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COX-1/COX-2 inhibition assays and histopathological study of the new designed anti-inflammatory agent with a pyrazolopyrimidine core

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ABSTRACT

Four pyrazolopyrimidine series were prepared with a substitution at position- 4 by Schiff base, triazole, oxadiazole and pyrazole moieties (**7a-f**, **8a,b**, **9a-f**, **10a,b** and **13a,b**), respectively. All the synthesized compounds were evaluated *in vitro* against COX-2 and *in vivo* against carrageenan-induced rat paw edema as anti-inflammatory agents. Regarding the anti-inflammatory activity (AI) compounds **7c**, **7f**, **8a**, and **9a** showed higher activity with respect to celecoxib. Compounds **9a**, **7d**, and **7f** were closely selective to celecoxib. Also, **7c** and **7d** were safer than indomethacin and similar to celecoxib as resulted from the histopathological study. In addition, the docking study that showed the binding mode of prominent pyrazolopyrimidine compounds inside the COX-2 receptor. Formation of unexpected pyrazole **13a** and **13b** was briefly discussed using 2D NMR.

1. Introduction

The inflammation process involves sequential activation of signaling molecules, pro-inflammatory mediators, such as prostaglandins, leukotrienes, and oxygen free radicals [1]. Two cyclooxygenases enzymes were responsible for activation of the production of prostaglandins (PG1s) and other inflammatory mediators. Cyclooxygenase-1 (COX-1) is a constitutive form which regulates the gastrointestinal cytoprotection and maintains the renal functions [2]. The other cyclooxygenase-2 isozyme (COX-2) catalyses the (PG1s) formation and is responsible for pain, fever and other inflammatory symptoms [3,4]. Traditional NSAIDs as ibuprofen, aspirin or indomethacin, suffered from many adverse effects including gastric ulceration, renal injury, and cardiotoxicity [5]. These drawbacks are due to non-selective inhibition of both COX-1 and COX-2 isozymes [6,7]. Selective COX-2 inhibitors like coxibs have been developed to limit those adverse effects. In spite of coxibs safety, they are still suffering from cardiovascular side effects. Regarding that, there is a continual need for more selective and safer COX-2 inhibitors. The selective COX-2 inhibitors, coxibs family and its members celecoxib (I), rofecoxib (II) and valdecoxib (III) (Fig. 1), all candidates contain a pyrazole core with a diaryl substituted with a sulphamoyl or methanesulphonyl group as selective COX-2 pharmacophores [8,9].

Also, the pyrazole and their fused pyrazolopyrimidine drug cores are of increasing interest. These drugs containing molecules based on the pyrazolo[3,4-*d*]pyrimidine ring system exhibited a multitude of wide pharmacological properties including anti-inflammatory agent [10,11], anticancer [12–14], tuberculostatic [15,16] and antimicrobial [17,18]. Recently, several novel series of pyrazolo[3,4-*d*]pyrimidine derivative (IV) have been prepared and showed a comparable anti-inflammatory activity (AI) to that of ketorolac against carrageenan-induced rat paw edema [10]. Also, the sulfamoyl pyrazolopyrimidine derivative (V) exhibited good anti-inflammatory activity compared to celecoxib (dose = 25 mg/kg) [19]. Furthermore, compound (VI) with an amino substitution at position-4 was reported to inhibit selectively and potently COX-2 activity in human monocytes (IC_{50} = 0.9 nM for COX-2 vs. IC_{50} = 59.6 nM for COX-1) with an anti-angiogenic activity as well [20]. Moreover, different pharmacophores such as pyrazole [21,22], triazole [23–25], oxadiazole [26,27] and Schiff base [28,29] were Found to have anti-inflammatory activity. Due to the presence of additional side pocket at COX-2 active site increase its volume to accommodate more bulky structures. Guided by the previously mentioned studies and to a continuation of previous work [30–33], our strategy is to synthesize new pyrazolo[3,4-*d*]pyrimidine derivatives aiming to be more selective COX-2 inhibitors. The presence of an additional side pocket on COX-2 active site increases its volume to accommodate more bulky structures. So the design of the synthesized compounds depends on the presence of a more bulky pyrazolopyrimidine core than pyrazole, central ring of coxibs in a way to increase fitting with larger COX-2 receptor site. This pyrazolopyrimidine central ring substituted with

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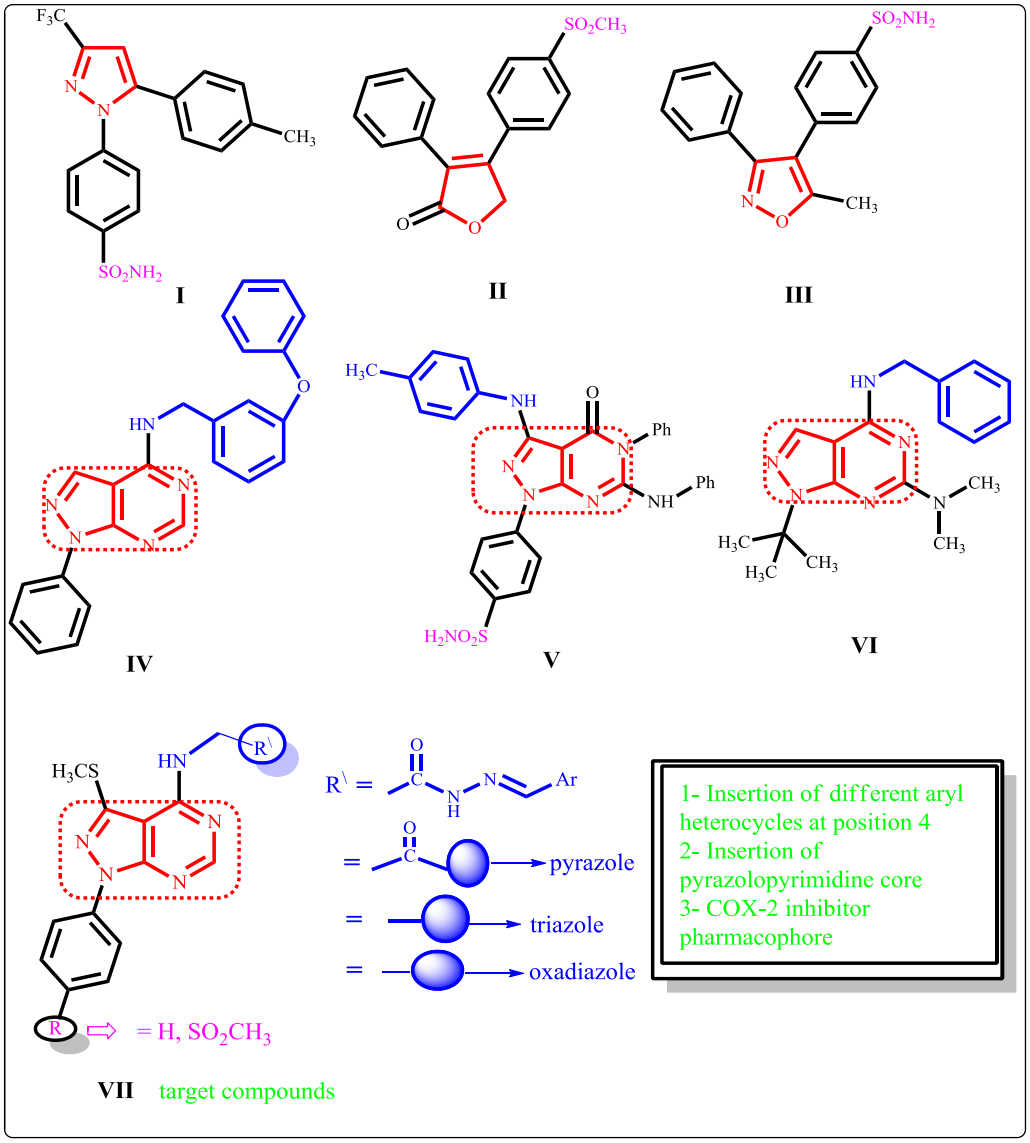


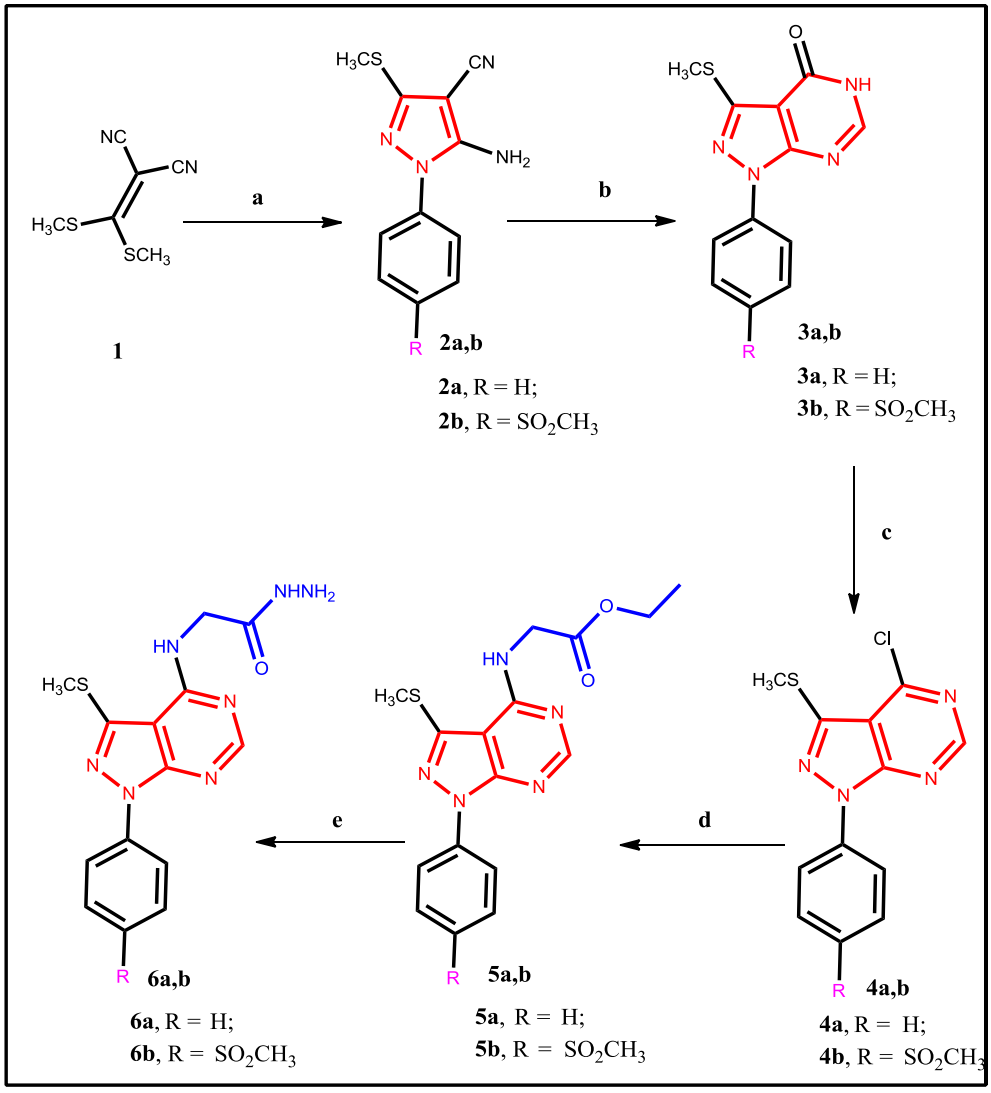
Fig. 1. Chemical structures of selective COX-2 inhibitors, celecoxib (I), rofecoxib (II), valdecoxib (III), some reported pyrazolo[3,4-*d*]pyrimidines (IV, V, VI) and design for the target compounds (VII).

phenyl ring or p-methylsulfonyl (COX-2 pharmacophore) phenyl at N1. Another amino substitution at position- 4 was placed to increase the incidence of hydrogen bonds with receptor. In addition, this amino group was decorated with different moieties such as Schiff bases (**7a-f**), triazoles (**8a**, **8b**, and **9a-f**), oxadiazoles (**10a** and **10b**) and pyrazoles (**13a** and **13b**). The resulted compounds were evaluated against COX-1\COX-2. Also, carrageenan-induced rat paw edema model and histopathological study was operated to determine their AI and gastric safety. In addition, docking study was performed to predict the mode of action of the target compounds inside the COX-2 active site aiming to get new anti-inflammatory compounds with improved activity and minor drawbacks.

2. Results and discussion

2.1. Chemistry

In a way, to synthesize the new pyrazolopyrimidine series, the precursor 5-Amino-pyrazole-4-carbonitrile **2a** and **2b** were prepared through the reaction of ketene dithioacetal 1 [34] with either PhNHNH2 or p-methylsulfonyl phenylhydrazine, respectively. Elemental analysis and spectral data confirmed the chemical structure of compound **2b**. The IR spectrum showed an absorption band at 3332, 3444 cm^{-1} refer to (NH_2) group and another band detected at 2214 cm^{-1} due to the (C≡N) group. Its 1H NMR revealed two singlet signals at δ 2.52 and 3.27 ppm corresponding to (SCH_3) and (SO_2CH_3), in sequent. Moreover, an additional singlet signal appeared at δ 7.12 ppm, which is exchangeable with D_2O due to (NH_2) protons. This key intermediates **2a** and **2b** were heated and fused with formic acid to get one reported pyrazolo[3,4-*d*]pyrimidine **3a** [35] and the other new one **3b** respectively. The IR spectrum of **3b** showed an absorption band at 3333 cm^{-1} refer to (NH) group and another strong band for (C=O) group at 1678 cm^{-1}. The 1H NMR spectrum of **3b** showed singlet signal at δ 8.23 ppm characteristic to the pyrimidine 6-H, in addition to a D_2O exchangeable singlet signal appeared at δ 12.61 ppm due to (NH) proton. Subsequently, chlorination of **3a** and **3b** with phosphorus oxychloride yielded the chloro derivative **4a** [35] and **4b**. The structure of 4b was illustrated via elemental analysis and the spectral data. IR spectrum of **4b** exhibited the disappearance of both absorption bands due to (NH) and (C=O) groups in the parent compound **3b**. The mass spectrum of **4b** showed the molecular ion peak at *m/z* 355 (M+., 17.42%) and *m/z* 357 (M+.+2, 6.04%) due to the natural abundance

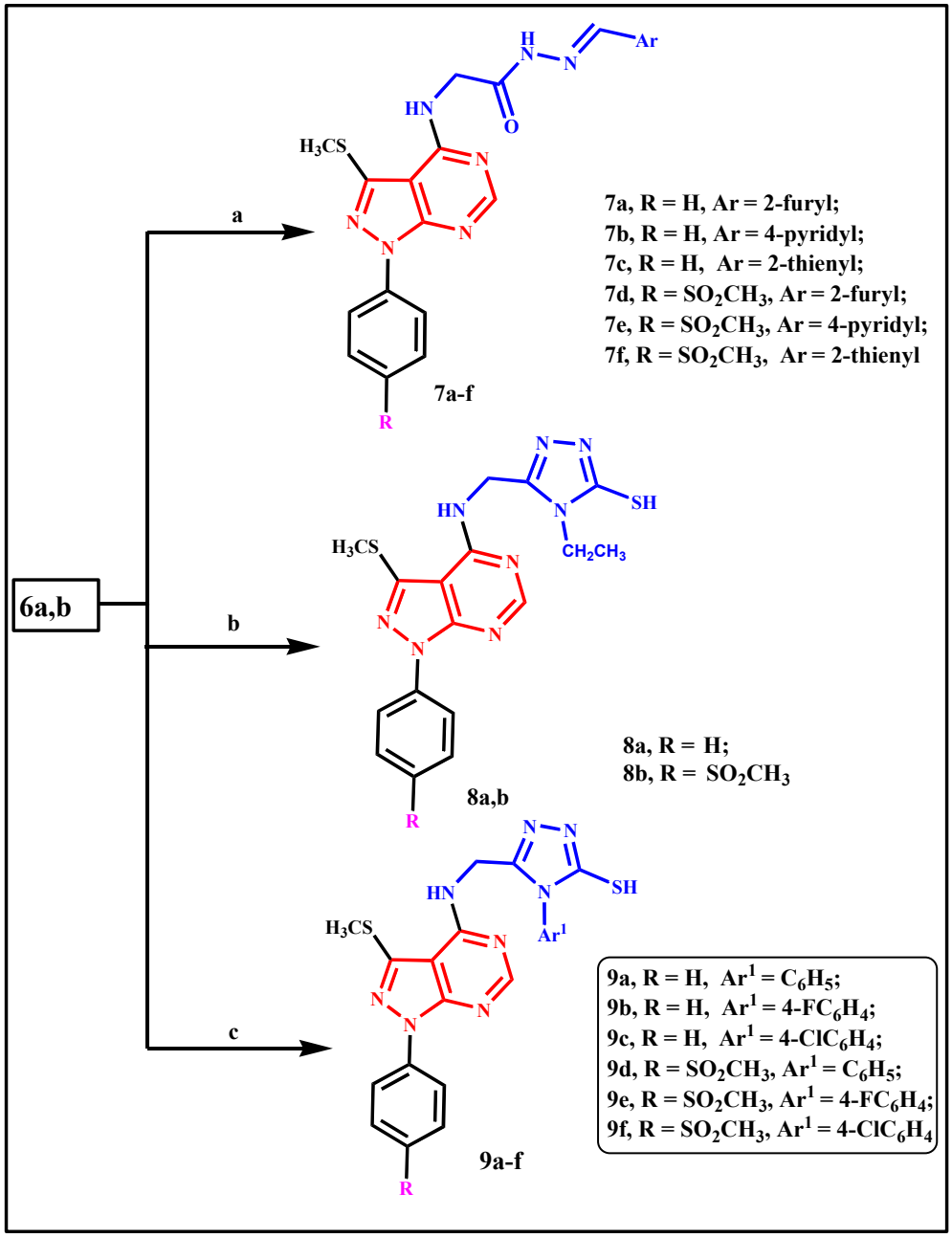


Scheme 1. Reagents and reaction conditions: (a) phenylhydrazine hydrochloric (R = H) and p-methanesulfonyl hydrochloride (R = SO_2CH_3), sodium acetate, 95% ethanol, reflux 5 h, (b) formic acid (85%), reflux 10 h, (c) $POCl_3$, DMF, reflux 4 h, (d) glycine ethyl ester hydrochloride, TEA, absolute ethanol, reflux 5–6 h, (e) hydrazine hydrate, ethanol, reflux 10 h.

of chlorine and sulfur isotopes in the **4b** molecule. The ester derivatives **5a** and **5b** were accomplished through induction of glycine ester moiety to the chloro compounds **4a** and **4b**. The latter **4a** and **4b** underwent a nucleophilic displacement with glycine ethyl ester hydrochloride. The structure of **5a** and **5b** was confirmed by their elemental analysis and spectral data. The ^{1}H NMR spectrum of **5a** showed the pronounced ethyl ester pattern as triplet signal at δ 1.22 ppm due to ($OCH_2\underline{CH_3}$) protons and another quartet signal at δ 4.15 ppm for ($O\underline{CH_2}CH_3$) protons. Additionally, a doublet signal at δ 4.32 ppm due to (NCH_2) protons. Also, a D_2O exchangeable triplet signal appeared at δ 7.32 ppm corresponding to (NH) proton of **5a**. Finally, hydrazinolysis of the ester group in compounds **5a** and **5b** using hydrazine hydrate yielded the key intermediates acetohydrazide **6a** and **6b**. The structure of **6a** and **6b** was established on the basis of elemental analysis and spectral data. The ^{1}H NMR spectrum of **6a** showed the absence of the ethyl ester protons and the appearance of a singlet signal at δ 4.28 ppm for (NH_2) protons. Another one was appeared at δ 9.24 ppm due to (NH) proton, both of them were exchanged with D_2O (Scheme 1).

The acetohydrazide intermediates **6a** and **6b** were reacted with a series of various aldehydes to yield the hydrazones **7a-f**. The ^{1}H NMR spectrum of **7a** showed a singlet signal at δ 8.41 ppm attributed to azomethine proton (N=CH). Also, condensation of the acetohydrazide **6a** and **6b** with ethyl isothiocyanate furnished triazole-3-thiol derivatives **8a** and **8b**, respectively. The ^{1}H NMR spectrum of **8a** showed triplet and quartet signals belong to an ethyl group at δ 1.25 and 4.10 ppm with a coupling constant 7.2 Hz. Besides the appearance of a singlet signal at δ 13.57 attributed to thiol proton (SH). The DEPTQ^{13}C NMR spectrum of **8a** showed the appearance of a signal at δ_{13c} 166.96 ppm corresponding to (C-SH) carbon. Similarly, compounds **9a-f** were prepared by the reaction of acetohydrazide **6a** and **6b** with phenyl isothiocyanate derivatives. The obtained data established the chemical structure of compounds **9a-f**. The ^{1}H NMR spectrum of **9a** revealed the absence of signals for (NH_2) and (NH) protons as in the parent compound **6a**, the appearance of a singlet signal at δ 13.80 ppm attributed to thiol (SH) proton and additional multiplet signal at δ 7.35–7.55 ppm due to phenyl protons in **9a** (Scheme 2).

Moreover, heating of the acid hydrazide **6a** and **6b** with an equivalent amount of CS_2 and KOH gave the new pyrazolo [3,4-*d*] pyrimidine derivatives **10a** and **10b** bearing oxadiazole scaffold at position 4. From **10a** NMR spectrum data an exchangeable singlet signal appeared at δ 14.55 ppm that attributed to a thiol (SH) proton. Additionally, oxadiazole C-5H and C-2H appeared at δ_{13c} 161.89 and 178.16 ppm, sequentially in ^{13}C NMR spectrum of **10b**. Finally, on the way to get pyrazolo-5-one (**11**) via cyclization of the acid hydrazide **6a**



Scheme 2. Synthesis of Schiff bases 7a-f and triazoles (8a,b and 9a-f). Reagent and reaction conditions: (a) appropriate aldehyde, absolute ethanol, gl. acetic acid, reflux 4 h, (b) ethyl isothiocyanate, absolute ethanol, TEA, reflux 3 h, (c) appropirate phenyl or 4-substituted phenyl isothiocyanate, absolute ethanol, TEA, reflux 3 h.

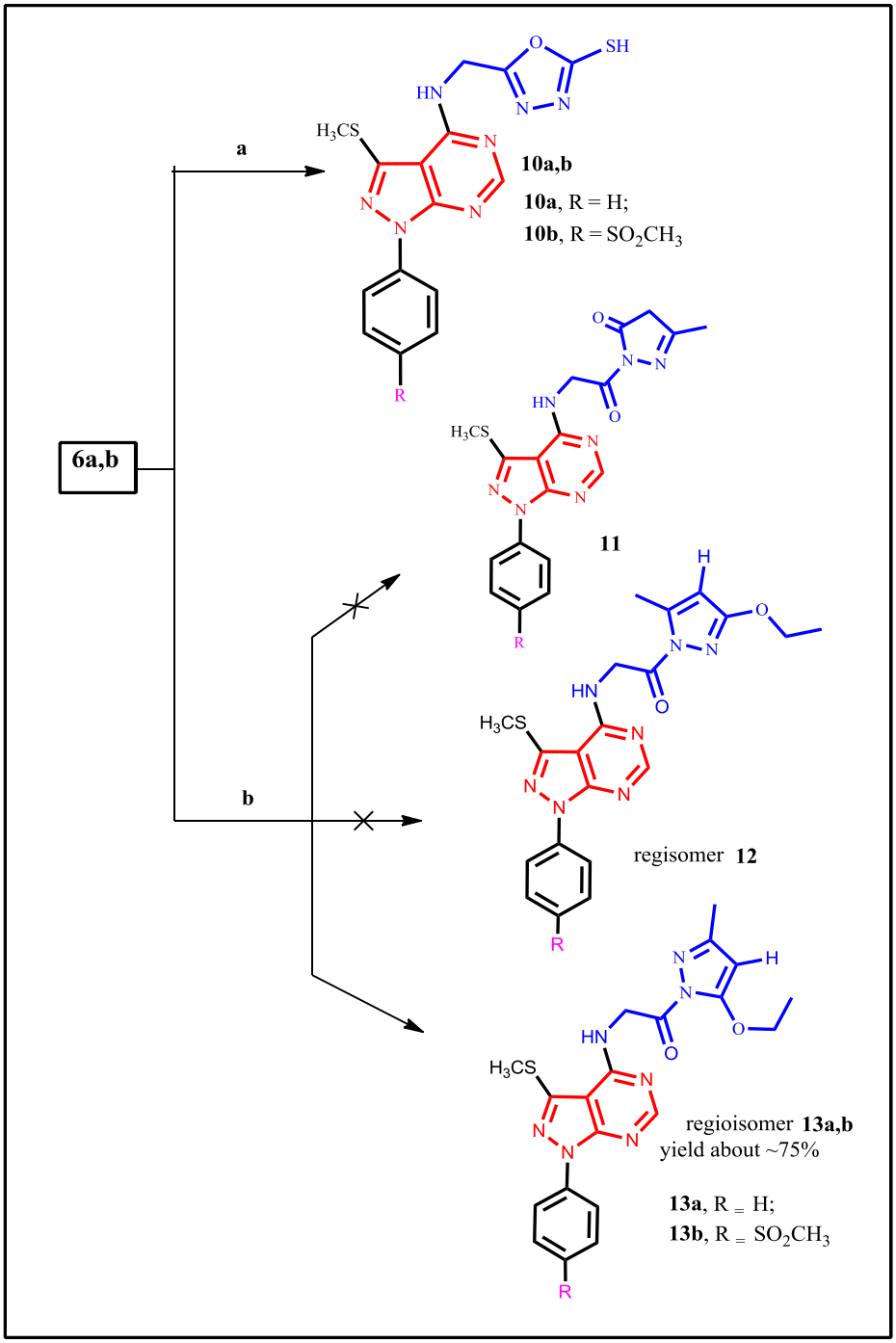
or **6b** with ethylacetoacetate afforded the ethoxy pyrazole derivatives **13a** or **13b**, respectively. Elemental analysis and spectral data confirmed the structure of **13a** and **13b**. Thus, the ^{1}H NMR spectrum of **13a** exhibited triplet and quartet peaks due to ethyl ether protons at δ 1.21 and 4.14 ppm. No evidence for the presence of THE (CH_2) pyrazolone ring in structure (**11**). Also, ^{13}C NMR spectrum of **13a** confirmed the construction of the pyrazole ring rather than pyrazolone. Thus, pyrazole C-4H appeared at δ_{13c} 89.35 ppm and the ether carbons (CH_3) and (CH_2) at δ_{13c} 15.35 and 61.11 ppm, respectively (Scheme 3). The mechanism of formation **13a** and **13b** is illustrated in (Fig. 2).

The pyrazole cyclization of compounds formation**13a** and **13b** could have occurred through two different pathways through the formation of two intermediate **14** or **15**. All spectral data (IR, ^{1}H NMR and ^{13}C NMR) were not enough to illustrate which intermediate is formed either **12** or **13**. Additional spectral NMR experiment [NOESY] (Fig. 3) is operated for elucidation of structure **13a**. Upon 3D studying of two regioisomers **12** and **13**, it was found that there is a proton-proton correlation between CH_3 of pyrazole and CH_2 of glycinyl moiety in form **12** while regioisomer **13** don't show this NOE correlation. The NOESY experiment revealed that no NOE correlation existed between CH_3 and CH_2 in **13a** that confirmed its structure. **13a** regioisomer was achieved through the condensation reaction of NH_2 of hydrazide **6a** with the carbonyl group of acetate moiety followed by another condensation of NH of **6a** with the carbonyl of ethyl ester moiety belong to ethylacetoacetate molecule as illustrated in (Fig. 2).

2.2. Biological anti-inflammatory activity

2.2.1. COX-1 and COX-2 inhibition assays

The tested compounds were subjected to *in vitro* inhibition of ovine COX-1/COX-2 to determine their selectivity through the determination of the minimum inhibitory dose causing 50% activity (IC_{50}). In addition



Scheme 3. Synthesis of oxadiazoles 10a,b and pyrazoles 13a,b. Reagent and reaction conditions: (a) CS_2, KOH, absolute ethanol, reflux 3 h, (b) ethylacetoacetate, absolute ethanol, reflux 10 h.

the COX-2 selectivity index (S.I.) that defines as IC_{50} (COX-1)/IC_{50} (COX-2) were determined. These values were monitored by using *N, N, N′, N′*-tetramethyl-*p*-phenylenediamine at 590 nm and the enzyme immunoassay (EIA) kit [4]. The data calculated and compared with standard drugs (celecoxib, diclofenac sodium and indomethacin). Compounds **7a-f-13a** and **13b** were evaluated and the obtained results were listed in Table 1 and represented in Fig. 4. The results revealed that all the tested compounds showed inhibitory activity on COX-2 enzyme with IC_{50} range (IC_{50} = 0.10 – 0.38 μM) more than that on COX-1 isoform (IC_{50} = 5.28 – 13.11 μM). The most active compounds on COX-2 were **7d**, **7f**, **9a**, **13a** and **7c** (IC_{50} = 0.10 – 0.11 μM range) if compared to celecoxib (IC_{50} = 0.049 μM). The COX-2 selectivity index for all the tested compounds (S.I. = 14.84 – 131.10) was higher than that of both indomethacin (S.I. = 0.080) and diclofenac sodium (S.I. = 4.52). The results revealed that Schiff's derivatives **7c-f** were weak COX-1enzyme inhibitors (IC_{50} = 10.24 – 12.31 μM range) in comparison with celecoxib (IC_{50} = 8.1 μM) and showed high potency against COX-2 enzyme (IC_{50} = 0.10 – 0.12 μM range). Among them the appreciated thienyl **7c** and **7f** and the furyl **7d** with *p*-methyl sulfonyl substituted phenyl ring. Also the triazole derivative **9a** with a COX-1 inhibitory activity (IC_{50} = 13.11 μM), a high potency against COX-2 (IC_{50} = 0.10 μM), and was the most selective (SI = 131.10) close to celecoxib (SI = 165.30). On the other hand compound, **9c** with a 4-chlorophenyl triazole moiety was the unsuccessful choice that showed a

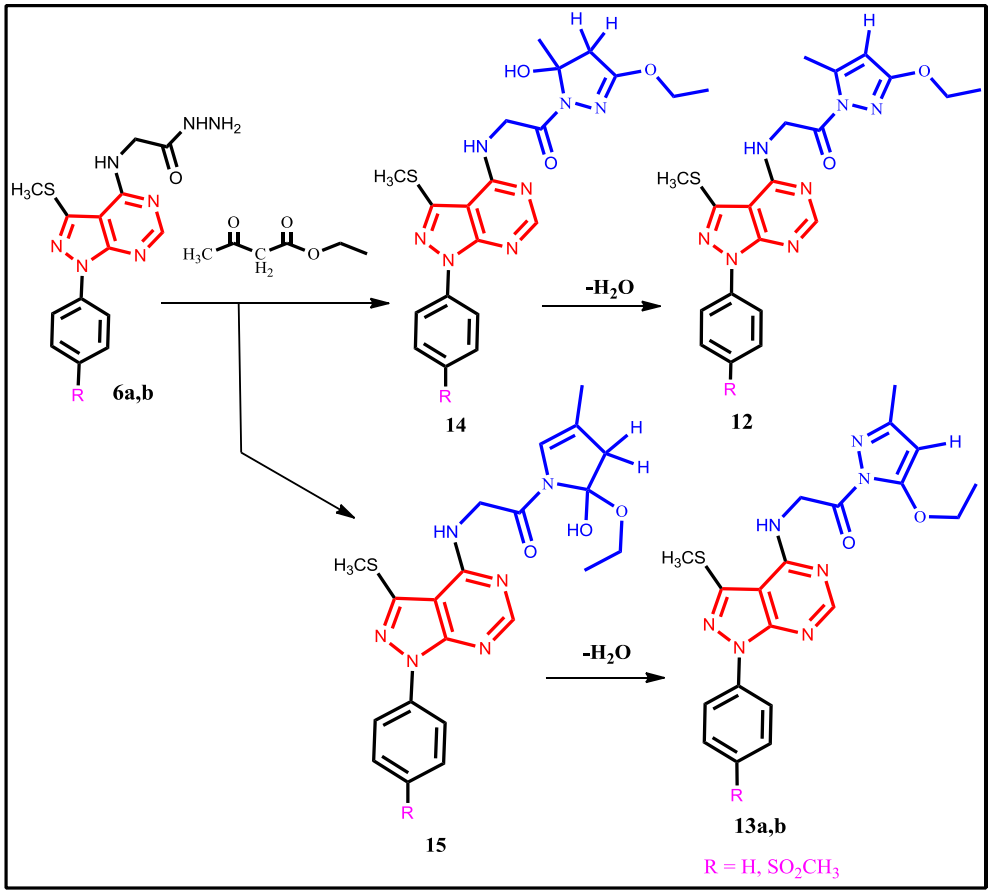


Fig. 2. Plausible mechanism formation of compounds **13a** and **13b**.

lower selectivity index as the COX-2 inhibitor (SI = 14.84).

2.3. *In vivo AI assay*

Carrageenan-induced rat paw edema method [30] was carried out to evaluate synthesized compounds AI activity. The change in edema volume was measured after 1, 3 and 5 h compared to standard drugs celecoxib and indomethacin (Table 2 and Fig. 5) the results revealed that after one hour, compound **10a** (oxadiazole derivative) and **13a** (pyrazole derivative) showed inhibitory activity (59% and 82%), respectively that more than that of celecoxib (50%). After three hours, **7c-f**, **10b**, and **13a** showed AI (75 – 94% range). The long-lasting anti-inflammatory activity was observed in Schiff's compounds **7c** (with a thienyl derivative), **7d-f** (bearing SO_2CH_3 group), ethyl triazole derivative **8a**, phenyl triazole derivative **9a** and pyrazole derivative **13a** in the range 88–96%. For most of the tested compounds, % of anti-inflammatory activity was increased after five hours than three hours and one hour except compound **13a**.

2.4. *Histopathological study*

Examination of histopathological lesions was proceeded to evaluate the ulcerogenic effect for the most active compounds (**7c-f**, **9a** and **13a**) on both glandular and non-glandular portions of the stomach and to compare the severity of lesions with those induced by celecoxib and indomethacin as a standard (Table 3). The control negative group received 2.5% tween 80, normal histological structure of the stomach, including glandular and non-glandular portions. In the former, normal mucosal lining, submucosa and mucosa layers (Fig. 6 (Ia)). In the later, normal histological structure could be Found (Fig. 6 (Ib)). Administrations of indomethacin were associated with severe pathological lesions in the form of degenerative change and necrotic changes in the glandular and non-glandular stomach. The former portion showed erosive and ulcerative changes associated with lymphocytic infiltration, edema and congestion in the submucosal layer. The muscular layer showed severe hyalinosis and diffuse leuckocyte infiltration (Fig. 6 (IIa)). The later portion (non-glandular stomach) was suffering from severe hyperkeratosis accompanied by focal erosive and ulcerative lesions (Fig. 6 (IIb)). Administrations of celecoxib showed mild to moderate lesions in the glandular stomach, mainly degenerative changes of the mucosal lining and mild leuckocyte infiltration in the submucosal layer (Fig. 6 (IIIa)). Additionally, the mucosal lining of the non-glandular stomach portion was suffering from mild hyperkeratosis (Fig. 6 (IIIb)). For Schiff bases, **7c** (H substituted, 2-thienyl) and **7d** (-SO_2CH_3 substituted, 2-furyl) administrations showed similar histopathological lesions in the stomach to celecoxib. Moderate degenerative changes of the mucosal lining of the glandular portion of the stomach in both treatments could be Found associated with mild necrotic changes in the treatment **7c** and moderate lesions in that treated with **7d** (Fig. 6 (IVa, Va)). The submucosal layer showed moderate congestion, leuckocytic infiltration, and edema in the submucosal layer. In addition to, mild hyalinosis and leuckocytic infiltration of the muscular layer of the glandular stomach. The non-glandular stomach showed more or less normal histological structure except for mild focal hyperkeratosis in treatment **7d** (Fig. 6 (IVb, Vb)).

For **9a** (N4 substituted, phenyltriazole) administrations revealed the presence of multifocal areas of degeneration in the lining epithelium in association with moderate necrotic changes. The submucosal layer showed moderate to severe congestion, leukocytic infiltration, hyalinosis and moderate leuckocytic infiltration in the muscular layer (Fig. 7 (VIa)). Moderate hyperkeratosis of the non-glandular stomach could be detected and absence of any erosive or ulcerative lesions (Fig. 7 (VIb)).

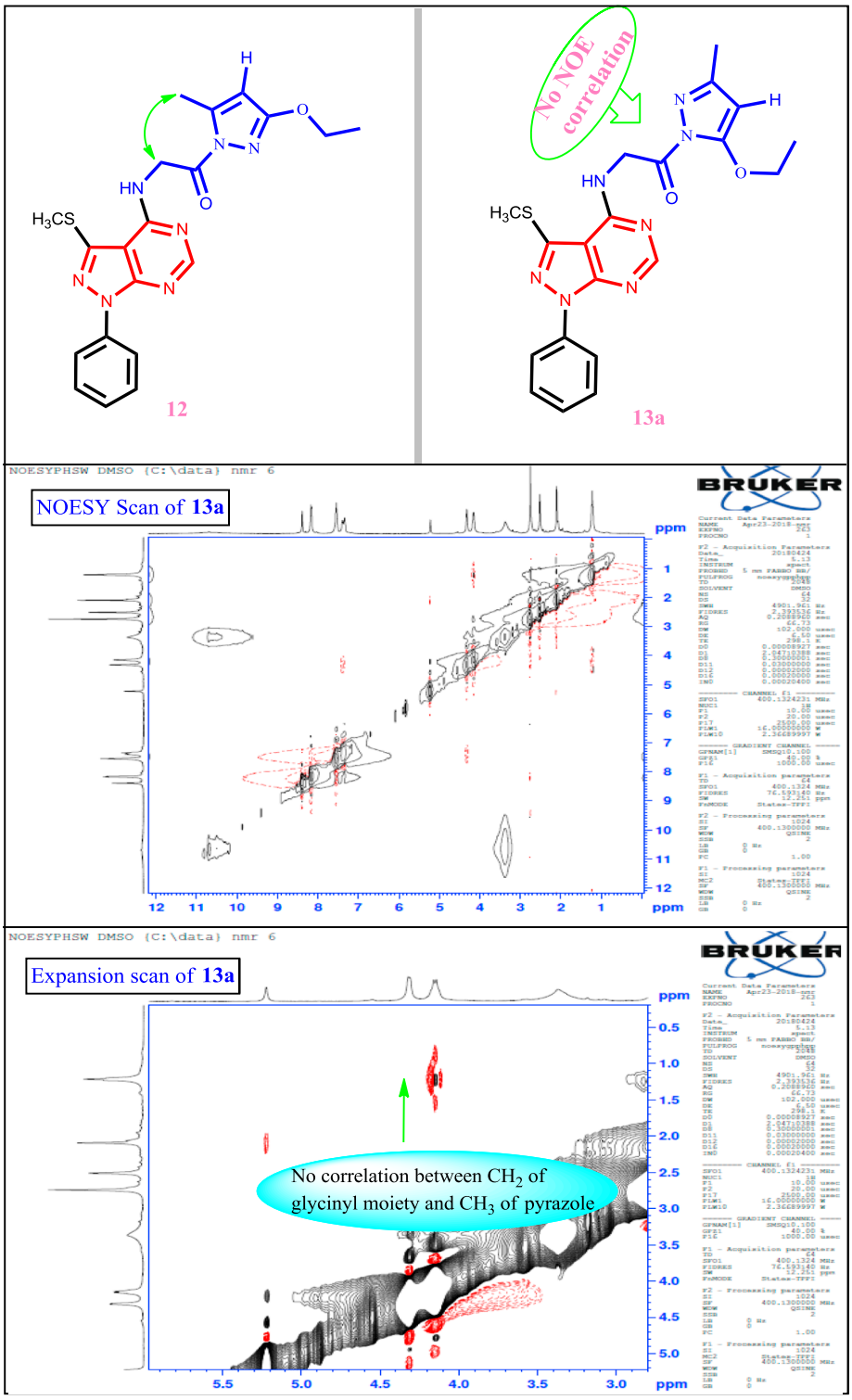


Fig. 3. NOESY scan and expansion of compound **13a**.

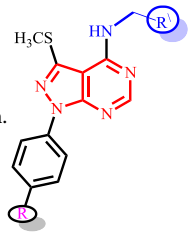
For **7e** (Schiff base -SO_2CH_3 substituted, 2-pyridyl) and **13a** (H substituted, pyrazole) administrations were associated with severe degenerative changes, necrosis of the glandular portion of the stomach in certain areas (Fig. 7 (VIIa, VIIIa)), massive leuckocytic infiltration and congestion of the muscular layer which showed moderate hyalinosis and the presence of focal areas of hyperkeratosis and degenerative changes in the non-glandular stomach (Fig. 7 (VIIb, VIIIb)). For **7f** (Schiff base -SO_2CH_3 substituted, 2-thienyl) administration revealed the presence of the highest pathological lesions in the form of severe degenerative changes, necrosis and ulceration in the glandular stomach which accompanied by congestion, leuckocytic infiltration in the submucosal and mucosal layer (Fig. 7 (IXa)). Moderate erosive and ulcerative changes and hyperkeratosis could be detected in the non-glandular stomach (Fig. 7 (IXb)).

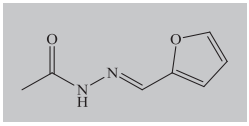
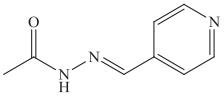
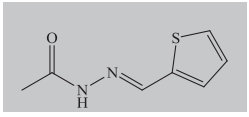
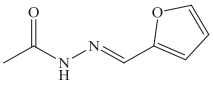
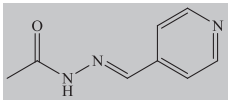
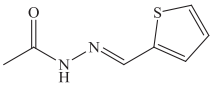
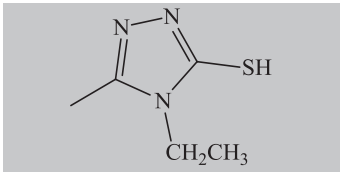
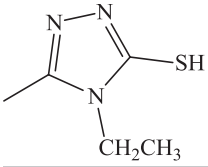
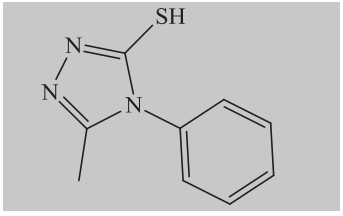
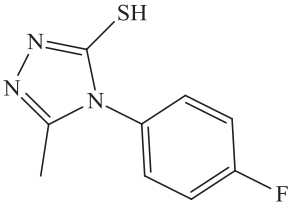
2.5. Docking study

The docking study was performed for **7c**, **7d**, **7f**, **9a** and **13a** as the

Table 1

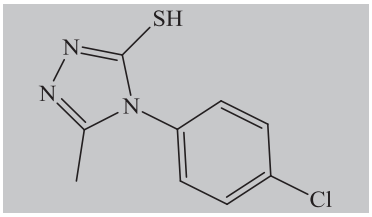
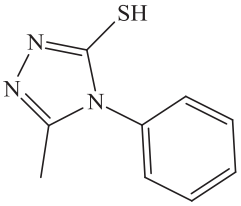
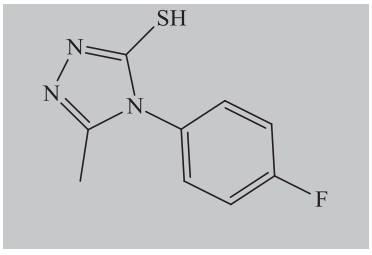
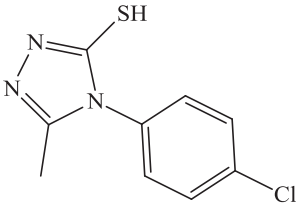
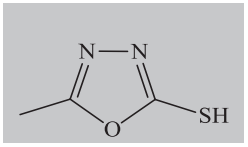
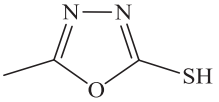
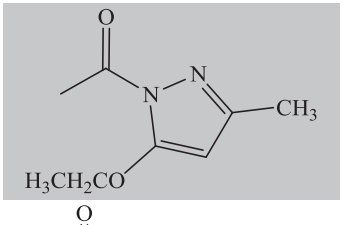
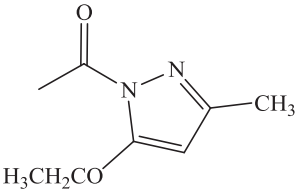
In vitro IC_{50} of COX-1 and COX-2 and selectivity index of the tested compounds, celecoxib, diclofenac sodium and indomethacin.



Compound No	R	R\	COX-1 IC_{50} μM[a]	COX-2 IC_{50} μM[a]	COX-2 S.I.[b]
7a	H		6.51	0.33	19.72
7b	H		9.11	0.19	47.94
7c	H		10.4	0.11	94.54
7d	SO_2CH_3		12.31	0.10	123.1
7e	SO_2CH_3		10.24	0.12	85.33
7f	SO_2CH_3		12.34	0.10	123.40
8a	H		7.35	0.31	23.70
8b	SO_2CH_3		9.52	0.23	41.39
9a	H		13.11	0.10	131.10
9b	H		6.97	0.26	26.80

(*continued on next page*)

Table 1 (*continued*)

Compound No	R	R\	COX-1 IC_{50} μM[a]	COX-2 IC_{50} μM[a]	COX-2 S.I.[b]
9c	H		5.64	0.38	14.84
9d	SO_2CH_3		8.79	0.24	36.62
9e	SO_2CH_3		5.28	0.29	18.20
9f	SO_2CH_3		8.74	0.36	24.27
10a	H		6.78	0.29	23.37
10b	SO_2CH_3		8.24	0.24	34.33
13a	H		11.32	0.10	113.20
13b	SO_2CH_3		9.54	0.16	59.62
Celecoxib		8.1	0.049	165.30	
Diclofenac sodium		3.8	0.84	4.52	
Indomethacin		0.041	0.51	0.080	

[a] IC_{50}: The concentration causing 50% COX inhibition.
[b] S.I.: selectivity index (IC_{50} COX-1/IC_{50} COX-2).

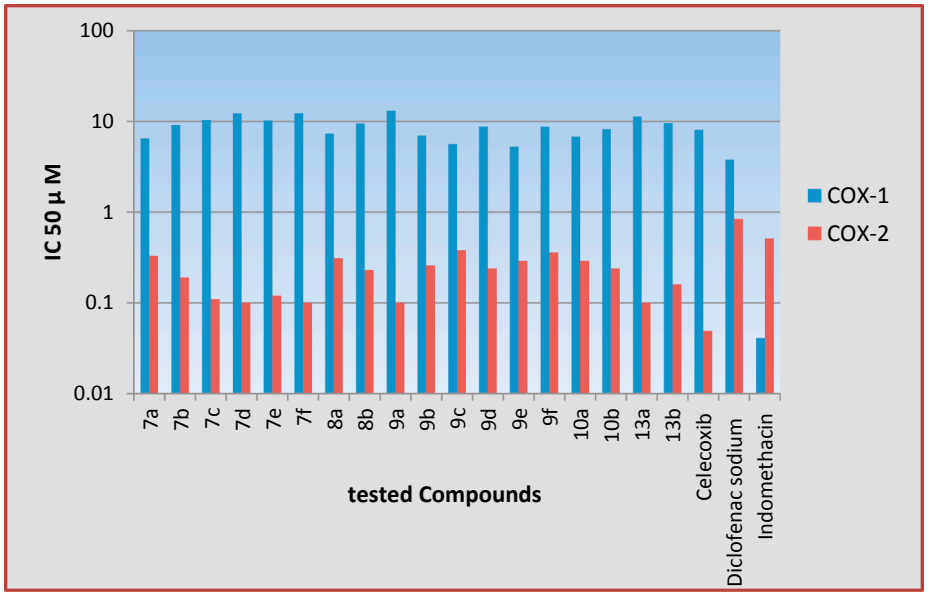


Fig. 4. Graphical representation of IC_{50} values (COX-1 and COX-2) of the tested compounds, celecoxib, diclofenac sodium and indomethacin *in vitro*.

most *in vivo* active compounds in a way to illustrate the possible binding mode of the newly synthesized pyrazolopyrimidines within the binding site of COX-2. The Molecular Operating Environment (MOE) version 2008.10 modeling software was used in this study. The crystal structure of **SC-558** bound at COX-2 active site was deposited in the protein data bank with code (PDB: 1CX2) [36]. It was reported that the presence of an additional side pocket on COX-2 active site increase its volume to accommodate the bulky structures. This pocket allows more interaction with Arg513 that replaced by His513 in COX-1 [37]. It was observed that the ligand **SC-558** (bromocelecoxib) bound to the COX-2 active site by forming two hydrogen bonds (HBs) between (His90 and Arg513) amino acids and $–SO_2CH_3$ group in a distance of 2.35 and 2.47 A°, respectively (Fig. 8). In addition the energy score was −13.39 Kcal/mol.

Regarding the tested compounds, Schiff bases **7c**, **7d**, **7f**, triazole derivative **9a** and pyrazole containing compound **13a** were docked inside the COX-2 active site (Fig. 9, 10, 11, 12 and 13, in the order). All the tested compounds exhibited good selectivity toward COX-2 enzyme by forming 1 – 4 hydrogen bonds with different amino acids, namely, His90, Arg513 (as with SC-558), Arg120, Ser530 and Tyr355 with energy scores between −13.46: −16.71 Kcal/mol. The results of docking were in accordance with that of both *in vivo* anti-inflammatory evaluation and COX-2 inhibitory activity. Thus, the most active compounds **7c** and **7d** showed % of anti-inflammatory activity 4, 81 and 96% in **7c** and 2, 79 and 88% for **7d** if compared to celecoxib (50, 73 and 89%) after 1, 3 and 5 h, sequentially. Moreover, IC_{50} for **7c** and **7d** toward COX-2 was 0.11 and 0.10 µM, respectively, and S.I equal to 94.54 and

Table 2
In vivo anti-inflammatory activity of the tested compounds, celecoxib and indomethacin against carrageenan-induced rat paw edema.

Compound No	Edema (mm) ± SEM			% of anti-inflammatory activity (AI)		
	1 h	3 h	5 h	1 h	3 h	5 h
7a	0.1550[bc] ± 0.01848	0.1950[abc] ± 0.02021	0.1800[abc] ± 0.03894	–	26	37
7b	0.0400[a] ± 0.02517	0.1600[ab] ± 0.04041	0.1133[ab] ± 0.03333	–	39	60
7c	0.1100[c] ± 0. 02,121	0.0500[ac] ± 0.01155	0.0100[ac] ± 0.00577	4	81	96
7d	0.1125[c] ± 00.01931	0.0533[ac] ± 0.00333	0.0333[a] ± 0.00882	2	79	88
7e	0.0875 ± 00.03119	0.0533[ac] ± 0.01202	0.0333[a] ± 0.00333	23	79	88
7f	0.0900 ± 0.01780	0.0300[ac] ± 0.01291	0.0133[ac] ± 0.00667	21	88	95
8a	0.1375[bc] ± 0.02780	0.0933[ac] ± 0.02333	0.0250[a] ± 0.00645	–	64	91
8b	0.1600[bc] ± 0.04708	0.1633[ab] ± 0.02333	0.1533[abc] ± 0.02028	–	38	46
9a	0.0925[c] ± 0.03449	0.0200[ac] ± 0.02000	0.0200[a] ± 0.01225	19	92	93
9b	0.2333[abc] ± 0.02186	0.1133[a] ± 0.02028	0.0667[a] ± 0.00667	–	57	76
9c	0.1150[c] ± 0.02021	0.0800[a] ± 0.00577	0.0600[a] ± 0.00000	–	69	79
9d	0.1875[abc] ± 0.02810	0.0725[ac] ± 0.02496	0.0575[a] ± 0.02016	–	72	80
9e	0.0767 ± 0.02028	0.0933[a] ± 0.03180	0.0767[a] ± 0.01764	33	64	73
9f	0.1967[abc] ± 0.01453	0.1967[abc] ± 0.01333	0.1533[abc] ± 0.00882	–	25	46
10a	0.0467 ± 0.01333	0.0967[a] ± 0.01764	0.0667[a] ± 0.00882	59	63	76
10b	0.0700 ± 0.00913	0.0650[ac] ± 0 0.00957	0.0500[a] ± 0.00816	39	75	82
13a	0.0200[ac] ± 0.01155	0.0150[abc] ± 0.00500	0.0275[a] ± 0.01601	82	94	90
13b	0.0925 ± 0.01493	0.0800[a] ± 0.02000	0.0725[a] ± 0.01601	19	69	74
Celecoxib	0.0575 ± 0.02213	0.0700[a] ± 0.02739	0.0300[a] ± 0.01528	50	73	89
Indomethacin	0.0275[a] ± 0.00750	0.1300[ab] ± 0.01472	0.0733[a] ± 0.02603	76	50	74
control	0.1150 ± 0.01190	0.2650 ± 0.02754	0.2875 ± 0.02839	–	–	–

Values represent the mean ± SEM (n = 4), Significance levels.
[a] Significantly different from control at $P < 0.05$.
[b] Significantly different from celebrex at $P < 0.05$.
[c] Significantly different from indomethacin at $P < 0.05$.

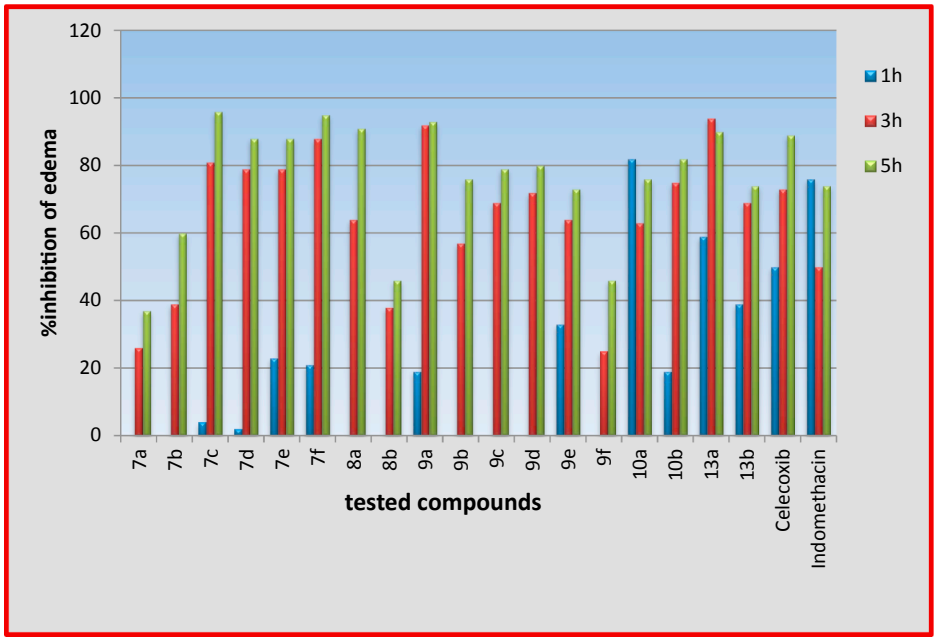


Fig. 5. Graphical representation for % of the anti-inflammatory activity of the tested compounds, celecoxib and indomethacin at 1 h, 3 h and 5 h against carrageenan-induced rat paw edema.

123.1, in the order. Also, the histopathological study confirmed the above results as, both **7c** and **7d** showed more or less normal glandular stomach layers (mucosa, submucosa, and muscolosa) and also in the non-glandular stomach if compared to the reference celecoxib. It is important to observe that His90 and Arg513 exhibited two hydrogen bonds with C=O (Schiff base moiety) in **7c** and $-SO_2CH_3$ group in **7d**, while, additional amino acids Ser530 made an additional hydrogen bond with C=O (Schiff base moiety). The same amino acids His90 and Arg513 were the target amino acids for the ligand **SC-558** inside the COX-2 active site. All the obtained data (amino acids, energy scores and HB lengths) were listed in Table 4.

3. Conclusion

In this research, several anti-inflammatory agents with pyrazolo [3,4-*d*]pyrimidine cores were synthesized. Such choice of large core than regular pyrazole was valid as a proper modification. That most of the prepared compounds showed excellent AI activity. In addition, substitution in position-4 with a variety of active phramacophores exhibited a good activity. Pyrazole **13a** its result was more potent and significant than celecoxib. The long lasting anti-inflammatory activity was observed in Schiff's derivatives especially that bearing SO_2CH_3 pharmacophores. Moreover the triazole derivative showed a good inhibitory activity when compared to celecoxib. For gastric safety, many compounds were safe as celecoxib as obtained from the histopathological study.

4. Experimental

4.1. Chemistry

The Griffin apparatus was used to determine the melting points and were uncorrected. Shimadzu IR-435 spectrophotometer was used for recording IR spectra using KBr discs and the obtained data was

Table 3
Showing scoring of different pathological lesions caused by the tested compounds (**7c-f**, **9a** and **13a**) on both glandular and non-glandular portions of the stomach compared with those induced by celecoixb and indomethasin as standard.

Lesion	7c	7d	7e	7f	9a	13a	Celecoxib	Indomethasin	negative
Glandular stomach									
Mucosa									
Degeneration	+	+	++	++	++	++	+	+++	−/+
Nuclear pyknosis	+	++	+++	++	++	++	+	+++	–
Erosion	+	+	++	++	++	+++	+	+++	–
Ulcer	–	–	++	++	+	++	–	+++	–
Submucosa									
Congestion	+	+	++	++	++	+++	+	+++	–
Leuckocytic infiltration	+/++	++	++	++	++	+++	+	+++	–
Edema	+	++	++/+++	+++	++	+++	+	+++	–
Muscolosa									
Degeneration	+	+	++	++	++	++	+	+++	–
Hyalinosis	+	+	++	++	++	++	+	+++	–
Leuckocytic infiltration	+	+	++	++	++	++	+	+++	–
Non-glandular stomach									
Erosion	–	–	+	+	–	+	–	+	–
Ulcer	–	–	−/+	+	–	−/+	–	−/+	–
Hyperkeratosis	–	+	+++	+++	++	+++	+	+++	−/+

−/+ minimal, +/mild, ++/moderate, +++/severe.

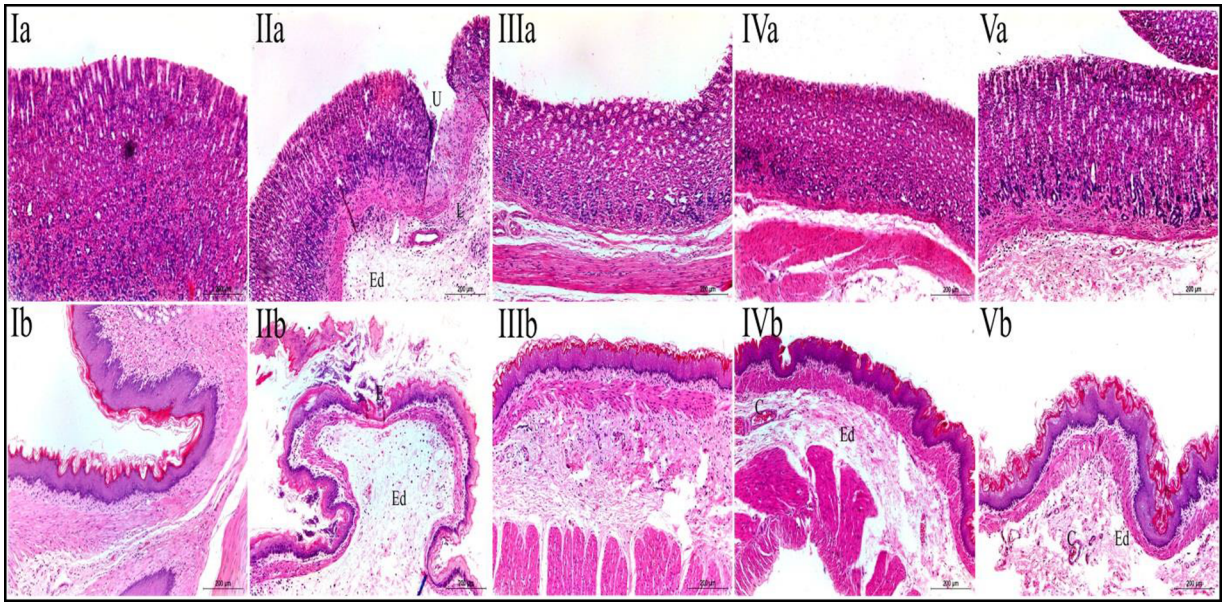


Fig. 6. Histopathological alterations of glandular stomach (1st raw) and non-glandular stomach (2nd raw) in control negative group (Ia, Ib), Indomethacin (IIa, IIb), celecoxib (IIIa, IIIb), compound 7c (IVa, IVb) and compound 7d (Va, Vb).

represented in cm^{-1}. Bruker spectrophotometer was used to carry out ^{1}H NMR spectra (at 400 MHz) and ^{13}C NMR spectra (at 100 MHz) at (Faculty of pharmacy, Beni-Suef University, Egypt). DMSO-d_6 and D_2O with TMS as an internal standard, chemical shift was recorded in ppm on δ scale while the coupling constant (J) values were estimated in Hertz (Hz). Mass spectra (MS) were run on a Hewlett Packard 5988 spectrophotometer (Palo Alto, CA). Microanalysis for C, H, N (within ± 0.4% of the theoretical value), was proceeded at the regional center for Mycology and Biotechnology (Al-Azhar University, Egypt). All reactions were monitored by thin layer chromatography (TLC) using a UV lamp. All other reagents and compound **1** were purchased from Acros Chemical Company.

*4.1.1. General procedure for preparation of **2a** and **2b***

A mixture of ketene dithioacetal **1** (0.9 g, 0.005 mol), appropriate phenylhydrazine hydrochloride (0.005 mol) of each and sodium acetate (0.6 g, 0.005 mol) was dissolved in methanol or ethanol 95%. The reaction mixture was heated under reflux for 4–6 h. After cooling, the formed precipitate was filtered, dried and crystallized from methanol to give crystals of **2a** [34] or **2b**.

*4.1.1.1. 5-Amino-3-(methylthio)-1-(4-methylsulphonylphenyl)-1H-pyrazole-4-carbonitrile (**2b**)*. yellow crystals; 90% yield; mp: 182–184 °C; IR: 3444, 3332 (NH_2), 3008 (aromatic C—H), 2924 (aliphatic C—H), 2214 (C≡N), 1384, 1145 (SO_2); ^{1}H NMR: δ 2.52 (s, 3H, SCH_3), 3.27 (s, 3H, SO_2CH_3), 7.12 (br s, 2H, NH_2, exchange with D_2O), 7.82 (d, J = 8.4 Hz, 2H, phenyl H-3, H-5), 8.07 (d, J = 8.4 Hz, 2H, phenyl H-2, H-6); ^{13}C NMR: δ 13.58 (SCH_3), 43.98 (SO_2CH_3), 74.38 (pyrazole C-4), 114.15(C≡N), 124.53 (phenyl C-2, C-6), 128.97 (phenyl C-3, C-5), 139.63 (phenyl C-4), 141.83 (phenyl C-1), 150.72 (pyrazole C-3), 153.30 (pyrazole C-5); MS (m/z, %): 308 [$(M)^{+\cdot}$, 100%]; Anal.Calcd for $C_{12}H_{12}N_4O_2S_2$: C, 46.74; H, 3.92; N, 18.17.

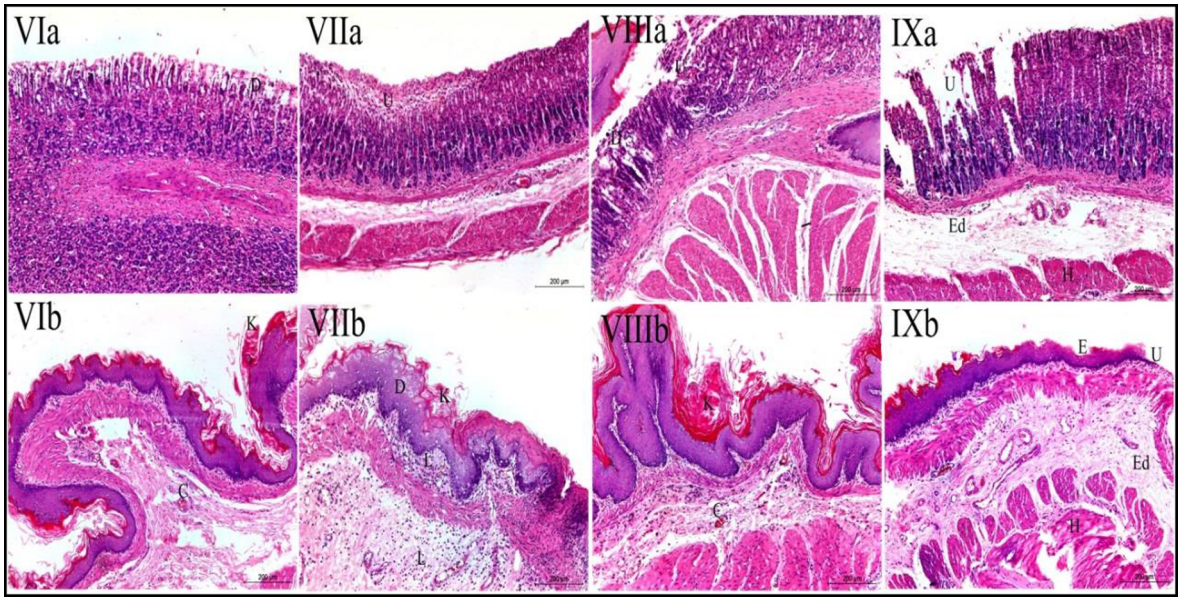


Fig. 7. Histopathological alterations of glandular stomach (1st raw) and non-glandular stomach (2nd raw) in compound 9a (VIa, VIb), compound 7e (VIIa, VIIb), compound 13a (VIIIa, VIIIb) and compound 7f (IXa, IXb). C: congestion, D: degenerative changes, E: erosion, Ed: edema, H: hyalinosis, K: hyperkeratosis, L: leuckocytic infiltration, U: ulcer.

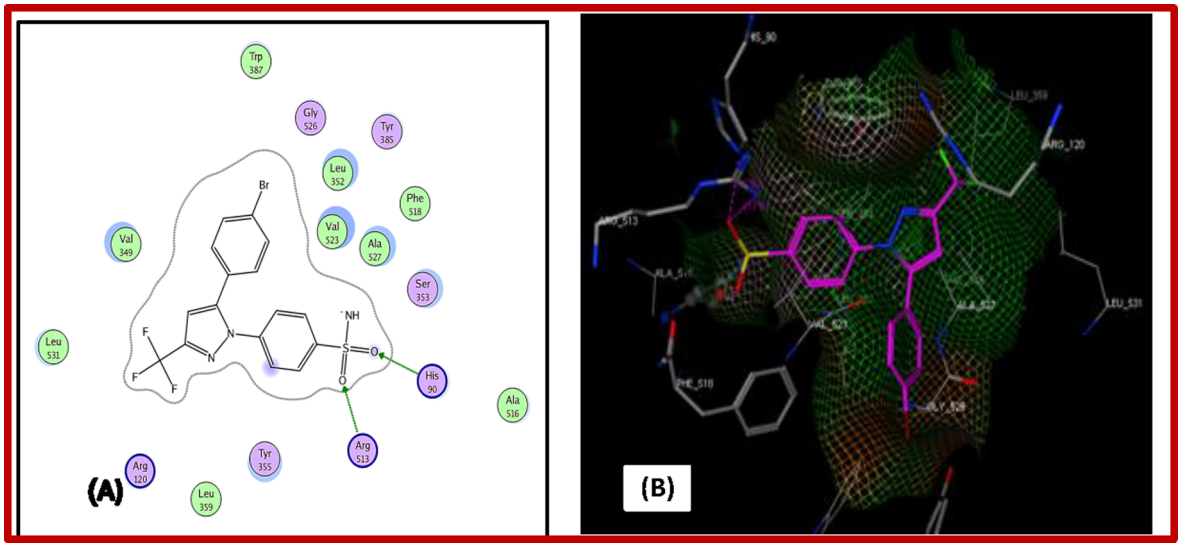


Fig. 8. X-ray crystallographic structure of SC-558 (bromocelecoxib) co-crystallized within COX-2 active site (PDB:1CX2). (A) 2D structure of SC-558, it forms two H-bonds with His90 and Arg513 amino acids; (B) 3D structure of SC-558.

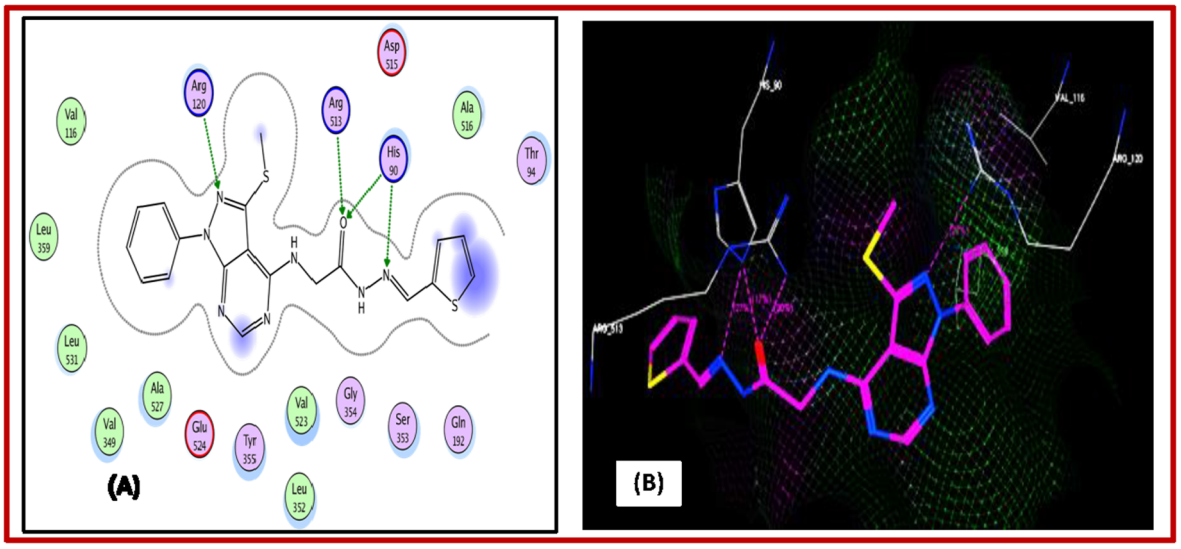


Fig. 9. Binding of the most active compound 7c inside COX-2 active site. (A) 2D interaction of the proposed binding mode of 7c inside the active site of COX-2 resulting from docking, it forms four H-bonds with His90, Arg513 and Arg120 amino acids; (B) 3D interaction of 7c.

Table 4
Molecular modeling data for compounds for **7c, 7f, 9a, 13a** and **SC-558** during docking in the COX-2 receptor.

compound No	E-score Kcal/mol	No of hydrogen bonds	Hydrogen bonding residues	Distance (A°)	Functional group
7c	−14.56	4	His90 Arg513 Arg120 His90	2.56 3.01 2.77 2.98	C=O C=O N3(pyrazole) N (Schiff'sbase)
7d	−15.44	3	His90 Arg513 Ser530	2.46	SO_2CH_3
				3.17	SO_2CH_3
				2.65	C=O
7f	−16.71	2	His90 Tyr355	2.57 3.16	SO_2CH_3 N3(pyrazole)
9a	−14.71	1	Arg513	2.77	N3(pyrazole)
13a	−13.46	3	Tyr355 Arg513 Tyr355	2.37 2.39 2.74	N3(pyrazole) C=O N5(pyrimidine)
SC-558	−13.39	2	Arg513	2.47	SO_2CH_3
			His90	2.35	SO_2CH_3

Found: C, 46.86; H, 4.15; N, 18.43.

*4.1.2. General procedure for preparation of **3a** and **3b***

A solution of the appropriate pyrazole derivative **2a** or **2b** (0.01 mol) of each in formic acid (30 ml, 85%) was heated under reflux for 10 h. The reaction mixture was cooled and poured into ice-cooled water. The separated solid was filtered off, dried and crystallized from ethanol 95% to give crystals of **3a** [35] or **3b**.

*4.1.2.1. 3-Methylthio-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d] pyrimidine-4(5H)-one (**3b**).* White crystals; 74% yield; mp: 312–314 °C; IR: 3333 (NH), 3044 (aromatic C—H), 2924 (aliphatic C—H), 1678 (C=

O), 1369, 1149 (SO_2); ^{1}H NMR: δ 2.78 (s, 3H, SCH_3), 3.26 (s, 3H, SO_2CH_3), 8.07 (d, J = 8.4 Hz, 2H, phenyl H-3, H-5), 8.23 (s, IH, pyrimidine C—H), 8.36 (d, J = 8.4 Hz, 2H, phenyl H-2, H-6), 12.61 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 13.17 (SCH_3), 44.13 (SO_2CH_3), 106.53 (pyrazolopyrimidine C-3a), 121.13 (phenyl C-2, C-6), 128.89 (phenyl C-3, C-5), 138.32 (phenyl C-4), 142.34 (pyrazolopyrimidine C-7a), 147.44 (phenyl C-1), 150.52 (pyrazolopyrimidine C-6), 154.32 (pyrazolopyrimidine C-3), 157.26 (C═O); MS (*m/z*, %): 336 [$(M)^{+\cdot}$, 100%]; Anal.Calcd for $C_{13}H_{12}N_4O_3S_2$: C, 46.42; H, 3.60; N, 16.66. Found: C, 46.76; H, 3.74; N, 16.98.

*4.1.3. General procedure for preparation of **4a** and **4b***

The appropriate pyrazolopyrimidinone derivative **3a** or **3b** (0.24 mol) of each was suspended in phosphorusoxychloride (50 ml). The mixture was heated under reflux for 4 h. The excess phosphorusoxychloride was removed under reduced pressure. The obtained residue was triturated with ice-cold water (250 ml). The aqueous suspension was extracted with chloroform (3 × 60 ml). The solvent was evaporated and the residue obtained was dried and crystallized from methylene chloride/methanol (8:2) to obtain pure crystals of **4a** [35] or **4b**.

*4.1.3.1. 4-Chloro-3-methylthio-1-(4-methylsulphonylphenyl)-1H-pyrazolo [3,4-d]pyrimidine (**4b**)*. Buff crystals; 92% yield; mp: 363–365 °C; IR: 3048 (aromatic C—H), 2924 (aliphatic C—H), 1354, 1145 (SO_2); ^{1}H NMR: δ 2.78 (s, 3H, SCH_3), 3.26 (s, 3H, SO_2CH_3), 8.07 (d, J = 8.4 Hz, 2H, phenyl H-3, H-5), 8.23 (s, IH, pyrimidine C—H), 8.36 (d, J = 8.4 Hz, 2H, phenyl H-2, H-6); ^{13}C NMR: δ 13.17 (SCH_3), 44.13 (SO_2CH_3), 106.53 (pyrazolopyrimidine C-3a), 121.13 (phenyl C-2, C-6), 128.81 (phenyl C-3, C-5), 138.32 (phenyl C-4), 142.34 (pyrazolopyrimidine C-7a), 147.44 (phenyl C-1), 150.52 (pyrazolopyrimidine C-6), 154.32 (pyrazolopyrimidine C-3), 158.36 (pyrazolopyrimidine C-4); MS (*m/z*, %): 355 [$(M)^{+\cdot}$, 17.42%], 348 [100%]; Anal.Calcd for $C_{13}H_{11}Cl\ N_4O_2S_2$: C, 44.00; H, 3.12; N, 15.79. Found: C, 43.89; H, 3.30; N, 16.12.

*4.1.4. General procedure for preparation of **5a** and **5b***

To a mixture of the appropriate chloro-derivative **4a** or **4b** (0.01 mol) of each and glycine ethyl ester hydrochloride (1.39 g, 0.01 mol) in absolute ethanol (30 ml), a catalytic amount of triethylamine was added. The reaction mixture was heated under reflux for 5–6 h. The reaction mixture was evaporated under reduced pressure. The solid obtained was crystallized from ethanol 95% to afford compound **5a** or **5b**.

*4.1.4.1. Ethyl-2-[3-(methylthio)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine-4-ylamino]acetate (**5a**)*. Yellow crystals; 86% yield; mp: 175–177 °C; IR: 3387 (NH), 2928 (aliphatic C—H), 1736 (C═O); ^{1}H NMR: δ 1.22 (t, J = 6.8 Hz, 3H, $OCH_2\underline{CH_3}$), 2.74 (s, 3H, SCH_3), 4.15 (q, J = 6.8 Hz, 2H, $O\underline{CH_2}CH_3$), 4.32 (d, J = 4.8 Hz, 2H, $NHCH_2$), 7.32 (t, J = 4.8 Hz, 1H, NH, exchange with D_2O), 7.35 (t, J = 7.2 Hz, 1H, phenyl H-4), 7.54 (t, J = 7.2 Hz, 2H, phenyl H-3, H-5), 8.17 (d, J = 7.6 Hz, 2H, phenyl H-2, H-6), 8.39 (s, IH, pyrimidine C—H); ^{13}C NMR: δ 14.54 (SCH_3), 15.38 ($CH_2\underline{CH_3}$), 42.87 ($NHCH_2$), 61.10 ($\underline{CH_2}CH_3$), 101.15 (pyrazolopyrimidine C-3a), 120.99 (phenyl C-2, C-6), 126.62 (phenyl C-4), 129.59 (phenyl C-3, C-5), 138.54 (phenyl C-1), 141.55 (pyrazolopyrimidine C-7a), 153.50 (pyrazolopyrimidine C-3), 154.45 (pyrazolopyrimidine C-4), 156.69 (pyrazolopyrimidine C-6), 169.58 (C═O); MS (*m/z*, %): 343 [$(M)^{+\cdot}$, 25.65%], 258 [100%]; Anal.Calcd for $C_{16}H_{17}N_5O_2S$: C, 55.96; H, 4.99; N, 20.39. Found: C, 56.23; H, 5.08; N, 20.61.

*4.1.4.2. Ethyl-2-[3-(methylthio)-1-(4-methylsulphonyphenyl)-1H-pyrazolo [3,4-d]pyrimidine-4-ylamino]acetate (**5b**)*. Buff powder; 85% yield; mp: 365–367 °C; IR: 3422 (NH), 2928 (aliphatic C—H), 1736 (C═O), 1377, 1296 (SO_2); ^{1}H NMR: δ 1.21 (t, J = 7.2 Hz, 3H, $OCH_2\underline{CH_3}$), 2.78 (s, 3H, SCH_3), 3.26 (s, 3H, SO_2CH_3), 4.16 (q, J = 7.2 Hz, 2H, $O\underline{CH_2}CH_3$), 4.31 (d, J = 5.6 Hz, 2H, $NHCH_2$), 7.47 (t, J = 5.6 Hz, 1H, NH, exchange with D_2O), 8.09 (d, J = 8.8 Hz, 2H, phenyl H-3, H-5), 8.46 (s, IH, pyrimidine C—H), 8.52 (d, J = 8.8 Hz, 2H, phenyl H-2, H-6); ^{13}C NMR: δ 14.55 (SCH_3), 14.90 ($CH_2\underline{CH_3}$), 42.93 ($NHCH_2$), 44.18 (SO_2CH_3), 61.15 ($\underline{CH_2}CH_3$), 101.41 (pyrazolopyrimidine C-3a), 120.42 (phenyl C-2, C-6), 128.95 (phenyl C-3, C-5), 137.66 (phenyl C-4), 142.74 (phenyl C-1), 143.64 (pyrazolopyrimidine C-7a), 155.34 (pyrazolopyrimidine C-3), 156.68 (pyrazolopyrimidine C-4), 157.51 (pyrazolopyrimidine C-6), 170.12 (C═O); MS (*m/z*, %): 421 [$(M)^{+\cdot}$, 69.87%], 348 [100%]; Anal.Calcd for $C_{17}H_{19}N_5O_4S_2$: C, 48.44; H, 4.54; N, 16.62. Found: C, 48.67; H, 4.72; N, 16.89.

*4.1.5. General procedure for preparation of **6a** and **6b***

A mixture of compound **5a** or **5b** (0.01 mol) of each and hydrazine hydrate (99.9%) (2.5 ml, 0.05 mol) in absolute ethanol (30 ml) was heated under reflux for 5 h. The solid formed was filtered while hot, dried and crystallized from dioxane to obtain compounds **6a** or **6b**.

*4.1.5.1. 2-[3-(Methylthio)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine-4-ylamino]acetohydrazide (**6a**)*. White powder; 85% yield; mp: 210–212 °C; IR: 3446, 3368 (NH_2), 3318, 3217 (2NH), 3024 (aromatic C—H), 2928 (aliphatic C—H), 1686 (C═O); ^{1}H NMR: δ 2.74 (s, 3H, SCH_3), 4.15 (d, J = 5.6 Hz, 2H, $NHCH_2$), 4.28 (br s, 2H, NH_2, exchange with D_2O), 7.17 (t, J = 5.6 Hz, 1H, $NHCH_2$, exchange with D_2O), 7.34 (t, J = 7.6 Hz, 1H, phenyl H-4), 7.55 (t, J = 7.6 Hz, 2H, phenyl H-3, H-5), 8.16 (d, J = 7.6 Hz, 2H, phenyl H-2, H-6), 8.38 (s, IH, pyrimidine C—H), 9.24 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 15.64 (SCH_3), 42.75 ($NHCH_2$), 101.50 (pyrazolopyrimidine C-3a), 120.93 (phenyl C-2, C-6), 126.57 (phenyl C-4), 129.61 (phenyl C-3, C-5), 139.04 (phenyl C-1), 141.57 (pyrazolopyrimidine C-7a), 154.36 (pyrazolopyrimidine C-3), 156.50 (pyrazolopyrimidine C-4), 157.31 (pyrazolopyrimidine C-6), 168.36 (C═O); MS (*m/z*, %): 329 [$(M)^{+\cdot}$, 99.92%], 298 [100%]; Anal.Calcd for $C_{14}H_{15}N_7OS$: C, 51.05; H, 4.59; N, 29.77. Found: C, 51.32; H, 4.32; N, 29.85.

*4.1.5.2. 2-[3-(Methylthio)-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidine-4-ylamino]acetohydrazide (**6b**)*. White powder; 85% yield; mp: 220–222 °C; IR: 3446, 3368 (NH_2), 3318, 3217 (2NH), 3024(aromatic C—H), 2928 (aliphatic C—H), 1686 (C═O), 1380, 1142 (SO_2); ^{1}H NMR: δ 2.77 (s, 3H, SCH_3), 3.26 (s, 3H, SO_2CH_3), 4.16 (s, 2H, $NH\underline{CH_2}$), 4.37 (br s, 2H, NH_2, exchange with D_2O), 7.20 (br s, 1H, $NHCH_2$, exchange with D_2O), 8.10 (d, J = 6.6 Hz, 2H, phenyl H-3, H-5), 8.44 (s, IH, pyrimidine C—H), 8.54 (d, J = 6.6 Hz, 2H, phenyl H-2, H-6), 9.24 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 14.50 (SCH_3), 42.92 ($NHCH_2$), 44.20 (SO_2CH_3), 101.65 (pyrazolopyrimidine C-3a), 120.25 (phenyl C-2, C-6), 128.88 (phenyl C-3, C-5), 137.50 (phenyl C-4), 142.77 (phenyl C-1), 143.66 (pyrazolopyrimidine C-7a), 155.14 (pyrazolopyrimidine C-3), 156.62 (pyrazolopyrimidine C-4), 157.55 (pyrazolopyrimidine C-6), 168.31 (C═O); MS (*m/z*, %): 407 [$(M)^{+\cdot}$, 9.26%], 348 [100%]; Anal.Calcd for $C_{15}H_{17}N_7O_3S_2$: C, 44.21; H, 4.21; N, 24.06. Found: C, 44.49; H, 4.30; N, 24.34.

*4.1.6. General procedure for preparation of **7a-f***

A mixture of acid hydrazide derivative **6a** or **6b** (0.005 mol) of each and the appropriate aromatic aldehyde (0.005 mol) in absolute ethanol (30 ml) containing a catalytic amount of glacial acetic acid (0.2 ml) was heated under reflux for 3 h. The solid precipitated on hot was filtered off, dried and crystallized from acetic acid 96% to give compounds **7a-f**.

*4.1.6.1. N′-(furyl-2-ylmethylene)-2-[3-(Methylthio)-1-phenyl-1H-pyrazolo [3,4-d]pyrimidine-4-ylamino]acetohydrazide (**7a**)*. white powder; 79% yield; mp: 233–235 °C; IR: 3441, 3362 (2NH), 3047 (aromatic C—H), 2934 (aliphatic C—H), 1682 (C═O); ^{1}H NMR: δ 2.76 (s, 3H, SCH_3), 4.65 (s, 2H, $NHCH_2$), 6.64 (br s, 1H, $NHCH_2$, exchange with D_2O), 6.93 (t,

J = 3.6 Hz, 1H, furyl H-4), 7.19 (d, J = 3.6 Hz, 1H, furyl H-3), 7.35 (t, J = 6.8 Hz, 1H, phenyl H-4), 7.55 (t, J = 6.8 Hz, 2H, phenyl H-3, H-5), 7.85 (s, IH, pyrimidine C—H), 7.94 (d, J = 3.6 Hz, 1H, furyl H-5), 8.19 (d, J = 7.2 Hz, 2H, phenyl H-2, H-6), 8.41 (s, 1H, N = CH azomethine), 11.65 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 15.49 (SCH_3), 42.65 ($NHCH_2$), 101.26 (pyrazolopyrimidine C-3a), 112.63 (furyl C-4), 114.36 (furyl C-3), 120.92 (phenyl C-2, C-6), 126.58 (phenyl C-4), 129.59 (phenyl C-3, C-5), 137.15 (N = CH azomethine), 139.00 (phenyl C-1), 141.43 (pyrazolopyrimidine C-7a), 145.56 (furyl C-5), 149.40 (furyl C-2), 154.34 (pyrazolopyrimidine C-3), 156.67 (pyrazolopyrimidine C-4), 157.35 (pyrazolopyrimidine C-6), 170.22 (C═O); MS (*m/z*, %): 407 [$(M)^{+\cdot}$, 28.37%], 298 [100%]; Anal.Calcd for $C_{19}H_{17}N_7O_2S$: C, 56.01; H, 4.21; N, 24.06. Found: C, 56.43; H, 4.32; N, 24.38.

*4.1.6.2. N′-(pyridyl-4-ylmethylene)-2-[3-(Methylthio)-1-phenyl-1H-pyrazolo [3,4-d]pyrimidine-4-ylamino]acetohydrazide (**7b**)*. White powder; 85% yield; mp: 234–236 °C; IR: 3358, 3239 (2NH), 3048 (aromatic C—H), 2941 (aliphatic C—H), 1702 (C═O); ^1H NMR: δ 2.76 (s, 3H, SCH_3), 4.76 (d, J = 5.2 Hz, 2H, $NHCH_2$), 7.25 (t, J = 5.2 Hz, 1H, $NHCH_2$, exchange with D_2O), 7.35 (t, J = 7.6 Hz, 1H, phenyl H-4), 7.56 (t, J = 7.6 Hz, 2H, phenyl H-3, H-5), 7.69 (d, J = 5.2 Hz, 2H, pyridyl H-3, H-5), 8.04 (s, IH, pyrimidine C—H), 8.19 (d, J = 8 Hz, 2H, phenyl H-2, H-6), 8.41 (s, 1H, N = CH azomethine), 8.67 (d, J = 5.2 Hz, 2H, pyridyl H-2, H-6), 11.94 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 15.54 (SCH_3), 42.67 ($NHCH_2$), 101.26 (pyrazolopyrimidine C-3a), 121.10 (phenyl C-2, C-6), 121.32 (pyridyl C-3, C-5), 126.74 (phenyl C-4), 129.67 (phenyl C-3, C-5), 138.96 (phenyl C-1), 141.57 (pyridyl C-4), 142.09 (N = CH azomethine), 150.72 (pyridyl C-2, C6) 151.31 (pyrazolopyrimidine C-7a), 154.43 (pyrazolopyrimidine C-3), 156.93 (pyrazolopyrimidine C-4), 157.39 (pyrazolopyrimidine C-6), 170.22 (C═O); MS (*m/z*, %): 418 [$(M)^{+\cdot}$, 100%]; Anal.Calcd for $C_{20}H_{18}N_8OS$: C, 57.40; H, 4.34; N, 26.78. Found: C, 57.78; H, 4.41; N, 26.96.

*4.1.6.3. N′-(thienyl-2-ylmethylene)-2-[3-(Methylthio)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine-4-ylamino]acetohydrazide (**7c**)*. Yellow powder; 85% yield; mp: 235–237 °C; IR : 3457, 3368 (2NH), 3080 (aromatic C—H), 2930 (aliphatic C—H), 1679 (C═O); ^1H NMR: δ 2.76 (s, 3H, SCH_3), 4.65 (d, J = 5.2 Hz, 2H, $NHCH_2$), 7.15 (t, J = 3.6 Hz, 1H, thienyl H-4), 7.20 (t, J = 5.2 Hz, 1H, $NHCH_2$, exchange with D_2O), 7.35 (t, J = 7.6 Hz, 1H, phenyl H-4), 7.46 (d, J = 3.6 Hz, 1H, thienyl H-3), 7.56 (t, J = 7.6 Hz, 2H, phenyl H-3, H-5), 7.66 (d, J = 3.6 Hz, 1H, thienyl H-5), 8.19 (d, J = 8 Hz, 2H, phenyl H-2, H-6), 8.23 (s, IH, pyrimidine C—H), 8.42 (s, 1H, N = CH azomethine), 11.66 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 15.56 (SCH_3), 42.50 ($NHCH_2$), 102.26 (pyrazolopyrimidine C-3a), 121.01 (phenyl C-2, C-6), 126.64 (N = CH azomethine), 128.44 (phenyl C-4), 129.07 (thienyl C-4), 129.64 (thienyl C-5), 131.15 (phenyl C-3, C-5), 139.05 (phenyl C-1), 139.66 (thienyl C-3), 141.55 (thienyl C − 2), 148.67 (pyrazolopyrimidine C-7a), 154.46 (pyrazolopyrimidine C-3), 156.85 (pyrazolopyrimidine C-4), 158.21 (pyrazolopyrimidine C-6), 170.01 (C═O); MS (*m/z*, %): 423 [$(M)^{+\cdot}$, 22.98%], 298 [100%]; Anal.Calcd for $C_{19}H_{17}N_7OS_2$: C, 53.88; H, 4.05; N, 23.15. Found: C, 53.94; H, 3.98; N, 22.82.

*4.1.6.4. N′-(furyl-2-ylmethylene)-2-[3-(Methylthio)-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidine-4-ylamino] acetohydrazide (**7d**)*. Buff powder; 73% yield; mp: 259–261 °C; IR : 3458, 3374 (2NH), 3119 (aromatic C—H), 2926 (aliphatic C—H), 1684 (C═O), 1294, 1142 (SO_2); ^1H NMR: δ 2.75 (s, 3H, SCH_3), 3.26 (s, 3H, SO_2CH_3), 4.65 (d, J = 5.2 Hz, 2H, $NHCH_2$), 6.64 (t, J = 3.6 Hz, 1H, furyl H-4), 7.94 (d, J = 3.6 Hz, 1H, furyl H-3), 7.23 (t, J = 5.2 Hz, 1H, NH, exchange with D_2O), 7.85 (d, J = 3.6 Hz, 1H, furyl H-5), 7.93 (s, IH, pyrimidine C—H), 8.11 (d, J = 8.8 Hz, 2H, phenyl H-3, H-5), 8.48 (s, 1H, N = CH azomethine), 8.56 (d, J = 8.8 Hz, 2H, phenyl H-2, H-6), 11.64 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 15.10 (SCH_3), 42.72 ($NHCH_2$), 44.22 (SO_2CH_3), 101.60 (pyrazolopyrimidine C-3a), 112.63 (furyl C-4), 114.32 (furyl C-3), 120.39 (phenyl C-2, C-6), 128.99 (phenyl C-3, C-5), 134.62 (N = CH azomethine), 137.75 (phenyl C-4), 142.82 (phenyl C-1), 143.56 (pyrazolopyrimidine C-7a), 145.60 (furyl C-5), 149.45 (furyl C-2), 155.34 (pyrazolopyrimidine C-3), 156.79 (pyrazolopyrimidine C-4), 157.80 (pyrazolopyrimidine C-6), 170.11 (C═O); MS (*m/z*, %): 485 [$(M)^{+\cdot}$, 7.78%], 335 [100%]; Anal.Calcd for $C_{20}H_{19}N_7O_4S_2$: C, 49.47; H, 3.94; N, 20.19. Found: C, 49.19; H, 4.01; N, 20.52.

*4.1.6.5. N′-(pyridyl-4-ylmethylene)-2-[3-(Methylthio)-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidine-4-ylamino] acetohydrazide (**7e**)*. White powder; 82% yield; mp: 268–270 °C; IR : 3446, 3373 (2NH), 2932 (aromatic C—H), 2781 (aliphatic C—H), 1693 (C═O), 1397, 1146 (SO_2); ^1H NMR: δ 2.81 (s, 3H, SCH_3), 3.26 (s, 3H, SO_2CH_3), 4.76 (d, J = 4.8 Hz, 2H, $NHCH_2$), 7.21 (t, J = 4.8 Hz, 1H, $NHCH_2$, exchange with D_2O), 7.69 (d, J = 5.2 Hz, 2H, pyridyl H-3, H-5), 8.04 (s, IH, pyrimidine C—H), 8.12 (d, J = 8.4 Hz, 2H, phenyl H-3, H-5), 8.48 (s, 1H, N = CH azomethine), 8.56 (d, J = 8.4 Hz, 2H, phenyl H-2, H-6), 8.67 (d, J = 5.2 Hz, 2H, pyridyl H-2, H-6), 11.94 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 15.07 (SCH_3), 42.74 ($NHCH_2$), 44.20 (SO_2CH_3), 101.60 (pyrazolopyrimidine C-3a), 120.46 (pyridyl C-3, C-5), 121.31 (phenyl C-2, C-6), 128.98 (phenyl C-3, C-5), 137.72 (phenyl C-4), 141.53 (pyridyl C-4), 142.10 (N = CH azomethine), 143.63 (phenyl C-1), 148.01 (pyrazolopyrimidine C-7a), 150.75 (pyridyl C-2, C6), 155.34 (pyrazolopyrimidine C-3), 156.92 (pyrazolopyrimidine C-4), 157.82 (pyrazolopyrimidine C-6), 170.01 (C═O); MS (*m/z*, %): 496 [$(M)^{+\cdot}$, 5.18%], 376 [100%]; Anal.Calcd for $C_{21}H_{20}N_8O_3S_2$: C, 50.79; H, 4.06; N, 22.57. Found: C, 50.91; H, 4.09; N, 22.81.

*4.1.6.6. N′-(thienyl-2-ylmethylene)-2-[3-(Methylthio)-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidine-4-ylamino] acetohydrazide (**7f**)*. Yellow powder; 85% yield; mp: 245–247 °C; IR : 3374, 3250 (2NH), 3119 (aromatic C—H), 2926 (aliphatic C—H), 1682 (C═O), 1300, 1149 (SO_2); ^1H NMR: δ 2.80 (s, 3H, SCH_3), 3.26 (s, 3H, SO_2CH_3), 4.63 (d, J = 5.2 Hz, 2H, $NHCH_2$), 7.14 (t, J = 4.8 Hz, 1H, thienyl H-4), 7.22 (t, J = 5.2 Hz, 1H, $NHCH_2$, exchange with D_2O), 7.46 (d, J = 3.2 Hz, 1H, thienyl H-3), 7.66 (d, J = 4.8 Hz, 1H, thienyl H-5), 8.11 (d, J = 8.8 Hz, 2H, phenyl H-3, H-5), 8.23 (s, IH, pyrimidine C—H), 8.48 (s, 1H, N = CH azomethine), 8.53 (d, J = 8.8 Hz, 2H, phenyl H-2, H-6), 11.66 (br s, 1H, NH, exchange with D_2O); ^{13}C NMR: δ 15.08 (SCH_3), 42.56 ($NHCH_2$), 44.21 (SO_2CH_3), 101.58 (pyrazolopyrimidine C-3a), 120.37 (phenyl C-2, C-6), 128.44 (thienyl C-4), 129.08 (phenyl C-3, C-5), 131.15 (thienyl C-5), 137.71 (phenyl C-4), 139.09 (thienyl C-2), 139.70 (thienyl C-3), 142.55 (N = CH azomethine), 142.81 (phenyl C-1), 143.57 (pyrazolopyrimidine C-7a), 155.32 (pyrazolopyrimidine C-3), 156.81 (pyrazolopyrimidine C-4), 157.80 (pyrazolopyrimidine C-6), 169.90 (C═O); MS (*m/z*, %): 501 [$(M)^{+\cdot}$, 0.76%], 376 [100%]; Anal.Calcd for $C_{20}H_{19}N_7O_3S_3$: C, 47.89; H, 3.82; N, 19.55. Found: C, 47.81; H, 3.73; N, 19.38.

*4.1.7. General procedure for preparation of **8a** and **8b**.*

A mixture of acid hydrazide derivative **6a** or **6b** (0.003 mol) of each and ethyl isothiocyanate (0.26 g, 0.003 mol) in absolute ethanol (30 ml) containing a catalytic amount of TEA (3dps) was heated under reflux for 3 h. The precipitate formed on hot was filtered off, dried and crystallized from ethanol 95% to give compounds **8a** or **8b**.

*4.1.7.1. 4-Ethyl-5-{[3-methylthio-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4H-[1,2,4]triazole-3-thiol (**8a**)*. White powder; 75% yield; mp: 256–258 °C; IR : 3350 (NH), 3106 (aromatic C—H), 2935 (aliphatic C—H), 2347 (SH); ^1H NMR: δ 1.25 (t, J = 7.2 Hz, 3H, CH_2CH_3), 2.74 (s, 3H, SCH_3), 4.10 (q, J = 7.2 Hz, 2H, CH_2CH_3), 4.90 (d, J = 5.2 Hz, 2H, $NHCH_2$), 7.35 (t, J = 6.8 Hz, 1H, NH, exchange with D_2O), 7.53–7.57 (m, 3H, phenyl H-3, H-4, H-5), 8.17 (d, J = 8 Hz, 2H,

phenyl H-2, H-6), 8.41 (s, IH, pyrimidine C—H), 13.57 (s, 1H, triazole SH); ^{13}C NMR: δ 13.81 (SCH_3), 15.36 ($CH_2\underline{C}H_3$), 36.34 ($\underline{C}H_2CH_3$), 38.79 ($NHCH_2$), 101.29 (pyrazolopyrimidine C-3a), 121.09 (phenyl C-2, C-6), 128.70 (phenyl C-4), 129.63 (phenyl C-3, C-5), 138.95 (phenyl C-1), 141.58 (pyrazolopyrimidine C-7a), 150.14 (pyrazolopyrimidine C-3), 154.54 (triazole C-5), 156.69 (pyrazolopyrimidine C-4), 157.17 (pyrazolopyrimidine C-6), 166.96 (triazole C-3); MS (*m/z*, %): 398 [$(M)^{+\cdot}$, 100%]; Anal.Calcd for $C_{17}H_{18}N_8S_2$: C, 51.24; H, 4.55; N, 28.12. Found: C, 51.55; H, 4.49; N, 28.41.

4.1.7.2. 4-Ethyl-5-{[3-methylthio-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4H-[1,2,4]triazole-3-thiol ***(8b)***. Buff powder; 64% yield; mp: 335–337 °C; IR : 3350 (NH), 3106 (aromatic C—H), 2935 (aliphatic C—H), 2630 (SH), 1300, 1145 (SO_2); ^{1}H NMR: δ 1.25 (t, J = 6.8 Hz, 3H, $CH_2\underline{CH_3}$), 2.77 (s, 3H, SCH_3), 3.25 (s, 3H, SO_2CH_3), 4.09 (q, J = 6.8 Hz, 2H, $\underline{CH_2}CH_3$), 4.88 (d, J = 4.8 Hz, 2H, $NHCH_2$), 7.63 (t, J = 4.8 Hz, 1H, NH, exchange with D_2O), 8.10 (d, J = 8.4 Hz, 2H, phenyl H-2, H-6), 8.46 (s, IH, pyrimidine C—H), 8.52 (d, J = 8.4 Hz, 2H, phenyl H-3, H-5), 13.59 (s, IH, triazole SH); ^{13}C NMR: δ 13.81 (SCH_3), 14.93 ($CH_2\underline{C}H_3$), 36.41 ($\underline{C}H_2CH_3$), 38.79 ($NHCH_2$), 44.21 (SO_2CH_3), 101.59 (pyrazolopyrimidine C-3a), 120.42 (phenyl C-2, C-6), 128.94 (phenyl C-3, C-5), 137.75 (phenyl C-4), 142.71 (phenyl C-1), 143.61 (pyrazolopyrimidine C-7a), 150.02 (pyrazolopyrimidine C-3), 155.40 (triazole C-5), 156.60 (pyrazolopyrimidine C-4), 157.50 (pyrazolopyrimidine C-6), 166.98 (triazole C-3); MS (*m/z*, %): 477 [$(M)^{+\cdot}$, 1.25%], 368 [100%]; Anal.Calcd for $C_{18}H_{20}N_8O_2S_3$: C, 45.36; H, 4.23; N, 23.51. Found: C, 45.62; H, 4.39; N, 23.79.

4.1.8. General procedure for preparation of ***9a-f***.

A mixture of acid hydrazide derivative **6a** or **6b** (0.003 mol) of each and the appropriate phenyl isothiocyanate derivatives (0.003 mol) in absolute ethanol (30 ml) containing a catalytic amount of TEA (3dps) was heated under reflux for 3 h. The solid separated on hot was filtered off, dried and crystallized from ethanol 95% to give compounds ***9a-f***.

4.1.8.1. 5-{[3-Methylthio-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4-phenyl-4H-[1,2,4]triazole-3-thiol ***(9a)***. White powder; 75% yield; mp: 262–264 °C; IR : 3332 (NH), 3090 (aromatic C—H), 2924 (aliphatic C—H), 2347 (SH); ^{1}H NMR: δ 2.71 (s, 3H, SCH_3), 4.65 (d, J = 5.6 Hz, 2H, $NH\underline{CH_2}$), 7.31 (t, J = 5.6 Hz, 1H, NH, exchange with D_2O), 7.34 (t, J = 7.6 Hz, 1H, phenyl H-4), 7.47–7.55 (m, 7H, pyrazolpyrimidine phenyl H-2, H-3, H-4, H-5, H-6 & phenyl H-3, H-5), 8.15 (d, J = 8 Hz, 2H, phenyl H-2, H-6), 8.33 (s, IH, pyrimidine C—H), 13.81 (s, 1H, triazole SH); ^{13}C NMR: δ 15.49 (SCH_3), 36.69 ($NHCH_2$), 101.24 (pyrazolopyrimidine C-3a), 121.10 (pyrazolopyrimidine phenyl C-2, C-6), 126.73 (pyrazolopyrimidine phenyl C-4), 128.56 (pyrazolopyrimidine phenyl C-3, C-5), 129.64 (phenyl C-3, C-5), 129.82 (phenyl C-4), 129.92 (phenyl C-2, C-6), 133.82 (pyrazolopyrimidine phenyl C-1), 138.91 (phenyl C-1), 141.51 (pyrazolopyrimidine C-7a), 150.26 (pyrazolopyrimidine C-3), 154.36 (triazole C-5), 156.36 (pyrazolopyrimidine C-4), 157.01 (pyrazolopyrimidine C-6), 168.46 (triazole C-3); MS (*m/z*, %): 446 [$(M)^{+\cdot}$, 100%]; Anal. Calcd for $C_{21}H_{18}N_8S_2$: C, 56.48; H, 4.06; N, 25.09. Found: C, 56.54; H, 3.95; N, 24.97.

4.1.8.2. 5-{[3-Methylthio-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4-(4-florophenyl)-4H-[1,2,4]triazole-3-thiol ***(9b)***. Buff powder; 75% yield; mp: 260–262 °C; IR : 3332 (NH), 3090 (aromatic C—H), 2924 (aliphatic C—H), 2367 (SH); ^{1}H NMR: δ 2.74 (s, 3H, SCH_3), 4.66 (s, 2H, $NH\underline{CH_2}$), 7.33–7.38 (m, 4H, florophenyl H-3, H-5, pyrazolopyrimidine phenyl H-4 & NH (exchange with D_2O)), 7.54–7.59 (m, 4H, florophenyl H-2, H-6 & pyrazolpyrimidine phenyl H-3, H-5), 8.14 (d, J = 8 Hz, 2H, pyrazolopyrimidine phenyl H-2, H-6), 8.31 (s, IH, pyrimidine C—H), 13.80 (s, 1H, triazole SH); ^{13}C NMR: δ 15.43 (SCH_3), 36.62 ($NHCH_2$), 101.19 (pyrazolopyrimidine C-3a), 116.57 (florophenyl C-3, C-5), 121.05 (pyrazolopyrimidine phenyl C-2, C-6), 126.66 (pyrazolopyrimidine phenyl C-4), 129.60 (pyrazolopyrimidine phenyl C-3, C-5), 130.98 (florophenyl C-2, C-6), 138.94 (pyrazolopyrimidine phenyl C-1), 141.48 (florophenyl C-1), 150.33 (pyrazolopyrimidine C-7a), 154.35 (pyrazolopyrimidine C-3), 156.28 (triazole C-5), 156.94 (pyrazolopyrimidine C-6), 161.41 (pyrazolopyrimidine C-4), 163.86 (florophenyl C-4), 168.60 (triazole C-3); MS (*m/z*, %): 464 [$(M)^{+\cdot}$, 9.91%], 433 [100%]; Anal.Calcd for $C_{21}H_{17}FN_8S_2$: C, 54.30; H, 3.69; N, 24.12. Found: C, 54.62; H, 3.84; N, 24.37.

4.1.8.3. 5-{[3-Methylthio-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4-(4-chlorophenyl)-4H-[1,2,4]triazole-3-thiol ***(9c)***. White powder; 75% yield; mp: 265–267 °C; IR : 3375 (NH), 3063 (aromatic C—H), 2928 (aliphatic C—H), 2376 (SH); ^{1}H NMR: δ 2.71 (s, 3H, SCH_3), 4.69 (d, J = 4.8 Hz, 2H, $NH\underline{CH_2}$), 7.30 (br s, 1H, NH, exchange with D_2O), 7.34 (t, J = 8.4 Hz, 1H, pyrazolpyrimidine phenyl H-4), 7.47–7.51 (m, 4H, pyrazolopyrimidine phenyl H-2, H-3, H-5, H-6), 7.56 (d, J = 8 Hz, 2H, chlorophenyl H-3, H-5), 8.14 (d, J = 8 Hz, 2H, chlorophenyl H-2, H-6), 8.31 (s, IH, pyrimidine C—H), 13.87 (s, 1H, triazole SH); ^{13}C NMR: δ 15.38 (SCH_3), 36.59 ($NHCH_2$), 101.13 (pyrazolopyrimidine C-3a), 121.05 (pyrazolopyrimidine phenyl C-2, C-6), 126.64 (pyrazolopyrimidine phenyl C-4), 129.60 (chlorophenyl C-2, C-6), 129.76 (chlorophenyl C-3, C-5), 130.56 (pyrazolopyrimidine phenyl C-3, C-5), 132.79 (chlorophenyl C-4), 134.54 (pyrazolopyrimidine phenyl C-1), 138.95 (chlorophenyl C-1), 141.49 (pyrazolopyrimidine C-7a), 150.25 (pyrazolopyrimidine C-3), 154.32 (triazole C-5), 156.20 (pyrazolopyrimidine C-4), 156.90 (pyrazolopyrimidine C-6), 168.37 (triazole C-3); MS (*m/z*, %): 480 [$(M)^{+\cdot}$, 100%]; Anal. Calcd for $C_{21}H_{17}ClN_8S_2$: C, 52.44; H, 3.56; N, 23.30. Found: C, 52.78; H, 3.80; N, 23.13.

4.1.8.4. 5-{[3-Methylthio-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4-phenyl-4H-[1,2,4]triazole-3-thiol ***(9d)***. White powder; 68% yield; mp: 275–277 °C; IR : 3386 (NH), 3116 (aromatic C—H), 2933 (aliphatic C—H), 2349 (SH), 1295, 1146 (SO_2); ^{1}H NMR: δ 2.75 (s, 3H, SCH_3), 3.25 (s, 3H, SO_2CH_3), 4.65 (d, J = 5.6 Hz, 2H, $NH\underline{CH_2}$), 7.39 (t, J = 5.6 Hz, 1H, NH, exchange with D_2O), 7.46–7.53 (m, 5H, phenyl-H), 8.08 (d, J = 8.8 Hz, 2H, pyrazolopyrimidine phenyl H-3, H-5), 8.39 (s, IH, pyrimidine C—H), 8.50 (d, J = 8.8 Hz, 2H, pyrazolopyrimidine phenyl H-2, H-6), 13.85 (s, 1H, triazole SH); ^{13}C NMR: δ 15.03 (SCH_3), 36.74 ($NHCH_2$), 44.20 (SO_2CH_3), 101.54 (pyrazolopyrimidine C-3a), 120.47 (pyrazolopyrimidine phenyl C-2, C-6), 128.58 (phenyl C-4), 128.97 (phenyl C-3, C-5), 129.81 (pyrazolopyrimidine phenyl C-3, C-5), 129.89 (phenyl C-2, C-6), 133.83 (pyrazolopyrimidine phenyl C-4), 137.77 (pyrazolopyrimidine phenyl C-1), 142.72 (phenyl C-1), 143.59 (pyrazolopyrimidine C-7a), 150.17 (pyrazolopyrimidine C-3), 155.26 (triazole C-5), 156.34 (pyrazolopyrimidine C-4), 157.37 (pyrazolopyrimidine C-6), 168.49 (triazole C-3); MS (*m/z*, %): 524 [$(M)^{+\cdot}$, 2.03%], 335 [100%]; Anal.Calcd for $C_{22}H_{20}N_8O_2S_3$: C, 50.36; H, 3.84; N, 21.36. Found: C, 50.07; H, 3.91; N, 21.44.

4.1.8.5. 5-{[3-Methylthio-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4-(4-florophenyl)-4H-[1,2,4]triazole-3-thiol ***(9e)***. Buff powder; 65% yield; mp: 280–282 °C; IR : 3386 (NH), 3116 (aromatic C—H), 2933 (aliphatic C—H), 2380 (SH), 1288, 1149 (SO_2); ^{1}H NMR: δ 2.72 (s, 3H, SCH_3), 3.25 (s, 3H, SO_2CH_3), 4.65 (d, J = 5.2 Hz, 2H, $NH\underline{CH_2}$), 7.29–7.36 (m, 3H, florophenyl H-3, H-5 & NH (exchange with D_2O)), 7.50, 7.51 (dd, J = 4.8 Hz, J = 8.4 Hz, 2H, florophenyl H-2, H-6), 8.08 (d, J = 8.8 Hz, 2H, pyrazolopyrimidine phenyl H-3, H-5), 8.34 (s, IH, pyrimidine C—H), 8.48 (d, J = 8.8 Hz, 2H, pyrazolopyrimidine phenyl H-2, H-6), 13.86 (s, 1H, triazole SH); ^{13}C NMR: δ 14.92 (SCH_3), 36.64 ($NHCH_2$), 44.19 (SO_2CH_3), 101.43 (pyrazolopyrimidine C-3a), 116.81 (florophenyl C-3, C-5), 120.35 (pyrazolopyrimidine phenyl C-2, C-6), 128.91 (pyrazolopyrimidine phenyl C-3, C-5), 130.96 (florophenyl C-2, C-6), 137.65

(pyrazolopyrimidine phenyl C-4), 142.68 (florophenyl C-1), 143.56 (pyrazolopyrimidine phenyl C-1), 150.24 (pyrazolopyrimidine C-7a), 155.17 (pyrazolopyrimidine C-3), 156.21 (triazole C-5), 157.25 (pyrazolopyrimidine C-6), 161.40 (pyrazolopyrimidine C-4), 163.86 (florophenyl C-4), 168.57 (triazole C-3); MS (*m/z*, %): 542 [$(M)^{+\cdot}$, 4.58%], 335 [100%]; Anal.Calcd for $C_{22}H_{19}FN_8O_2S_3$: C, 48.70; H, 3.53; N, 20.65. Found: C, 48.97; H, 3.65; N, 20.88.

*4.1.8.6. 5-{[3-Methylthio-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-4-(4-chlorophenyl)-4H-[1,2,4]triazole-3-thiol (**9f**)*. White powder; 65% yield; mp: 282–284 °C; IR : 3368 (NH), 3105 (aromatic C—H), 2932 (aliphatic C—H), 2380 (SH), 1287, 1149 (SO_2); ^{1}H NMR: δ 2.73 (s, 3H, SCH_3), 3.25 (s, 3H, SO_2CH_3), 4.69 (d, J = 4 Hz, 2H, NH$\underline{CH_2}$), 7.33 (t, J = 4 Hz, 1H, NH, exchange with D_2O), 7.45–7.51 (m, 4H, chlorophenyl H-2, H-3, H-5, H-6), 8.07 (d, J = 8.4 Hz, 2H, pyrazolopyrimidine phenyl H-3, H-5), 8.35 (s, IH, pyrimidine C—H), 8.47 (d, J = 8.4 Hz, 2H, pyrazolopyrimidine phenyl H-2, H-6); ^{13}C NMR: δ 14.92 (SCH_3), 36.61 ($NHCH_2$), 44.18 (SO_2CH_3), 101.41 (pyrazolopyrimidine C-3a), 120.38 (pyrazolopyrimidine phenyl C-2, C-6), 128.92 (chlorophenyl C-3, C-5), 129.75 (pyrazolopyrimidine phenyl C-3, C-5), 130.53 (chlorophenyl C-2, C-6), 132.76 (chlorophenyl C-4), 134.54 (pyrazolopyrimidine phenyl C-4), 137.64 (chlorophenyl C-1), 142.71 (pyrazolopyrimidine phenyl C-1), 143.57 (pyrazolopyrimidine C-7a), 150.13 (pyrazolopyrimidine C-3), 155.16 (triazole C-5), 156.15 (pyrazolopyrimidine C-4), 157.21 (pyrazolopyrimidine C-6), 168.39 (triazole C-3); MS (*m/z*, %): 558 [$(M)^{+\cdot}$, 2.82%], 464 [100%]; Anal.Calcd for $C_{22}H_{19}ClN_8O_2S_3$: C, 47.26; H, 3.43; N, 20.04. Found: C, 47.03; H, 3.60; N, 20.19.

*4.1.9. General procedure for preparation of **10a** and **10b***

A mixture of acid hydrazide derivative **6a** or **6b** (0.003 mol) of each, carbon disulphide (0.68 g, 0.009 mol) and KOH (0.17 g, 0.003 mol) in absolute ethanol (30 ml) was heated under reflux for 3 h. The reaction mixture was allowed to cool, then poured into ice-cold water. The obtained solution was acidified with dil. HCl (1 ml, 33%). The obtained precipitate was filtered off, washed with water, dried and crystallized from ethanol 95%to obtain compounds **10a** or **10b**.

*4.1.9.1. 5-{[3-Methylthio-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-[1,3,4]oxadiazole-2-thiol (**10a**)*. Yellow powder; 87% yield; mp: 215–217 °C; IR : 3333 (NH), 2994 (aromatic C—H), 2920 (aliphatic C—H), 2376 (SH); ^{1}H NMR: δ 2.73 (s, 3H, SCH_3), 4.86 (d, J = 5.6 Hz, 2H, NH$\underline{CH_2}$), 7.34 (t, J = 5.6 Hz, 1H, $NHCH_2$, exchange with D_2O), 7.53 (t, J = 8 Hz, 2H, phenyl H-3, H-5), 7.73 (t, J = 8 Hz, 1H, phenyl H-4), 8.12 (d, J = 8 Hz, 2H, phenyl H-2, H-6), 8.41 (s, IH, pyrimidine C—H), 14.55 (s, 1H, oxadiazole SH); ^{13}C NMR: δ 14.45 (SCH_3), 36.44 ($NHCH_2$), 101.39 (pyrazolopyrimidine C-3a), 121.22 (phenyl C-2, C-6), 126.78 (phenyl C-4), 129.58 (phenyl C-3, C-5), 138.89 (phenyl C-1), 141.64 (pyrazolopyrimidine C-7a), 154.48 (pyrazolopyrimidine C-3), 156.44 (pyrazolopyrimidine C-4), 156.85 (pyrazolopyrimidine C-6), 161.87 (oxadiazole C-5), 178.25 (oxadiazole C-2); MS (*m/z*, %): 371 [$(M)^{+\cdot}$, 20.42%], 336 [100%]; Anal.Calcd for $C_{15}H_{13}N_7OS_2$: C, 48.50; H, 3.53; N, 26.40. Found: C, 48.82; H, 3.61; N, 26.74.

*4.1.9.2. 5-{[3-Methylthio-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]-methyl}-[1,3,4]oxadiazole-2-thiol (**10b**)*. Yellow powder; 85% yield; mp: 225–227 °C; IR : 3333 (NH), 2994 (aromatic C—H), 2920 (aliphatic C—H), 2380 (SH), 1299, 1140 (SO_2); ^{1}H NMR: δ 2.73 (s, 3H, SCH_3), 3.25 (s, 3H, SO_2CH_3), 4.83 (d, J = 5.6 Hz, 2H, NH$\underline{CH_2}$), 7.71 (t, J = 5.6 Hz, 1H, NH, exchange with D_2O), 8.04 (d, J = 8.8 Hz, 2H, phenyl H-3, H-5), 8.42 (s, IH, pyrimidine C—H), 8.46 (d, J = 8.8 Hz, 2H, phenyl H-2, H-6), SH (not observed); ^{13}C NMR: δ 14.92 (SCH_3), 36.48 ($NHCH_2$), 44.21 (SO_2CH_3), 101.63 (pyrazolopyrimidine C-3a), 120.52 (phenyl C-2, C-6), 128.98 (phenyl C-3, C-5), 137.85 (phenyl C-4), 142.70 (phenyl C-1), 143.64 (pyrazolopyrimidine C-7a), 154.48 (pyrazolopyrimidine C-3), 156.47 (pyrazolopyrimidine C-4), 157.50 (pyrazolopyrimidine C-6), 161.89 (oxadiazole C-5), 178.16 (oxadiazole C-2); MS (*m/z*, %): 449 [$(M)^{+\cdot}$, 0.83%], 64 [100%]; Anal.Calcd for $C_{16}H_{15}N_7O_3S_3$: C, 42.75; H, 3.36; N, 21.81. Found: C, 43.01; H, 3.49; N, 22.05.

*4.1.10. General procedure for preparation of **13a** and **13b***.

A mixture of acid hydrazide derivative **6a** or **6b** (0.003 mol) of each and ethylacetoacetate (0.39 g, 0.003 mol) in absolute ethanol (30 ml) was heated under reflux for 10 h. The reaction mixture was evaporated under reduced pressure. The solid obtained was crystallized from ethanol 95% to get compounds **13a** or **13b**.

*4.1.10.1. 1-(5-Ethoxy-3-methyl-1H-pyrazol-1-yl)-2-[3-methythio-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]ethanone (**13a**)*. Yellow needles; 75% yield; mp: 256–258 °C; IR : 3387 (NH), 3102 (aromatic C—H), 2924 (aliphatic C—H), 1740 (C═O); ^{1}H NMR: δ 1.21 (t, J = 7.2 Hz, 3H, $CH_2\underline{CH_3}$), 2.09 (s, 3H, pyrazole CH_3), 2.74 (s, 3H, SCH_3), 4.14 (q, J = 7.2 Hz, 2H, $\underline{CH_2}CH_3$), 4.30 (d, J = 5.6 Hz, 2H, NH$\underline{CH_2}$), 5.21 (s,1H, pyrazole H-4), 7.34 (t, J = 7.6 Hz, 1H, phenyl H-4), 7.40 (t, J = 5.6 Hz, 1H, $NHCH_2$, exchange with D_2O), 7.55 (t, J = 7.6 Hz, 2H, phenyl H-3, H-5), 8.15 (d, J = 7.6 Hz, 2H, phenyl H-2, H-6), 8.39 (s, IH, pyrimidine C—H); ^{13}C NMR: δ 11.63 (pyrazole CH_3), 14.55 (SCH_3), 15.35 ($CH_2\underline{C}H_3$), 42.87 ($NHCH_2$), 61.11 ($\underline{C}H_2CH_3$), 89.35 (pyrazole C-4), 101.13 (pyrazolopyrimidine C-3a), 120.78 (phenyl C-2, C-6), 126.67 (phenyl C-4), 129.62 (phenyl C-3, C-5), 138.94 (phenyl C-1), 141.56 (pyrazole C-3), 148.31 (pyrazolopyrimidine C-7a), 154.46 (pyrazolopyrimidine C-3), 156.71 (pyrazolopyrimidine C-4), 157.16 (pyrazolopyrimidine C-6), 162.53 (pyrazole C-5), 170.22 (C═O); MS (*m/z*, %): 423 [$(M)^{+\cdot}$, 0.94%], 343 [100%]; Anal.Calcd for $C_{20}H_{21}N_7O_2S$: C, 56.72; H, 5.00; N, 23.15. Found: C, 56.52; H, 5.23; N, 23.40.

*4.1.10.2. 1-(5-Ethoxy-3-methyl-1H-pyrazol-1-yl)-2-[3-methythio-1-(4-methylsulphonylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino]ethanone (**13b**)*. Yellow needles; 74% yield; mp: 272–274 °C; IR : 3387 (NH), 3102 (aromatic C—H), 2924 (aliphatic C—H), 1740 (C═O), 1377, 1141 (SO_2); ^{1}H NMR: δ 1.21 (t, J = 6.8 Hz, 3H, $CH_2\underline{CH_3}$), 2.08 (s, 3H, pyrazole CH_3), 2.76 (s, 3H, SCH_3), 3.25 (s, 3H, SO_2CH_3), 4.13 (q, J = 6.8 Hz, 2H, $\underline{CH_2}CH_3$), 4.29 (d, J = 5.2 Hz, 2H, $NHCH_2$), 5.22 (s, IH, pyrazole H-4), 7.42 (t, J = 5.2 Hz, 1H, $NHCH_2$, exchange with D_2O), 8.07 (d, J = 8.4 Hz, 2H, phenyl H-3, H-5), 8.43 (s, IH, pyrimidine C—H), 8.49 (d, J = 8.4 Hz, 2H, phenyl H-2, H-6); ^{13}C NMR: δ 11.63 (pyrazole CH_3), 14.55 (SCH_3), 14.86 ($CH_2\underline{C}H_3$), 42.93 ($NHCH_2$), 44.18 (SO_2CH_3), 61.14 ($\underline{C}H_2CH_3$), 89.34 (pyrazole C-4), 101.38 (pyrazolopyrimidine C-3a), 120.33 (phenyl C-2, C-6), 128.93 (phenyl C-3, C-5), 137.62 (phenyl C-4), 141.13 (pyrazole C-3), 142.74 (phenyl C-1), 143.60 (pyrazolopyrimidine C-7a), 155.32 (pyrazolopyrimidine C-3), 156.64 (pyrazolopyrimidine C-4), 157.49 (pyrazolopyrimidine C-6), 162.54 (pyrazole C-5), 170.11 (C═O); MS (*m/z*, %): 501 [$(M)^{+\cdot}$, 3.68%], 43 [100%]; Anal.Calcd for $C_{21}H_{23}N_7O_4S_2$: C, 50.29; H, 4.62; N, 19.55. Found: C, 50.09; H, 4.90; N, 19.85.

4.2. Biological activity

4.2.1. Inhibition of COX-1 and COX-2 assays

The enzyme immune assay (EIA) kit was used to measure the ability of the tested compounds listed in table 1 to inhibit ovine COX-1 and COX-2 by using N, N, N′, *N′*-tetramethyl-*p*-phenylenediamine at 590 nm according to the method prescribed upon the kit [4].

4.2.2. In vivo AI activity

Regarding the care of laboratory animals guidelines, the experiment was performed in the morning. The AI activity of the tested compounds was determined *in vivo* by using Carrageenan-induced rat paw edema method [30]. Wister albino rats (body weight = 100–150 g) were kept

under controlled environment (temperature 27 ± 2 °C with humidity 60 ± 10%) with free access to food and water. Before the experiment, rats were fasted for 24 h with maintaining free access of water. Animals were divided into 24 groups (group = 4 animals), the first group was administered with vehicle (2.5% tween 80), the second one was administered with celecoxib (100 mg/kg), the third was administered with indomethacin (100 mg/kg) and the remaining groups were administered the tested compounds (**7a-f – 13a,b**) (100 mg/kg) orally one group per one compound. After one hour of administration, subcutaneous injection of carrageenan (1% in saline) was used to induce the paw edema in the left hind paw of each rat. After 1, 3 and 5 h of carrageenan injection, paw edema thickness for each rat was measured. The change in thickness and % of inhibition of edema was calculated.

4.3. Histopathological study

Ulcerogenic liability for the most active pyrazolopyrimidine compounds, celecoxib and indomethacin was evaluated. Twenty seven rats were divided into 9 groups and fasted for 18 h before drug administration. One group (control group, 4 animals) was administered with vehicle (2.5% tween 80) and the remaining groups were administered with the tested compounds, celecoxib and indomethacin (100 mg/kg) for three successive days (animals were fed after 2 h of each dose). After 2 h of the last dose, rats were sacrificed, the stomach of each rat was removed, opened along the greater curvature and rinsed within saline. The Glandular portion of the stomach and non-glandular one were collected, fixed at 10% buffered formalin for 48 h followed by routing histological processing and paraffin embedding adapting Bancroft and Gamble (2008) [33] (five micron tissue sections were stained with routine Heamtoxylin and Eosin stain).

4.4. Docking study

Molecular Operating Environment (MOE) version 2008.10 modeling software was used in this study to perform molecular docking for the most active compounds. Docking steps were summarized as follows: The crystal structure of **SC-558** (ligand) bound at COX-2 active site was obtained from the protein data bank with code (PDB: 1CX2) [36]. 3D protonation for ligand and the tested compound structures must be done firstly. London DG force and force field energy were used for the refinement of results. Docking for the ligand was carried out to study its energy score, root mean, standard deviation (rmsd) and interaction of different amino acids. Running conformational analysis using systemic search and selecting the least energetic conformer must be done before applying docking for the tested compounds. Results that obtained from docking amino acid interactions, HB lengths and energy scores were listed in Table 4.

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Appendix A. Supplementary material

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