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Design, synthesis and biological evaluation of some pyrazole derivatives as anti-inflammatory-antimicrobial agents

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Abstract—The synthesis of novel series of structurally related 1*H*-pyrazolyl derivatives is described. All the newly synthesized compounds were tested for their in vivo anti-inflammatory activity by two different bioassays namely; cotton pellet-induced granuloma and sponge implantation model of inflammation in rats. In addition, COX-1 and COX-2 inhibitory activities, ulcerogenic effects and acute toxicity were determined. The same compounds were evaluated for their in vitro antimicrobial activity against *Escherichia coli*, as an example of Gram negative bacteria, *Staphylococcus aureus* as an example of Gram positive bacteria, and *Candida albicans* as a representative of fungi. The combined anti-inflammatory data from local and systemic in vivo animal models showed that compounds **4**, **5**, **8**, **9**, **11** and **12a** exhibited anti-inflammatory activity comparable to that of indomethacin with no or minimal ulcerogenic effects and high safety margin ($LD_{50} > 500$ mg/Kg). In addition, compounds **4**, **7**, **10**, **12a** and **12b** displayed appreciable antibacterial activities when compared with ampicillin, especially against *S. aureus*. Compounds **4** and **12a** are the most distinctive derivatives identified in the present study because of their remarkable in vivo and in vitro anti-inflammatory potency and their pronounced antibacterial activities comparable to ampicillin against Gram positive. On the other hand, compound **12a** exhibited good selective inhibitory activity against COX-2 enzyme. Therefore, such compound would represent a fruitful matrix for the development of anti-inflammatory-antimicrobial candidates.

1. Introduction

The prescription of co-administration of multiple drugs for treatment of inflammatory conditions associated with some microbial infections may inflect added health problems especially in patients with impaired liver or kidney functions. A mono therapy of an anti-inflammatory drug with antimicrobial properties will be better from the pharmaco-economic point, this will enhance patient compliance and in case that this anti-inflammatory- antimicrobial agent shows minimum adverse effects and high safety margin, this drug will be highly desirable.

Among the already marketed COX-2 inhibitors that comprise the pyrazole nucleus, celecoxib; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (Fig. 1); occupies a unique position as a potent and GI safe anti-inflammatory and analgesic agent. It is considered as a typical model of the diaryl heterocycle template that is known to selectively inhibit the COX-2 enzyme.[1]

On the other hand, much attention has been focused towards pyrazoles as antimicrobial,[2,3] antiviral[4,5] and anticancer[6,7] agents after the discovery of the natural pyrazole C-glycoside, pyrazofurin; 4-hydroxy-3-β-D-ribofuranosyl-1*H*-pyrazole-5-carboxamide (Fig. 1). This antibiotic was reported to possess a broad spectrum of antimicrobial and antiviral activities in addition to being active against several tumor cell lines.[8]

Motivated by the afore-mentioned findings, and as a continuation of our on going program in the field of pyrazoles as anti-inflammatory- antimicrobial agent,[9–15] it was designed to synthesise novel series of pyrazole derivatives (**A** & **B**) that would act as dual anti-microbial- non-acidic anti-inflammatory agents with

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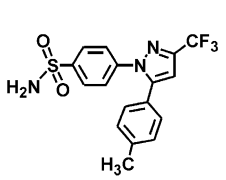
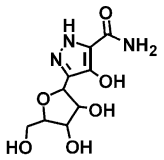
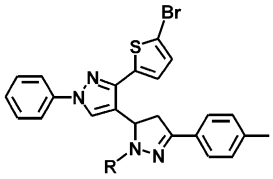


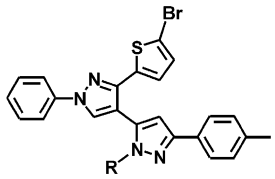
Celecoxib



Pyrazofurin



A



B

Figure 1. Structures of celecoxib, pyrazofurin and the novel series of pyrazole **A** and **B**.

minimal GI side effects and high safety margin. The substitution pattern of the pyrazole ring was rationalised so as to be correlated to the diaryl heterocycles template of compounds that are known to act selectively as COX-2 inhibitors such as celecoxib. It should be pointed out that, in addition to the targeted anti-inflammatory and antimicrobial activities, the ulcerogenic and acute toxicity profiles of the newly synthesised compounds were determined.

2. Chemistry

Synthesis of the intermediate and target compounds were performed by the reactions illustrated in Figure 2. Compound **2**, namely; 3-(5-bromo-2-thienyl)-4-[3-(4-methylphenyl)-3-oxopropenyl]-1-phenyl-1*H*-pyrazole; was synthesised in an excellent yield by condensing 3-(5-bromo-2-thienyl)-1-phenyl-1*H*-pyrazole-4-carboxaldehyde[10] **1** with 4-methylacetophenone in the presence of potassium hydroxide. This α,β-unsaturated ketone **2** was converted to the 3-(5-bromo-2-thienyl)-4-[1-formyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole **3** by the reaction with hydrazine hydrate in boiling formic acid. Analogously, heating **2** with hydrazine hydrate in boiling acetic acid yielded the corresponding 3-(5-bromo-2-thienyl)-4-[1-acetyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole **4.** Furthermore, the target compound **5**; 3-(5-bromo-2-thienyl)-4-[3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole; was obtained by cyclizing the intermediates **2** with hydrazine hydrate in boiling ethanol for 30 min. The pyrazoline derivative **5** was allowed to react with phenyl isothiocyanate, $NaNO_2$, benzoyl chloride, methanesulfonyl chloride or 4-toluenesulfonyl chloride to give rise to 3-(5-bromo-2-thienyl)-4-[1-phenylthiocarbamoyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole **6**, 3-(5-bromo-2-thienyl)-4-[1-nitroso-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole **7**, 3-(5-bromo-2-thienyl)-4-[1-benzoyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole **8**, 3-(5-bromo-2-thienyl)-4-[1-methanesulfonyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole **9** or 3-(5-bromo-2-thienyl)-4-[1-(4-methylphenylsulfonyl)-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole **10**. It is worth-mentioning that heating the intermediate **2** with hydrazine hydrate in boiling ethanol for 6 h afforded the pyrazole derivative **11**, namely; 3-(5-bromo-2-thienyl)-4-[3-(4-methylphenyl)-*1H*-pyrazol-5-yl]-1-phenyl-1*H*-pyrazole, which is the oxidation product of **5**. Compound **4** was also obtained by acetylation of compound **5**. Moreover, compound **11** could also obtained by oxidation of compound **5** using bromine.

On the other hand, compounds **12a,b**, namely; 3-(5-bromo-2-thienyl)-4-[1-aryl-3-(4-methylphenyl)-*1H*-pyrazol-5-yl]-1-phenyl-1*H*-pyrazole; were obtained in excellent yields by cyclizing the α,β-unsaturated ketone **2** with phenylhydrazine or p-tolylhydrazine respectively.

3. Results and discussion

3.1. Anti-inflammatory activity

3.1.1. Cotton pellet-induced granuloma bioassay. The anti-inflammatory activity of the synthesised compounds **2**–**11** and **12a**,**b** was evaluated by applying the cotton-pellet granuloma bioassay in rats[16] using indomethacin as a reference standard. The ED_{50} values were determined for each compound (Table 1).

Out of the compounds tested, compound **12a** displayed anti-inflammatory activity (ED_{50} 8.97 μmol) comparable to that of indomethacin (ED_{50} 9.17 μmol); (Table 1). Compounds **8**, **9**, **11** (ED_{50} 9.56, 9.57 and 9.23 μmol); were nearly equipotent with indomethacin at the same dose level and test conditions. Furthermore, compounds **4** and **5** exhibited moderate anti-inflammatory activity (Table 1). The rest of the compounds were found to be weakly active or inactive as anti-inflammatory agents (ED_{50} values range 13.00–17.46 μmol).

Table 1. The anti-inflammatory activity (ED_{50}, μmol), ulcerogenic effects and LD_{50} values of the test compounds

Test compound	ED_{50} (μmol)	% Ulceration	LD_{50} (mg/kg)
Control	—	0.0	—
Phenylbutazone	—	60	
Indomethacin	9.17±1.19	100	—
2	17.46±1.25	NT[a]	NT
3	12.64±1.55	10	>500
4	11.75±1.27	10	>500
5	10.10±1.08	0.0	>500
6	13.68±1.10	NT	NT
7	14.82±1.40	NT	NT
8	9.56±0.94	10	>500
9	9.57±1.18	0.0	>500
10	15.76±1.49	NT	NT
11	9.23±1.01	10	>500
12a	8.97±0.87	0.0	>500
12b	14.82±1.14	NT	NT
P	<0.001		

[a] NT = not tested.

3.2. Sponge implantation model of inflammation in rats

The anti-inflammatory activity of the newly synthesised compounds **2**–**11** and **12a**,**b** was further evaluated in vivo using the sponge implantation model of inflammation in rats with indomethacin as a standard. Polyester sponge implantation, the animal model chosen in this study, has the merit of triggering a non-immune acute type of inflammatory response. This model was used to assess the possibility of some of the synthesised compounds altering the course of inflammation and they have been claimed to potentially modulate inflammation. This was assessed by determining their effects on the inflammatory exudate parameters: exudate volume, Total Leucocyte Cell Count (TLC), Differential Leucocyte Cell Count (DLC), reduced cytochrome C and interleukin-1 beta (IL-1β) levels. Indomethacin was used as a standard anti-inflammatory drug (Tables 2 and 3).

The neutrophil phagocytic function was determined as one of the most important indices used to assess the different neutrophilic functions. This is because it is the ultimate and major step in neutrophilic response to acute inflammation. The main step in this function is the respiratory burst, that reflects an important face of immune system integrity. This was evaluated by the cytochrome C reduction test, where the reduction of cytochrome C by superoxide anion will change its light absorption properties, as detected spectrophotometrically.

The superoxide radical ($O^{2\cdot-}$) is a highly reactive intermediate produced in biological system by one-electron reduction of oxygen. It is generated by phagocytes during the respiratory burst. It is an intermediate in the formation of H_2O_2 and HOCl which are considered powerfull antimicrobial agents. The detection of $O^{2\cdot-}$ is based on its ability to reduce cytochrome C.

3.3. Effect on exudate volume

Results presented in Table 2 revealed that pre-treatment with indomethacin significantly reduced the exudate volume in comparison to the inflammatory control group ($p<0.001$). The mean values were 0.236 ± 0.012 and 0.362 ± 0.014 mL respectively.

Pre-treatment with the investigated compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** significantly reduced the exudate volume as compared to the inflammatory control group ($p<0.001$). The mean values were 0.238 ± 0.013, 0.282 ± 0.014, 0.256 ± 0.012, 0.274 ± 0.015, 0.251 ± 0.014, 0.251 ± 0.015 and 0.244 ± 0.013 mL respectively, compared to a control mean value of 0.362 ± 0.014 mL. On the other hand, pre-treatment with the investigated compounds **2**, **6**, **7**, **10** and **12b** did not significantly alter the exudate volume compared to the inflammatory control group ($p>0.05$).

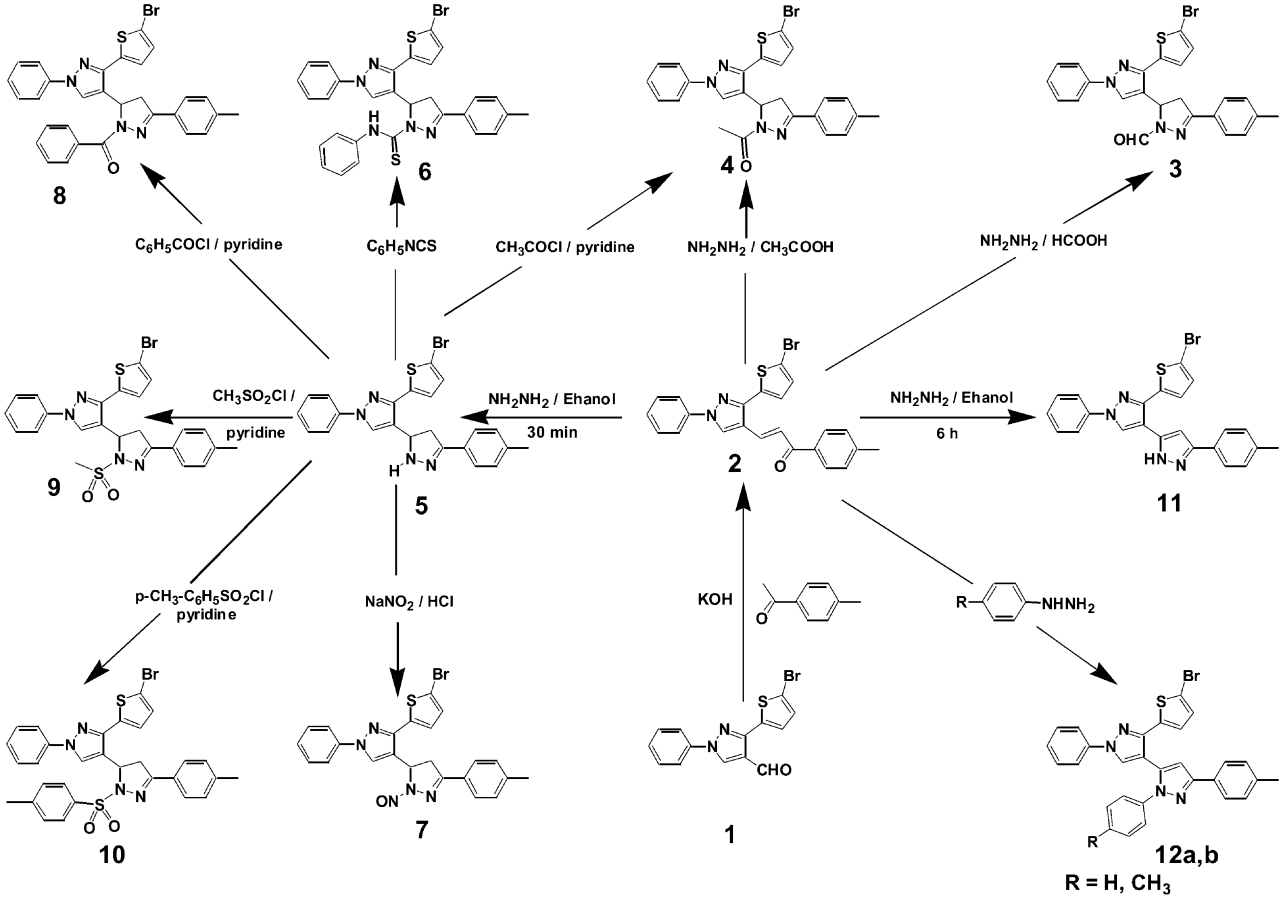


Figure 2. Synthesis of the intermediate and target compounds.

3.4. Effect on total leucocyte cell count (TLC)

Results presented in Table 2 showed that pre-treatment with indomethacin significantly reduced the exudate TLC, as compared to the inflammatory control group ($p < 0.001$). The mean value was 126.60 ± 14.28 compared to a control mean value of 274.40 ± 15.22 cell/cm^3. Pre-treatment with the investigated compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** significantly reduced the exudate TLC as compared to the inflammatory control group ($P < 0.001$). The mean values were 138.40 ± 12.56, 192.80 ± 15.26, 180.80 ± 14.16, 192.50 ± 13.68, 146.90 ± 13.82, 136.80 ± 13.24 and 148.40 ± 12.62 cell/cm^3 respectively, compared to a control mean value of 276.40 ± 15.22 cell/cm^3. However, pre-treatment with the investigated compounds **2**, **6**, **7**, **10** and **12b** did not significantly reduce the exudate TLC, in comparison to the control group ($p > 0.05$).

Table 2. The effect of inflammation induced by sponge implantation, in drug pre-treated rats (mean ± S.E., $n = 10$) on non-immunological parameters

Test compd	Exudate volume[a] X(±S.E.)	TLC[b] X(±S.E.)	DLC[c](neutrophil%) X(±S.E.)
Control	0.362(±0.014)	276.40(±15.22)	83.40(±3.16)
Indomethacin	0.236(±0.012)	126.60(±14.28)	56.60(±2.26)
2	0.312(±0.016)	252.20(±13.24)	72.40(±2.64)
3	0.238(±0.013)	138.40(±12.56)	56.40(±1.98)
4	0.282(±0.014)	192.80(±15.26)	63.60(±2.16)
5	0.256(±0.012)	180.80(±14.16)	60.20(±2.52)
6	0.336(±0.013)	240.40(±13.62)	76.40(±3.12)
7	0.321(±0.016)	262.30(±16.24)	81.20(±2.58)
8	0.274(±0.015)	192.50(±13.68)	62.40(±2.48)
9	0.251(±0.014)	146.90(±13.82)	59.80(±2.64)
10	0.336(±0.013)	202.60(±14.26)	68.40(±2.82)
11	0.251(±0.015)	136.80(±13.24)	59.20(±2.24)
12a	0.244(±0.013)	148.40(±12.62)	63.40(±2.36)
12b	0.347(±0.014)	248.20(±12.20)	71.20(±2.14)
P	<0.001	<0.001	<0.001

[a] Exudate volume (expressed in mL).
[b] Total leucocytic count (TLC) (expressed as cell/cm^3).
[c] Differential leucocytic count (DLC) (expressed as exudate neutrophil%).

3.5. Effect on differential leucocyte cell count (DLC)

Results presented in Table 2 showed that pre-treatment with indomethacin significantly reduced the exudate neutrophil% as compared to the inflammatory control group ($p < 0.001$). The mean value was $56.60 \pm 2.26\%$ compared to a control mean value of $83.40 \pm 3.16\%$. Pre-treatment with the investigated compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** significantly reduced the exudate neutrophil% as compared to the inflammatory control group ($p < 0.001$). The mean value were 56.40 ± 1.98, 63.60 ± 2.16, 60.20 ± 2.52, 62.40 ± 2.48, 59.80 ± 2.64, 59.20 ± 2.24 and $63.40 \pm 2.36\%$, compared to a control mean value of $83.40 \pm 3.16\%$. However, pre-treatment with the investigated compounds **2**, **6**, **7**, **10** and **12b** did not significantly alter the exudate neutrophil%, as compared to the control group ($p > 0.05$).

3.6. Effect on neutrophil phagocyte function

The neutrophil phagocyte function was measured using the cytochrome C reduction test. In this test, both the basal level of reduced cytochrome C (unstimulated), as well as its level after stimulation of granulocytes by means of Zymosan particles (stimulated) were estimated. The results were expressed as nmol $O^{\cdot -2}$/2×10^6 PMN/h. Results presented in Table 3 showed that pre-treatment with indomethacin significantly reduced the unstimulated reduced cytochrome C level as compared to the inflammatory control group ($p < 0.001$). The mean value was 0.74 ± 0.02 compared to a control mean value of 1.42 ± 0.02. Similarly, the results showed that pre-treatment with the investigated compounds **3**, **4**, **5**, **6**, **8**, **9**, **10**, **11**, **12a** and **12b** significantly reduced the cytochrome C level as compared to the inflammatory control group ($p < 0.001$). The mean values were 0.82 ± 0.04, 0.87 ± 0.04, 0.79 ± 0.04, 0.92 ± 0.02, 0.72 ± 0.02, 0.86 ± 0.03, 0.94 ± 0.01, 0.81 ± 0.03, 0.84 ± 0.03 and 0.98 ± 0.01 respectively, compared to a control value of 1.42 ± 0.02. However, pre-treatment with the investigated compounds **2** and **7**

Table 3. The effect of inflammation induced by sponge implantation, in drug pre-treated rats (mean ± S.E., $n = 10$) on the immunological parameters[a,b]

Test compd	Unstimulated reduced cytochrome C level X(±S.E.)	Stimulated reduced cytochrome C level X(±S.E.)	Unstimulated IL-1β level X(±S.E.)	LPS stimulated IL-1β level X(±S.E.)
Control	1.422(±0.018)	1.522(±0.036)	196.40(±15.42)	281.60(±16.24)
Indomethacin	0.742(±0.016)	0.868(±0.022)	102.20(±13.26)	126.20(±13.22)
2	1.17(±0.02)	1.29(±0.02)	172.60(±12.34)	260.40(±12.40)
3	0.82(±0.04)	0.96(±0.05)	116.40(±14.24)	146.40(±12.82)
4	0.87(±0.04)	1.11(±0.03)	122.80(±13.28)	178.40(±14.28)
5	0.79(±0.04)	0.89(±0.04))	134.40(±14.32)	179.40(±14.42)
6	0.92(±0.02)	1.24(±0.05)	182.40(±14.62)	268.20(±13.24)
7	1.36(±0.04)	1.42(±0.03)	172.20(±16.28)	262.40(±15.34)
8	0.72(±0.02)	1.04(±0.07)	145.40(±14.28)	161.80(±13.68)
9	0.86(±0.03)	0.98(±0.02)	116.30(±14.42)	138.40(±14.66)
10	0.94(±0.01)	1.21(±0.03)	160.60(±15.24)	206.80(±13.98)
11	0.81(±0.03)	0.89(±0.03)	114.40(±12.38)	138.40(±14.12)
12a	0.84(±0.03)	0.97(±0.05)	138.40(±13.42)	176.30(±13.62)
12b	0.98(±0.01)	0.99(±0.03)	185.30(±14.20)	265.20(±15.30)
P	<0.001	<0.001	<0.001	<0.001

[a] Neutrophil Phagocyte Function (expressed in nmol $O^2/2 \times 10^6$ PMN/h) on both unstimulated and stimulated reduced cytochrome C level.
[b] The assay of Interleukin-1β (IL-1β) (expressed in pg/mL) on both unstimulated IL-1β level and LPS stimulated IL-1β level.

did not significantly reduce the unstimulated reduced cytochrome C level as compared to the control group ($p > 0.05$).

The results presented in Table 3 also showed that pre-treatment with indomethacin significantly reduced the stimulated cytochrome C level as compared to the inflammatory control group ($p < 0.001$). The mean value was 0.87 ± 0.02 compared to control value of 1.52 ± 0.04. Pre-treatment with the investigated compounds **3**, **4**, **5**, **8**, **9**, **11**, **12a** and **12b** significantly decreased the stimulated reduced cytochrome C level in comparison to the inflammatory control group ($p < 0.001$). Their mean value were 0.96 ± 0.05, 1.11 ± 0.03, 0.89 ± 0.04, 1.04 ± 0.07, 0.98 ± 0.02, 0.89 ± 0.03, 0.97 ± 0.05 and 0.99 ± 0.03 respectively, compared to a control mean value of 1.52 ± 0.04. On the other hand, pre-treatment with the investigated compounds **2**, **6**, **7** and **10** did not significantly alter the stimulated reduced cytochrome C level as compared to the control group ($p > 0.05$).

3.7. Effect on interleukin-1β (IL-1β) level

The assay of Interleukin-1β using an IL-1β ELISA Kit was done by estimating both the unstimulated level of IL-1β [spontaneously released as well as the lipo-polysaccharide (LPS) stimulated release of IL-1β]. The results were expressed in pg/mL. The results presented in Table 3 showed that pre-treatment with indomethacin significantly decreased the unstimulated IL-1β level as compared to the inflammatory control group ($p < 0.001$). The mean value was 102.20 ± 13.26 compared to the control value of 196.40 ± 15.42. Pre-treatment with the investigated compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** significantly decreased the unstimulated IL-1β levels in comparison to the inflammatory control group ($p < 0.001$). The mean values were 116.40 ± 14.24, 122.80 ± 13.28, 134.40 ± 14.32, 145.40 ± 14.28, 116.30 ± 14.42, 114.40 ± 12.38 and 138.60 ± 13.42 respectively, compared to a control mean value of 196.40 ± 15.42. On the other hand, pre-treatment with the investigated compounds **2**, **6**, **7**, **10** and **12b** did not significantly alter the unstimulated IL-1β levels as compared to the control group ($p > 0.05$).

Results presented in Table 3 also showed that pre-treatment with indomethacin significantly decreased the LPS-stimulated IL-1β levels as compared to the inflammatory control group ($p < 0.001$). The mean value was 126.20 ± 13.22 compared to a control mean value of 281.60 ± 16.24. Pre-treatment with the investigated compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** significantly decreased the LPS-stimulated IL-1β levels in comparison to the inflammatory control group ($p < 0.001$). The mean values were 146.40 ± 12.82, 178.40 ± 14.28, 179.40 ± 14.24, 161.80 ± 13.68, 138.40 ± 14.66, 138.40 ± 14.12 and 176.30 ± 13.62 respectively, compared to a control mean value of 281.60 ± 16.24. However, pre-treatment with the investigated compounds **2**, **6**, **7**, **10** and **12b** did not significantly alter the LPS-stimulated IL-1β levels as compared to the control group ($p > 0.05$).

The combined anti-inflammatoty results showed that it is not easy to draw structure–activity relationship. However, The types of the newly synthesised ring system whether pyrazoline (compounds **3**, **4**, **5**, **8**, **9**) or pyrazole (compounds **11**, **12a**) did not much affect the anti-inflammatory activity. In addition, substitution of $N^1$of the pyrazoline or pyrazole ring has only a minor influence on the activity. When N^1 is substituted by formyl (**3**), acetyl (**4**), phenylthiocarbamoyl (**6**), nitroso (**7**), 4-methylbenzenesulfonyl (**10**) or 4-methylphenyl (**12b**), the recorded anti-inflammatoty activity was found to be less than that of indomethacin. Whereas compounds unsubstituted on N^1 (**5**, **11**), N^1-benzoyl (**8**), N^1-methanesulfonyl (**9**) or N^1-phenyl (**12a**), showed anti-inflammatoty activity comparable to that of indomethacin.

3.8. Human COX-1 and COX-2 enzymatic assay

Compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** that exhibited moderate to potent anti-inflammatory profiles in the pre-mentioned animal models were further tested for their ability to inhibit human COX-2 and COX-1 enzymes in vitro appling the methodology of Wakitani et al.[17] COX-1 assay was carried out using platelets microsome fraction. Human platelets were prepared from NSAID-free normal human volunteers according to the method of Hammarström and Falardeau.[18] COX-2 assay was performed utilising human recombinant COX-2 (hrCOX-2) purchased from Sigma-Aldrich. The concentration of the compound causing 50% enzyme inhibition (IC_{50} μmol) was estimated. The results are recorded in Table 4. The results showed that the test compounds exhibited weak inhibitory activity against COX-1 enzyme (IC_{50} values between 76.8 and >100 μmol) when compared with indomethacin ($IC_{50} = 0.26$ μmol), but several were more potent than celecoxib ($IC_{50} = >100$ μmol). In addition, the test compounds showed higher inhibitory profile against COX-2 when compared with indomethacin. All test compounds showed approximate selectivity ratio (COX-1/COX-2) lower than that of celecoxib. In general, pyrazoline derivatives (compounds **3**, **4**, **5**, **8**, **9**) showed lower selectivity toward COX-2 enzyme than pyrazole derivative (compound **12a**), although, pyrazole compound **11** showed low selectivity. Substitution of

Table 4. In vitro human COX-2[a] and COX-1[b] enzymes inhibitory activities of compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a**

Test compd	COX-2 IC_{50} (μM)[c]	COX-1 IC_{50} (μM)[c]	Approximate Selectivity Ratio COX-1/COX-2
Indomethacin	2.63	0.26	0.098
Celecoxib	<0.3	>100	333
3	1.48	>100	67.
4	1.32	84.71	64
5	1.72	76.83	44
8	0.82	96.35	117
9	0.98	>100	102
11	1.52	78.46	51
12a	0.37	>100	270

[a] Human recombinant COX-2 enzyme.
[b] Human COX-1 enzyme from human platelets.
[c] Values are means of at least four experiments.

$N^1$of the pyrazoline or pyrazole ring affects the selectivity. When N^1 is substituted by benzoyl (**8**), methanesulfonyl (**9**) or phenyl (**12a**), the recorded selectivity ratio was found higher than other test compounds, but lower than that of celecoxib. Compound **12a** is the most selective COX-2 inhibitor (approximate selectivity ratio = 220) in the present study as compared with celecoxib (approximate selectivity ratio > 333).

3.9. Ulcerogenic effects

Compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** that exhibited moderate to potent anti-inflammatory profiles in the pre-mentioned animal models were evaluated for their ulcerogenic potential in rats.[19] Phenylbutazone and indomethacin were tested as reference drugs. All the active compounds revealed a superior GI safety profiles (0–10% ulceration) when compared with phenylbutazone (60% ulceration) or indomethacin (100% ulceration) under the same experimental conditions (Table 1).

Gross observation of the isolated rat stomach showed a normal stomach texture for compounds **5**, **9** and **12a** (0% ulceration), whereas the others showed slight hyperemia (10% ulceration).

3.10. Acute toxicity

The biologically significant compounds were further evaluated for their approximate LD_{50} in male mice using a literature method.[20,21] The results (Table 1) indicated that most of the tested compounds proved to be non-toxic and well tolerated by the experimental animals as evidenced by their LD_{50} values (> 500 mg/Kg).

3.11. In vitro antimicrobial activity

Compounds **2**–**11** and **12a,b** have been evaluated for their in vitro antimicrobial activity against *Escherichia coli* (*E. coli* ATCC 25922), as an example of Gram negative bacteria, *Staphylococcus aureus* (*S. aureus* ATCC 19433) as an example of Gram positive bacteria, and *Candida albicans* (*C. albicans*) as a representative of fungi. The microdilution susceptibility test in Müller-Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) were used for the determination of antibacterial and antifungal activity.[22] The minimal inhibitory concentrations (MICs, μg/mL) of the tested compounds are recorded in Table 5.

The results revealed that most of the newly synthesised compounds exhibited promising antibacterial activities but they showed poor antifungal activity. Generally, the test compounds showed better activity against the Gram positive bacteria (Table 5). Out of the compounds tested, compound **12a** (MIC 50 μg/mL) exhibited moderate antibacterial activity against the Gram negative *E. coli* as compared with the broad spectrum antibiotic ampicillin (MIC 25 μg/mL). In addition, compounds **4**, **12a** and **12b** (MIC 25 μg/mL) was 50% as active as ampicillin (MIC 25 μg/mL) against *S. aureus* (Table 4). It is worth-mentioning that compound **7** showed equal activity as ampicillin against *S. aureus*. The rest of the tested compounds were weakly active against both organisms with MIC values ranging between 100–> 200 μg/mL (Table 5). All the tested compounds showed weak antifungal activity against *C. albicans* (MIC values of 50–> 200 μg/mL) when compared with the reference antifungal agent clotrimazole (Canesten®, Bayer) (MIC 12.5 μg/mL).

Table 5. Minimal inhibitory concentrations (MIC μg/mL) of test compounds

Test compd	*E. coli*	*S. aureus*	*C. albicans*
2	> 200	> 200	> 200
3	200	100	> 200
4	200	25	> 200
5	> 200	> 200	100
6	100	50	> 200
7	100	12.5	200
8	> 200	> 200	> 200
9	200	100	> 200
10	100	50	> 200
11	200	> 200	100
12a	50	25	> 200
12b	200	25	50
Ampicillin	25	12.5	—
Clotrimazole	—	—	12.5

4. Conclusion

As the results obtained from cotton pellet-induced granuloma bioassay are comparable to that revealed from sponge implantation model of inflammation, one can conclude from this study that the sponge implantation model of inflammation seemed to be a reliable method for collection of an adequate amount of exudate necessary for the evaluation of different inflammatory markers. Moreover, it can be concluded from the present study that pre-treatment with the investigated compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** significantly altered the inflammatory markers, not only, the non-immunological parameters (exudate volume, TLC and DLC), but also the immunological parameters (reduced cytochrome C and IL-1β levels). Therefore, the investigated compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** were capable of modulating the inflammatory response and were assumed to have in vivo anti-inflammatory activity similar to that of indomethacin. Moreover, they showed minimum ulcerogenic activity as compared to phenylbutazone and indomethacin. Meanwhile, they displayed distinct in vitro inhibitory activity against COX-2 than COX-1 when compared with indomethacin and celecoxib. Moreover, they are well tolerated by experimental animals and showed high safety margin as revealed from their LD50 values (> 500 mg kg^{-1}). These speculations remain to be further expanded in other models of experimental inflammation and confirmed by clinical trials before a final drug design is to be made. On the other hand, compounds **4** and **12a** showed moderate antimicrobial activity against *S. aureus*. The latter compounds would represent a fruitful matrix for the development of a new class of dual non-acidic anti-inflammatory-antimicrobial agents that would deserve

further investigation and derivatization. It is worth-mentioning that compound **12a** showed anti-inflammatory activity profile similar to our priviously published compound (3-phenyl-2-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidene-hydrazono]-5-thioxo-2,3-dihydrothiazolo[4,5-*d*]pyrimidin-7(6*H*)-ones),[14] but with less antimicrobial activity. In addition, compound **12a** exhibited good selectivity against COX-2 enzyme.

5. Experimental

5.1. Chemistry

All chemicals used in this study were purchased from E. Merck, Fluka AG and Aldrich companies. Melting points were determined in open-glass capillaries using Thomas capillary melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on 470-Shimadzu infrared spectrophotometer using the KBr disc technique. The ^{1}H NMR spectra were recorded on a Varian XL-200 MHz Spectrometer, and the chemical shifts are given in δ (ppm) down field from tetramethylsilane (TMS) as an internal standard. Splitting patterns were designated as follows: s: singlet; d: doublet; m: multiplet. Elemental analyses were performed at the Microanalytical Unit, Faculty of Science, Cairo University, Cairo, Egypt, and the found values were within ±0.4% of the theoretical values. Follow up of the reactions and checking the homogeneity of the compounds were made by TLC on silica gel-protected aluminum sheets (Type 60 F254, Merck) and the spots were detected by exposure to UV-lamp at λ 254 nm for few seconds.

5.2. 3-(5-bromo-2-thienyl)-4-[3-(4-methylphenyl)-3-oxopropenyl]-1-phenyl-1H-pyrazole (2)

An equimolar mixture of 3-(5-bromo-2-thienyl)-1-phenyl-1*H*-pyrazole-4-carboxaldehyde[10] **1** (3.33 g, 10 mmol) and *p*-methylacetphenone (1.34 g, 10 mmol) in 30 mL ethanolic KOH (3%) was stirred at room temperature for 4 h. The precipitate formed was filtered, washed with ethanol, dried and crystallized from chloroform/ethanol (3:1). Yield: 94%; mp 159–161 °C. IR (cm^{-1}): 1660 (C=O), 1625 (C=N). ^{1}H NMR ($CDCl_3$): δ 2.41 (s, 3H, C*H*$_3$), 7.11 (d, *J* = 3.96 Hz, 1H, thioph C-3-H), 7.21-7.77 (m, 11H, Phenyl-H & C<u>H</u>=C<u>H</u>), 7.80 (d, *J* = 3.96 Hz, 1H, thioph C-4-H), 8.47 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{23}H_{17}BrN_2OS$ (449.364).

5.3. 3-(5-Bromo-2-thienyl)-4-[1-formyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole 3 and 3-(5-bromo-2-thienyl)-4-[1-acetyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole (4)

A mixture of **2** (2.2 g, 5 mmol) and hydrazine hydrate (1.6 mL, 5 mmol) in formic acid or acetic acid (15 mL) was heated under reflux for 6 h. The solid product obtained on cooling was filtered, washed with ethanol and crystallized from ethanol. Physical and analytical data of compound **3**; yield: 76%; mp 216–218 °C. IR (cm^{-1}): 1646 (C=O), 1622 (C=N). ^{1}H NMR ($CDCl_3$): δ 2.40 (s, 3H, C*H*$_3$), 2.94-3.03 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 3.34–3.44 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 5.08–5.16 (dd, J_1 = 10 Hz, J_2 = 10 Hz, 1H, pyrazoline-C-5-H), 7.11 (d, *J* = 3.96 Hz, 1H, thioph C-3-H), 7.21–7.77 (m, 9H, Phenyl-H), 7.80 (d, *J* = 3.96 Hz, 1H, thioph C-4-H), 8.43 (s, 1H, pyrazol C-5-H)) 8.43 (s, 1H, CHO). Analysis calculated for $C_{24}H_{19}BrN_4OS$ (491.404). Physical and analytical data of compound **4**; yield: 83%; mp 208–209 °C. IR (cm^{-1}): 1652 (C=O), 1628 (C=N). ^{1}H NMR ($CDCl_3$): δ 2.38 (s, 3H, C*H*$_3$), 2.43 (s, 3H, C*H*$_3$), 2.98–3.07 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 3.32–3.46 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 5.11–5.17 (dd, J_1 = 10 Hz, J_2 = 10 Hz, 1H, pyrazoline-C-5-H), 7.12 (d, *J* = 3.96 Hz, 1H, thioph C-3-H), 7.18-7.82 (m, 9H, Phenyl-H), 7.83 (d, *J* = 3.96 Hz, 1H, thioph C-4-H), 8.38 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{25}H_{21}BrN_4OS$ (505.430).

5.4. 3-(5-Bromo-2-thienyl)-4-[3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1H-pyrazole (5)

A mixture of **2** (2.2g, 5 mmol) and hydrazine hydrate (1.6 mL, 5 mmol) in ethanol (15 mL) was heated under reflux for 30 min. The white solid product obtained on cooling was filtered, washed with ethanol and recrystallized from ethanol. Yield: 87%; mp 134–136 °C. IR (cm^{-1}): 3380 (NH), 1630 (C=N). ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H, C*H*$_3$), 2.96–3.08 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 3.33–3.46 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 5.07–5.15 (dd, J_1 = 10 Hz, J_2 = 10 Hz, 1H, pyrazoline-C-5-H), 7.13 (d, *J* = 3.96 Hz, 1H, thioph C-3-H), 7.17–7.81 (m, 10H, phenyl-H, NH), 7.82 (d, *J* = 3.96 Hz, 1H, thioph C-4-H), 8.44 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{23}H_{19}BrN_4S$ (463.393).

5.5. 3-(5-Bromo-2-thienyl)-4-[1-phenylthiocarbamoyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole (6)

To a solution of **5** (2.31 g, 5 mmol) in dry ether (30 mL) was added an equal amount of phenyl isothiocyanate. The reaction mixture was stirred for 5 h. The solid product separated was filtered, washed with ethanol, dried and crystallized from ethanol. Yield: 78%; mp 215–216 °C. IR (cm^{-1}): 1630 (C=N), 1524, 1315, 1140 and 945 (NCS). ^{1}H NMR ($CDCl_3$): δ 2.38 (s, 3H, C*H*$_3$), 2.94–3.09 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 3.34–3.46 (dd, J_1 = 10 Hz, J_2 = 9 Hz, 1H, pyrazoline-C-4-H), 5.05–5.16 (dd, J_1 = 10 Hz, J_2 = 10 Hz, 1H, pyrazoline-C-5-H), 7.15 (d, *J* = 3.96 Hz, 1H, thioph C-3-H), 7.18–7.80 (m, 15H, phenyl-H, NH), 7.82 (d, *J* = 3.96 Hz, 1H, thioph C-4-H), 8.43 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{30}H_{24}BrN_5S_2$ (598.581).

5.6. 3-(5-Bromo-2-thienyl)-4-[1-nitroso-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole (7)

To an ice cold solution of **5** (2.31 g, 5 mmol) in hydrochloric acid (50%, 20 mL) was added sodium nitrite (0.35 g, 5 mmol). The reaction mixture was stirred for

2 h. The solid product separated was filtered, washed with ethanol, dried and crystallized from ethanol. Yield: 54%; mp 163–164 °C. IR (cm^{-1}): absence of 3380 (NH), 1630 (C=N). ^1H NMR ($CDCl_3$): δ 2.37 (s, 3H, CH_3), 2.97–3.10 (dd, $J_1=10$ Hz, $J_2=9$ Hz, 1H, pyrazoline-C-4-H), 3.33–3.46 (dd, $J_1=10$ Hz, $J_2=9$ Hz, 1H, pyrazoline-C-4-H), 5.08–5.15 (dd, $J_1=10$ Hz, $J_2=10$ Hz, 1H, pyrazoline-C-5-H), 7.12 (d, $J=3.96$ Hz, 1H, thioph C-3-H), 7.18–7.81 (m, 9H, phenyl-H), 7.81 (d, $J=3.96$ Hz, 1H, thioph C-4-H), 8.42 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{23}H_{18}BrN_5OS$ (492.392).

5.7. 3-(5-bromo-2-thienyl)-4-[1-acetyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole 4, 3-(5-bromo-2-thienyl)-4-[1-benzoyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole 8, 3-(5-bromo-2-thienyl)-4-[1-methanesulfonyl-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole 9 or 3-(5-bromo-2-thienyl)-4-[1-(4-methylbenzenesulfonyl)-3-(4-methylphenyl)-2-pyrazolin-5-yl]-1-phenyl-1*H*-pyrazole (10)

To a solution of **5** (0.46 g, 1 mmol) in dry pyridine (5 mL) was added an equivalent amount of acetyl chloride, benzoyl chloride, 4-methanesulphonyl chloride or 4-toluenesulphonyl chloride. The reaction mixture was heated on a boiling water bath for 20 min, cooled and then poured onto crushed ice (30 g). The solid product separated was filtered, washed with water, dried and crystallised from ethanol. Physical and analytical data for compound **4**; yield: 93%, mp, IR and ^1H NMR were identical to the compound prepared by the afore-mentioned method. Physical and analytical data of compound **8**: yield 89%; mp 233–235 °C. IR (cm^{-1}): 1665 (C=O), 1632 (C=N). ^1H NMR ($CDCl_3$): δ 2.41 (s, 3H, CH_3), 2.95–3.09 (dd, $J_1=10$ Hz, $J_2=9$ Hz, 1H, pyrazoline-C-4-H), 3.33–3.45 (dd, $J_1=10$ Hz, $J_2=9$ Hz, 1H, pyrazoline-C-4-H), 5.06–5.15 (dd, $J_1=10$ Hz, $J_2=10$ Hz, 1H, pyrazoline-C-5-H), 7.12 (d, $J=3.96$ Hz, 1H, thioph C-3-H), 7.14–7.81 (m, 14H, phenyl-H), 7.83 (d, $J=3.96$ Hz, 1H, thioph C-4-H), 8.39 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{30}H_{23}BrN_4OS$ (567.500). Physical and analytical data of compound **9**; yield: 91%; mp 208–210 °C. IR (cm^{-1}): 1632 (C=N), 1364, 1165 (SO_2). ^1H NMR ($CDCl_3$): δ 2.40 (s, 3H, CH_3), 2.95–3.10 (m, 4H, pyrazoline-C-4-H & $C\underline{H}_3SO_2$), 3.32–3.48 (dd, $J_1=10$ Hz, $J_2=9$ Hz, 1H, pyrazoline-C-4-H), 5.06–5.14 (dd, $J_1=10$ Hz, $J_2=10$ Hz, 1H, pyrazoline-C-5-H), 7.11 (d, $J=3.96$ Hz, 1H, thioph C-3-H), 7.14–7.80 (m, 9H, phenyl-H), 7.82 (d, $J=3.96$ Hz, 1H, thioph C-4-H), 8.40 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{24}H_{21}BrN_4O_2S_2$ (541.485). Physical and analytical data of compound **10**; yield: 87%; mp 214–215 °C. IR (cm^{-1}): 1630 (C=N), 1368,1165 (SO_2). ^1H NMR ($CDCl_3$): δ 2.39 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 2.97–3.12 dd, $J_1=10$ Hz, $J_2=9$ Hz, 1H, pyrazoline-C-4-H), 3.33–3.44 (dd, $J_1=10$ Hz, $J_2=9$ Hz, 1H, pyrazoline-C-4-H), 5.05–5.17 (dd, $J_1=10$ Hz, $J_2=10$ Hz, 1H, pyrazoline-C-5-H), 7.11 (d, $J=3.96$ Hz, 1H, thioph C-3-H), 7.13-7.81 (m, 13H, phenyl-H), 7.82 (d, $J=3.96$ Hz, 1H, thioph C-4-H), 8.41 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{30}H_{25}BrN_4O_2S_2$ (617.581).

5.8. 3-(5-Bromo-2-thienyl)-4-[3-(4-methylphenyl)-1*H*-pyrazol-5-yl]-1-phenyl-1*H*-pyrazole (11)

A mixture of **2** (2.2g, 5 mmol) and hydrazine hydrate (1.6 mL, 5 mmol) in ethanol (15 mL) was heated under reflux for 6 h. The reddish white solid product obtained on cooling was filtered, washed with ethanol and crystallized from ethanol. Physical and analytical data of compound **11**; yield: 64%; mp 172–174 °C. IR (cm^{-1}): 3385 (NH), 1628 (C=N). ^1H NMR ($CDCl_3$): δ 2.38 (s, 3H, CH_3), 6.97 (s,1H, pyrazol-C-4-H), 7.12 (d, $J=3.96$ Hz, 1H, thioph C-3-H), 7.17–7.80 (m, 10H, phenyl-H, NH), 7.83 (d, $J=3.96$ Hz, 1H, thioph C-4-H), 8.42 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{23}H_{17}BrN_4S$ (461.378).

5.9. 3-(5-Bromo-2-thienyl)-4-[1-aryl-3-(4-methylphenyl)-1*H*-pyrazol-5-yl]-1-phenyl-1*H*-pyrazole (12a&b)

To a solution of **2** (2.2 g, 5 mmol) in absolute ethanol (20 mL) was added the proper arylhydrazine hydrochloride (5 mmol) and anhydrous sodium acetate (0.49 g, 5 mmol). The reaction mixture was heated under reflux for 6 h, then cooled and poured into cold water (50 mL). The precipitated solid was filtered, washed with water, dried and crystallized from ethanol. Physical and analytical data of compound **12a**; yield: 82%; mp 196–198 °C. IR (cm^{-1}): 1625 (C=N). ^1H NMR ($CDCl_3$): δ 2.39 (s, 3H, CH_3), 6.98 (s,1H, pyrazol-C-4-H), 7.13 (d, $J=3.96$ Hz, 1H, thioph C-3-H), 7.15–7.80 (m, 14H, phenyl-H), 7.82 (d, $J=3.96$ Hz, 1H, thioph C-4-H), 8.42 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{29}H_{21}BrN_4S$ (537.474). Physical and analytical data of compound **12b**; yield: 78%; mp 184–186 °C. IR (cm^{-1}): 1628 (C=N). ^1H NMR ($CDCl_3$): δ 2.39 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 6.97 (s,1H, pyrazol-C-4-H), 7.13 (d, $J=3.96$ Hz, 1H, thioph C-3-H), 7.14–7.81 (m, 13H, phenyl-H), 7.82 (d, $J=3.96$ Hz, 1H, thioph C-4-H), 8.41 (s, 1H, pyrazol C-5-H)). Analysis calculated for $C_{30}H_{23}BrN_4S$ (551.500).

5.10. Anti-inflammatory activity

5.10.1. Cotton pellet-induced granuloma bioassay. Adult male Sprague–Dawley rats (120–140 g) were used.[16] They were acclimated 1 week prior to use and allowed unlimited access to standard rat chow and water. Prior to the start of experiment, the animals were randomly divided into groups (6 rats each). Cotton pellets (35±1 mg) cut from dental rolls were impregnated with 0.2 mL (containing 10 μmol) of a solution of the test compound in chloroform or acetone and the solvent was allowed to evaporate. Each cotton pellet was subsequently injected with 0.2 mL of an aqueous solution of antibiotics (1 mg penicillin G and 1.3 mg dihydrostreptomycin/mL). Two pellets were implanted subcutaneously, one in each axilla of the rat, under mild general anesthesia. One group of animals received the standard reference indomethacin and the antibiotics at the same level. Pellets containing only the antibiotics were similarly implanted in the control rats. Seven days later, the animals were sacrificed and the two cotton pellets, with adhering granulomas, were removed, dried for 48 h at 60 °C and

weighed. The increment in dry weight (difference between the initial and final weights) was taken as a measure of granuloma ± SE. This was calculated for each group and the percentage reduction in dry weight of granuloma from control value was also calculated. The ED_{50} values were determined through dose–response curves, using doses of 4, 7, 10 and 15 μmol for each compound (Table 1).

5.11. Sponge implantation model of inflammation in rats

5.11.1. Material and methods. One hundred and thirty male albino rats weighing 150–200 g were used throughout the study. They were kept in the animal house under standard conditions of light and temperature with free access to food and water. The animals were randomly divided into thirteen groups each of ten rats as follows:

Group I (control group): received 2% gum acacia orally (suspending vehicle) and served as inflammatory control group, to estimate reference values of the parameters studied.

Group II (indomethacin pre-treated inflammatory group): received indomethacin (Indomethacin-Pharco Pharmaceuticals, Egypt) suspended in 2% gum acacia, in a dose of 10 mg/kg body weight/day orally, divided into two equal doses, for three successive days.[23] On the third day, the first dose was administered 30 min before sponge implantation and the second dose 1 h before its removal.

Group III–XIII (pre-treated inflammatory groups): each being treated with the appropriate newly synthesised investigational compounds **1**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12a**&**b** respectively. The compounds were suspended in 2% gum acacia, and were given in a dose of 10 mg/kg body weight/day, orally, divided into two equal doses, for three successive days, as indomethacin.

5.12. Induction of inflammation

Inflammation was induced by subcutaneous implantation of dry polyester sponge (type E_{41}-blue-kay Metzeler, UK).[24] The polyester sponge was cut to a standard size of 15 mm diameter, 4 mm thickness, and a weight of 17–17.5 mg. The sponges were sterilized in 70% (v/v) ethanol, washed thoroughly twice with distilled water and antiseptic solution (Cyteal-Pierre Fabre laboratories, France), and were then dried in an oven (Gallenkamp-Poland) at 100 °C for 1.5 h.

For implantation, the rats were anaesthetized with diethyl ether and the skin of the ventral aspect of the abdominal surface was shaved and swabbed with a 1% solution of savlon in 70% (v/v) ethanol/water.[24–26] Two small medial incisions each of 1 cm length were made on either side of the midline of the ventral abdominal surface, and two cavities were performed by blunt dissection, separating the dermis from the muscular layers. Lastly one dry sponge was implanted in each side, the incisions were then closed with silk sutures (Mersilk 310-sterile silk suture, Ethicon, UK)[24] and the rats were kept in their cages for 6 h.

5.13. Collection of exudates

After 6 h, the animals were reanaesthetized with ether. The ventral incisions were quickly opened and the sponges were carefully dissected out to avoid local bleeding. Both sponge were gently squeezed with a 5 mL syringe plunger and the total exudates was collected in polyethylene centrifuge tubes containing 0.1 mL of 1.5% EDTA.[24] This was used to determine non-immunological markers, namely: exudates volume, total and differential leucocyte cell counts, in addition to immunological markers, namely: cytochrome C reduction test and interleukin-1 beta levels.

5.14. Cell isolation and preparations

Granulocyte cell suspension was prepared using a modification of the method of Lehrer and Cline.[27]

5.15. Neutrophil phagocyte function estimation[27–31]

The isolated cells were resuspended in 1 mL Hank's balanced salt solution (HBSS, free from phenol red-Gibco, England) and were adjusted to a concentration of 2×10^5 cells/mL. This was stored at −20 °C until tested for neutrophilic function. This was evaluated by the cytochrome C reduction test, where the reduction of cytochrome C by superoxide anion will change its light absorbance properties, as detected spectrophotometrically.

5.16. Interleukin-1 beta level estimation (IL-1β)[32]

The granulocyte cell layer was washed 3 times in RPMI-1640 complete medium (Grand Island Biological Company, NY) containing 2 mmol glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 20 mmol N-1-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES buffer) (Nalge, Sybron Corporation-Rochester, NY). The supernatant was aspirated and the cell pellet was vigorously tapped and resuspended in a known volume of complete RPMI medium supplemented with 10% inactivated fetal bovine serum (FBS), heat inactivated at 56 °C for 30 min. (Biofluid-Inc. Tockville, USA).

The granulocyte count was then adjusted to 2×10^6 cells/mL by complete RPMI-1640 supplemented with 10% heat inactivated FBS (RPMI-HIFBS). This constituted the granulocyte cell suspension that was stored at −20 °C until tested for IL-1β levels.

5.17. Stimulation of granulocytes by lipopolysaccharide (LPS) as a mitogen (lyophilized *E. coli*, Sigma, USA[33]

100 μL of the prepared cell suspension were added to LPS (10 μg/mL) (reconstituted in 5 mL sterile complete RPMI-1640 medium and stored at −20 °C until used) in a flat-bottomed well of a microtitre plate. Another 100 μL of the cell suspension (without stimulant) were

pippetted into a flat-bottomed well to serve as a control. Each sample was placed in triplicate well to obtain enough supernatants for measuring IL-1β levels. Complete RPMI medium was added to all wells to obtain a final volume of 200 μL per well. The cell cultures were incubated for 24 h at 37 °C, 10% CO_2 and 100% humidity in a CO_2 jar (Angelantoni Scientifica, Italy). After 24 h, the cultures were centrifuged at 700×*g* for 10 min and the supernatant containing putative IL-1β were collected and stored at −20 °C until tested for the cytokine by ELISA kite.

5.18. Human COX-1 and COX-2 enzymatic assay

Human COX-1 and COX-2 activities were determined by applying the methodology described by Wakitani et al.[17] Human COX-1 (0.3 mg protein/assay) or COX-2 (1 mg protein/assay) was suspended in 0.2 mL of 100 mmol Tris_/HCl buffer (pH 8) containing hematin (2 mmol) and tryptophan (5 mmol) as cofactors. The reaction mixture was pre-incubated with each test compound for 5 min at 24 °C. [^{14}C] Arachidonic acid (100.00 dpm, 30 mmol) was then added to the mixture and incubated for 2 (COX-1) or 45 min (COX-2) at 24 °C. The reaction was stopped by the addition of 400 mL of a stop solution composed of Et_2O:MeOH:1M citric acid (30:4:1, v/v). After centrifugation of the mixture at 1700 X/*g* for 5 min at 4 °C, 50 mL of the upper phase was applied to a thin-layer chromatography (TLC) plate. TLC was performed at 4 °C with a solvent system consisting of Et_2O:MeOH:AcOH (90:2:0.1, v/v). Enzyme activity was calculated from the percent conversion of arachidonic acid to PGH2 and its decomposition products, using a radiometric photographic system. The concentration of the compound causing 50% inhibition (IC_{50}) was calculated.

5.19. Statistical methods

Data are expressed as means with their corresponding standard errors. Data were evaluated by the one way analysis of variance. Then, the data were subjected to the least significant difference 'LSD' test.[34]

5.20. Ulcerogenic effects

Compounds **3**, **4**, **5**, **8**, **9**, **11** and **12a** that exhibited moderate to potent anti-inflammatory profiles in the pre-mentioned animal models were evaluated for their ulcerogenic potential in rats.[19] Phenylbutazone and indomethacin were tested as reference drugs. One hundred male albino rats (150–200 g) were fasted for 12 h prior to drug administration. Water was given *ad libitum*. The animals were divided into ten groups each of ten rats as follows:

Group I (control group): received 2% gum acacia orally.

Group II: received phenylbutazone suspended in 2% gum acacia at a dose of 10 mg/kg/day orally.

Group III: received indomethacin suspended in 2% gum acacia at a dose of 10 mg/kg/day orally.

Groups IV–X: received test compounds suspended in 2% gum acacia at a dose of 10 mg/kg/day orally.

The drugs were administered orally in two equal doses at 0.0 and 12 h for three successive days. Animals were sacrificed after 6 h of the last dose. The stomach from each animal was removed. An opening at the greater curvature was made and the stomach was washed with cooled saline and inspected with 3× magnifying lens for any evidence for hyperemia and haemorrhage, definite haemorrhagic errosion or ulcer. The percentage ulceration for each group was calculated as follows:

$$\%\,\text{Ulceration} = \frac{\text{Number of animals bearing ulcer in a group}}{\text{Total number of animals in the same group}} \times 100$$

5.21. Acute toxicity

The biologically significant compounds were further investigated for their approximate LD_{50} in male mice.[20,21] Eight groups of mice each consisting of six animals, were used. The compounds were given orally in doses of 1, 10, 100, 200, 250, 500 mg / kg, respectively. Twenty four h later, the% mortality in each group and for each compound was recorded. The LD_{50} values (Table 1) were calculated using the method described by Litchfield and Wilcoxon.[21]

5.22. In vitro antimicrobial activity

The microdilution susceptibility test in Müller-Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) were used for the determination of antibacterial and antifungal activity.[22] The utilized test organisms were: *Escherichia coli* (*E. coli*) ATCC 25922 as an example of Gram-negative bacteria, *Staphylococcus aureus* (*S. aureus*) ATCC 19433 as an example of Gram-Positive bacteria and *Candida albicans* (*C. albicans*) as yeast-like fungi. Ampicillin trihydrate and clotrimazole were used as standard antibacterial and antifungal agents, respectively. Solutions of the test compounds, ampicillin trihydrate and clotrimazole were prepared in DMSO at concentration of 1600 μg/mL. The two-fold dilution of the compounds were prepared (800, 400,...6.25 μg/mL). The microorganism suspensions at 10^6 CFU/mL (Colony Forming Unit / mL) concentration were inoculated to the corresponding wells. Plates were incubated at 36 °C for 24 h to 48 h. The incubation chamber was kept sufficiently humid. At the end of the incubation period, the minimal inhibitory concentrations (MIC) were determined.

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