - Vo)\ \text{test compound}}{(Vt - Vo)\ \text{control}} \times 100$$

Where Vt is the mean volume of edema after 4 h at specific time interval (4 h) and Vo is the mean volume of edema at zero time interval.

Statistical differences between control and test groups were estimated using the F test (ANOVA), and pairwise comparison was done using Post Hoc Test (Tukey).[57] Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.[58]

4.2.2.2. Cotton pellet-induced granuloma assay (chronic inflammation model):

Adsorbent cotton wool was cut into pieces weighing 20 ± 1 mg and made into pellets. The pellets were then sterilized in a hot air oven at 120 ^{o}C for 2 h. The abdomen of the rat was shaved cleanly, swabbed with 70 % ethanol and two sterilized cotton pellets were implanted subcutaneously, one on each side of the abdomen of the animal under Ketamine/xylazine anesthesia (50 mg/kg and 9 mg/kg respectively intraperitoneal). Gentamycin injection (4 mg/kg intramuscular) was used for three days following the procedure to avoid post-operative infection. Test compounds, celecoxib (5 mg/kg), diclofenac sodium (5 mg/kg) or vehicle (DMSO) were administered orally using gastric gavage once daily throughout the experimental period of 7 consecutive days. On the 8th day after implantation, rats were anaesthetized under light ether anesthesia. The pellets were dissected, dried at 60 ^{o}C for 24 h and weighed after cooling. The mean weight of the dried cotton pellets of the control group, the test groups as well as of reference groups was calculated. Percentage of granuloma inhibition of the test compounds and reference compounds was calculated relative to control.

The net dry weights were determined as follows:

Dry granuloma weight = weight of the cotton pellet of test, reference and control groups (dry) - weight of the cotton pellet before the experiment.

Statistical differences between control and test groups were estimated using the F test (ANOVA), and pairwise comparison was done using Post Hoc Test (Tukey).[57] Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.[58]

4.2.3 Ulcerogenic activity:

Compounds were also evaluated for chronic gastric ulcerogenic effect[52, 53] on the same groups of rats. On the 8th day and after scarification and removal of cotton pellets, rats' stomachs were also removed and opened through the greater curvature, washed under running water and fixed in saline solution.

Gross examination was performed for any evidence of hyperemia, hemorrhage, definite hemorrhagic erosion or ulcer. In addition, histopathological examination was performed to confirm the degree of inflammatory reaction in the gastric mucosal layers of the treated rats' stomachs.

5. Conflict of Interest

The authors have declared no conflict of interest.

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Highlights

- Synthesis of new pyrazolo[3,4-d] pyrimidines linked to different functionalities at 5 and 6 positions
- *In vitro* COX-1/ COX-2 inhibitory assay
- *In vivo* formalin paw edema and cotton pellets induced granuloma assays
- Compounds 16a and 18 were highly effective in formalin paw edema assay (acute model)
- Compound 17b showed promising activity in cotton-pellets granuloma assay (chronic model)
- Compounds 16a, 17b and 18 showed safe gastrointestinal margin

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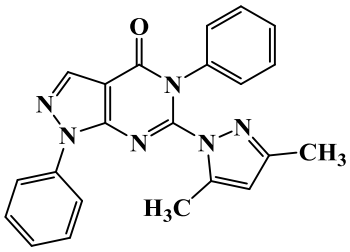
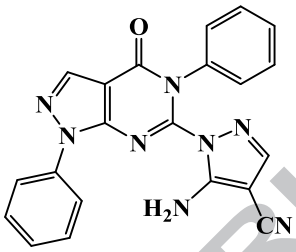


16a



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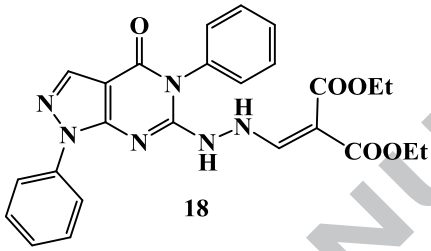

18

- These compounds showed promising anti-inflammatory activity with COX-2 selectivity comparable or higher than celecoxib with high safety gastrointestinal profile
- Compounds 16a and 18 were highly effective in formalin paw edema assay (acute model)
- Compound 17b showed promising activity in cotton-pellets granuloma assay (chronic model)

Highlights

- Synthesis of new pyrazolo[3,4-d] pyrimidines linked to different functionalities at 5 and 6 positions
- *In vitro* COX-1/ COX-2 inhibitory assay
- *In vivo* formalin paw edema and cotton pellets induced granuloma assays
- Compounds 16a and 18 were highly effective in formalin paw edema assay (acute model)
- Compound 17b showed promising activity in cotton-pellets granuloma assay (chronic model)
- Compounds 16a, 17b and 18 showed safe gastrointestinal margin