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Design and synthesis of some substituted 1*H*-pyrazolyl-thiazolo[4,5-*d*]pyrimidines as anti-inflammatory–antimicrobial Agents☆

Adnan A. Bekhit [a,*], Hesham T.Y. Fahmy [a], Sherif A.F. Rostom [a], Azza M. Baraka [b]

[a] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria 21521, Egypt*
[b] *Department of Pharmacology, Faculty of Medicine, University of Alexandria, Alexandria 21521, Egypt*

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Abstract

The synthesis of two novel series of structurally related 1*H*-pyrazolyl derivatives of thiazolo[4,5-*d*]pyrimidines is described. All the newly synthesised compounds were examined for their in vivo anti-inflammatory activity in two different bioassays namely; cotton pellet-induced granuloma and carrageenan-induced paw edema in rats. The in vitro inhibitory activity of the most active compounds towards human COX-1 and COX-2 enzymes was also estimated. In additon, the ulcerogenic effects and acute toxicity (LD_{50}) values of these compounds 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 results revealed that compounds **5a**, **9a**, **9b**, **10b** and **12a** exhibited anti-inflammatory activity comparable to that of indomethacin in both local and systemic in vivo animal models with no or minimal ulcerogenic effects (0–10%) and high safety margin ($LD_{50} > 500$ mg kg^{-1}). In addition, most of them displayed appreciable antibacterial activities when compared with ampicillin, especially against *S. aureus*. Compounds **9a** and **12a** are the most distinctive derivatives identified in the present study because of their remarkable in vivo and in vitro anti-inflammatory activity in addition to their pronounced antibacterial activities comparable to ampicillin against Gram positive and -negative bacteria. Therefore, they are considered as successful dual anti-inflammatory–antimicrobial candidates.

Keywords: 1*H*-Pyrazoles; Thiazolo[4,5-*d*]pyrimidines; Anti-inflammatory activity; Human COX-1 and COX-2 enzymatic activity; Ulcerogenic effect; Acute toxicity; Antimicrobial activity

1. Introduction

Fast and effective relief of pain and inflammation in the human being is continued to be a major challenge for the medicinal chemistry researchers. Non-steroidal anti-inflammatory drugs (NSAIDs) are important therapeutic agents for the alleviation of pain and inflammation associated with a number of pathological conditions. A major mechanism of action of NSAIDs is lowering prostaglandin (PG) production through the inhibition of cyclo-oxygenase (COX); a key enzyme in PG biosynthesis that catalyses the conversion of arachidonic acid into PG and thromboxane [1]. Because PG has dual function; mediation of inflammation and cytoprotection in the stomach and intestine, long term usage of NSAIDs to relieve the symptoms of inflammation and pain always results in gastrointestinal (GI) damage, ulceration, heamatologic effects and nephrotoxicity [2]. Over the last two decades, a major breakthrough in the anti-inflammatory research occurred when it was discovered that COX exists in two isoforms COX-1 and COX-2, which are regulated differently [3]. COX-1 is expressed constitutively and is responsible for the synthesis of gastroprotective PG in the GI and the proaggregatory thromboxane in blood platelets [4]. COX-2 is inducible and short-lived; its expression is stimulated in response to proinflammatory mediators such as endotoxin, cytokines and mitogens. It is believed to play a major role in PG biosynthesis in inflammatory cells (monocytes and macrophages) and in the central

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* Corresponding author.
E-mail address: adnbekhit@hotmail.com (A.A. Bekhit).

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nervous system (CNS) [5]. The discovery of the inducible enzyme COX-2, together with the differential tissue distribution of the two isoforms, and the accumulating evidence that it may be possible to separate its physiological role from the constitutive enzyme COX-1 spurred the search for a super NSAID with improved therapeutic potential. Therefore, a potent and selective COX-2 inhibitor should block the production of PGs in inflammatory cells while not interfering in the homeostatic and gastroprotective actions mediated by COX-1 [6]. This finding has been substantiated by the fact that, most of the currently available NSAIDs are known to inhibit both isoforms [7].

Among the already marketed COX-2 inhibitors that comprise the pyrazole nucleus, celecoxib; 4-[5-(4-methylphenyl)-3-(trifluorophenyl)-1*H*-pyrazol-1-yl]benzenesulphonamide (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 heterocycles template that is known to inhibit selectively the COX-2 enzyme [8].

On the other hand, much attention has been focussed towards pyrazoles as antimicrobial [9,10], antiviral [11,12] and anticancer [13,14] 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 tumour cell lines [15].

The concomitant use of several drugs to treat inflammatory conditions that might be associated with some microbial infections may cause health problems especially in patients with impaired liver or kidney functions. In addition, from the pharmaco-economic point of view, and for seeking a better patient compliance, an anti-inflammatory–antimicrobial agent with minimum adverse effects and high safety margin is highly desirable.

Motivated by the aforementioned findings, and as a continuation of our investigations in the field of pyrazoles [16–22], it was designed to synthesise two novel series of pyrazole derivatives namely; 3-aryl-6-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methyleneamino]-5-substituted-2-thioxo-2,3-dihydrothiazolo[4,5-*d*]pyrimidin-7(6*H*)-ones (**A**) and their structurally related analogues 3-aryl-2-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidenehydrazono]-5-substituted-2,3-dihydrothiazolo[4,5-*d*]pyrimidin-7(6*H*)-ones (**B**) (Fig. 1); that would act as dual antimicrobial–non-acidic anti-inflammatory agents with 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, rofecoxib and Dup-697 [4]. Furthermore, the 4-position in the pyrazole ring is linked to the thiazolo[4,5-*d*]pyrimidine ring system which is considered as 7-thia analogue of the natural purine bases, adenine and guanine. Therefore, such functionality may add some biological significance to the molecules as an antimetabolite that may improve the antimicrobial activity of the target products. Moreover, it was considered of interest to link the pyrazole moiety either to the 2- or 6-position of the bicyclic thiazolo[4,5-*d*]pyrimidine ring system through different linkages to investigate the influence of such structure variation on the anticipated biological activities. It should be pointed out that, in addition to the targeted anti-inflammatory and antimicrobial activities, the ulcerogenic and acute toxicity profiles of some of the newly synthesised compounds were determined.

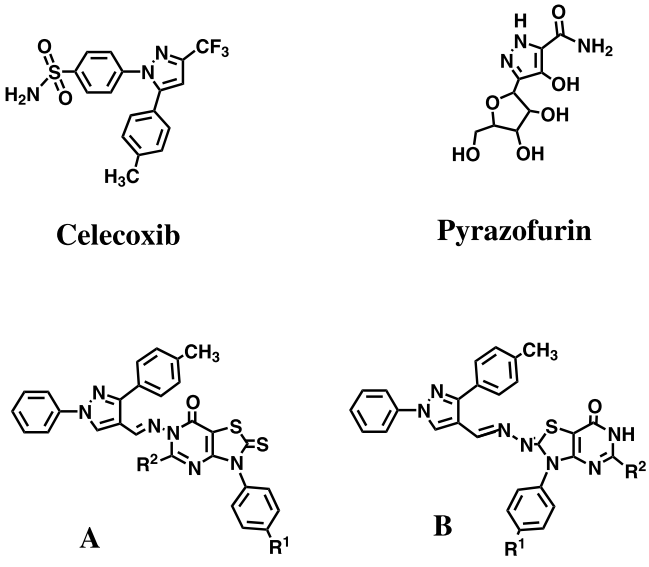

Fig. 1. Sructures of celecoxib, pyrazofurin and the novel series of pyrazole **A** and **B**.

2. Chemistry

The syntheses of the intermediate and target compounds were performed by the reactions illustrated in Fig. 2. Compound **2**, namely; *N*-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidene]-cyanoacetic acid hydrazide; was synthesised in an excellent yield by condensing 3-(4-methylphenyl)-1-phenyl-1*H*-pyrazole-4-carboxaldehyde (**1**) [23] with cyano-acetic acid hydrazide [24]. This intermediate was converted to the 4-amino-3-aryl-5-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidenehydrazinocarbonyl]-thiazole-2(3*H*)-thiones (**3a**,**b**) following the method described by Gewald [25]. It involved the reaction of the cyanoacetic acid hydrazide derivative **2** with sulphur and the appropriate aryl isothiocyanate in the presence of triethylamine as a basic catalyst. Cyclisation of **3a**,**b** to the 3-aryl-6-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-

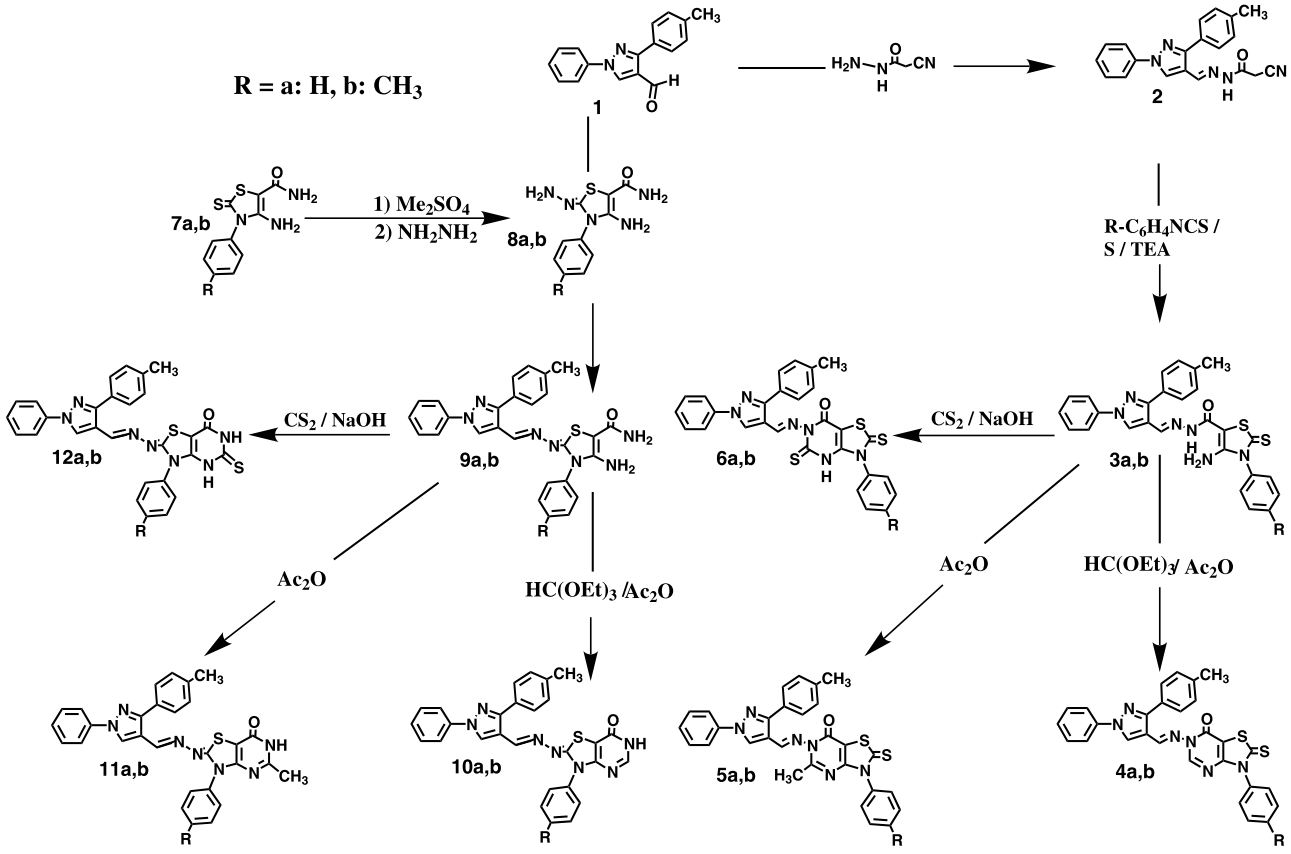

Fig. 2. Synthesis of the intermediate and target compounds.

4-methylideneamino]-2-thioxo-2,3-dihydrothiazolo[4,5-*d*]pyrimidin-7(6*H*)-ones (**4a**,**b**) was achieved by heating the former with a mixture of triethyl orthoformate and acetic anhydride (1:1). Analogously, heating **3a**,**b** with an excess of acetic anhydride yielded the corresponding 3-aryl-5-methyl-6-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylideneamino]-2-thioxo-2,3-dihydrothiazolo-[4,5-*d*]pyrimidin-7(6*H*)-ones (**5a**,**b**). Furthermore, the target compounds **6a**,**b**; 3-aryl-6-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylideneamino]-2,5-dithioxo-2,3-dihydrothiazolo[4,5-*d*]pyrimidin-7(6*H*)-ones; were obtained by cyclising the intermediates **3a**,**b** with carbon disulphide in the presence of sodium hydroxide at room temperature followed by acidification with diluted hydrochloric acid.

On the other hand, the intermediates **9a**,**b**, namely; 4-amino-3-aryl-2-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidenehydrazono]-2,3-dihydrothiazole-5-carboxamides; were obtained in excellent yields by condensing the pyrazole aldehyde **1** with the 4-amino-3-aryl-2-hydrazono-2,3-dihydrothiazole-5-carboxamides (**8a**,**b**). The latter compounds, in their turn, were synthesised following the procedure described by Gewald [26] which involves heating the 4-amino-3-aryl-2-thioxo-2,3-dihydrothiazole-5-carboxamides (**7a**,**b**) [25] with dimethyl sulphate in acetonitrile followed by reacting the intermediate thiazolium salt with hydrazine hydrate. In an analogous fashion, compounds **9a**,**b** were utilised to synthesise the thiazolo[4,5-*d*]pyrimidines **10a**,**b**, **11a**,**b** and **12a**,**b** following the same procedures adopted for the preparation of the structurally related analogues **4a**,**b**, **5a**,**b** and **6a**,**b**, 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 **3a**,**b**, **4a**,**b**, **5a**,**b**, **6a**,**b**, **9a**,**b**, **10a**,**b**, **11a**,**b** and **12a**,**b** was evaluated by applying the cotton-pellet granuloma bioassay in rats [27] using indomethacin as a reference standard. The ED_{50} values were determined for each compound (Table 1).

Out of the compounds tested, compound **10b** displayed anti-inflammatory activity (ED_{50} 8.23 μmol) superior to that of indomethacin (ED_{50} 9.17 μmol); (Table 1). Compound **12a** (ED_{50} 9.47 μmol) was nearly equipotent as indomethacin at the same dose level and test conditions. Furthermore, compounds **5b**, **6b** and **11b** exhibited moderate anti-inflammatory activities as

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^{-1})
Control	–	0.0	–
Indomethacin	9.17	100	–
3a	11.34	NT [a]	NT
3b	12.92	NT	NT
4a	12.46	NT	NT
4b	11.32	10	> 250
5a	11.60	NT	NT
5b	9.82	NT	NT
6a	13.22	NT	NT
6b	10.80	0.0	> 500
9a	7.56	10	> 500
9b	8.57	0.0	> 500
10a	13.76	NT	NT
10b	8.23	10	> 500
11a	11.52	10	> 500
11b	10.98	10	> 500
12a	9.47	0.0	> 500
12b	12.42	NT	NT

[a] NT, not tested.

revealed from their ED_{50} values (9.82, 10.8 and 10.98 μmol, respectively) (Table 1). Interestingly, the uncyclised 4-amino-3-aryl-2-[3-(4-methyl-phenyl)-1-phenyl-1*H*-pyrazol-4-methylidenehydrazono]-2,3-dihydrothiazole-5-carboxamides (**9a**,**b**) showed also distinctive anti-inflammatory pattern (ED_{50} 7.56 and 8.57 μmol, respectively) at the same dose level as compared with indomethacin (Table 1). The rest of the compounds were found to be weakly active or inactive as anti-inflammatory agents (ED_{50} values range 11.32–13.76 μmol).

3.1.2. Carrageenan-induced paw edema in rats

Compounds **5a**, **9a**, **9b**, **10b** and **12a** that showed anti-inflammatory activity comparable to that of indomethacin in the cotton pellet-induced granuloma bioassay, were further evaluated their in vivo systemic effect using the carrageenan-induced paw edema bioassay in rats [28]. The percentage protection against inflammation was calculated as follows:

Table 3
In vitro human COX-2 [a] and COX-1 [b] enzymes inhibitory activity of compounds **5a**, **9a**, **9b**, **10b** and **12a**

Test compound	COX-2 IC_{50} (μM) [c]	COX-1 IC_{50} (μM) [c]
Indomethacin	2.60	0.21
5a	0.36	95.0
9a	1.30	> 100
9b	0.17	82.6
10b	12.0	> 100
12a	0.29	> 100

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

$$V_c - V_d / V_c \times 100$$

where V_c is the increase in paw volume in the absence of test compound (control) and V_d is the increase of paw volume after injection of the test compound. Data were expressed as the mean ± S.E.M. Significant difference between the control and the treated groups was performed using Student's *t*-test and *P* values. The difference in results was considered significant when $P > 0.001$ The anti-inflammatory activity of the test compounds relative to that of indomethacin was also calculated. The results are recorded in Table 2.

The results revealed that compounds **5a**, **9a** and **10b** exhibited systemic anti-inflammatory activity (% protection 72.4, 67.8 and 70.1, respectively) comparable to that of indomethacin (% protection 75.9). On the other hand, compounds **9b** and **12a** were about 20% less active than indomethacin at the same dose level (Table 2).

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

Compounds **5a**, **9a**, **9b**, **10b** and **12a** that showed in vivo anti-inflammatory activity comparable to that of indomethacin in both cotton pellet-induced granuloma and carrageenan-induced paw edema in rats were further tested for their ability to inhibit human COX-2 and COX-1 enzymes in vitro applying the methodology of Wakitani et al. [29]. COX-1 assay was carried out using platelets microsome fraction. Human platelets

Table 2
Effect of compounds **5a**, **9a**, **9b**, **10b** and **12a** on carregeenan-induced rat paw edema (mL), percentage protection and activity relative to indomethacin

Test compound	Increase in paw edema (mL) ± S.E.M. [a,b]	% Protection	Activity relative to indomethacin
Control	0.87 ± 0.025	0.0	0.0
Indomethacin	0.21 ± 0.027	75.9	100
5a	0.24 ± 0.012	72.4	95.5
9a	0.28 ± 0.023	67.8	89.4
9b	0.34 ± 0.067	60.9	80.3
10b	0.26 ± 0.024	70.1	92.4
12a	0.31 ± 0.031	64.4	84.8

[a] S.E.M. denotes the standard error of the mean.
[b] All data are significantly different from control ($P > 0.001$).

were prepared from NSAID-free normal human volunteers according to the method of Hammarström and Falardeau [30]. 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 3.

The results clearly showed that the tested compounds exhibited very weak inhibitory activity against COX-1 enzyme (IC_{50} values between 82.6 and >100 μmol) when compared with indomethacin ($IC_{50} = 0.21$ μmol) (Table 3). On the other hand, compounds **5a**, **9a**, **9b** and **12a** revealed superior inhibitory profile against COX-2 enzyme as evidenced by their IC_{50} values (0.36, 1.3, 0.17 and 0.29 μmol, respectively), when compared with indomethacin ($IC_{50} = 2.60$ μmol) (Table 3).

3.1.4. Ulcerogenic effects

Compounds **4b**, **6b**, **9a**, **9b**, **10b**, **11a**, **11b** and **12a** that exhibited moderate to potent anti-inflammatory profiles in the pre-mentioned animal models were evaluated for their ulcerogenic potential in rats [31]. All the active compounds revealed a superior GI safety profiles (0–10% ulceration) in the population of the test animals at the oral doses of 30 μmol kg^{-1} per day, when compared with indomethacin; the reference standard drug; which was found to cause 100% ulceration under the same experimental conditions (Table 1). Although the uncyclised derivatives **9a** and **9b** displayed more potent anti-inflammatory profiles than indomethacin, yet they keep their GI safety characters (10 and 0% ulceration, respectively) (Table 1).

Gross observation of the isolated rat stomachs showed a normal stomach texture for compounds **6b**, **9b** and **12a** (0% ulceration), whereas the others showed slight hyperemia (10% ulceration).

Table 4
MICs (μg mL^{-1}) of test compounds

Test compound	*E. coli* ATCC 25922	*S. aureus* ATCC 19433	*C. albicans*
3a	200	>200	>200
3b	200	>200	>200
4a	>200	>200	>200
4b	200	100	>200
5a	100	50	>200
5b	25	12.5	200
6a	200	25	>200
6b	>200	>200	100
9a	50	12.5	>200
9b	200	100	>200
10a	50	50	>200
10b	200	>200	100
11a	200	100	100
11b	200	100	100
12a	12.5	12.5	>200
12b	200	25	50
Ampicillin	25	12.5	–
Clotrimazole	–	–	12.5

3.1.5. Acute toxicity

The biologically significant compounds were further evaluated for their approximate LD_{50} in male mice using a literature method [32,33]. 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^{-1}).

3.2. In vitro antimicrobial activity

Compounds **3a**,**b**, **4a**,**b**, **5a**,**b**, **6a**,**b**, **9a**,**b**, **10a**,**b**, **11a**,**b** 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 [34]. The minimal inhibitory concentrations (MICs, μg mL^{-1}) of the tested compounds are recorded in (Table 4).

The results revealed that most of the newly synthesised compounds exhibited promising antibacterial activities but they showed poor antifungal activity. Generally, compounds of the second series **9a**,**b**, **10a**,**b**, **11a**,**b** and **12a**,**b** exhibited more pronounced antibacterial potencies than members of the first series **4a**,**b**, **5a**,**b** and **6a**,**b**, with better activity against the Gram positive bacteria (Table 4). Out of the compounds tested, compounds **5b**, **9a**, **10a** and **12a** exhibited remarkable antibacterial activity against the Gram negative *E. coli* as compared with the broad spectrum antibiotic ampicillin (MIC 25 μg mL^{-1}). Compound **5b** was equipotent as ampicillin (MIC 25 μg mL^{-1}). The antibacterial activity of compounds **9a** and **10a** (MIC 50 μg mL^{-1}) were 50% lower than that of ampicillin against the same organism. It is worth-mentioning that, compound **12a** (MIC 12.5 μg mL^{-1}) was twice as active as ampicillin (MIC 25 μg mL^{-1}) against the same organism (Table 4).

On the other hand, compounds **5a**, **5b**, **6a**, **9a**, **10a**, **12a** and **12b** exhibited potential activity against the Gram positive *S. aureus* as compared with ampicillin (MIC 12.5 μg mL^{-1}). Compounds **5b**, **9a** and **12a** were equipotent as ampicillin (MIC 12.5 μg mL^{-1}). The antibacterial activities of compounds **6a** and **12b** (MIC 25 μg mL^{-1}) were 50% lower than the standard against the same organism. Moreover, compounds **5a** and **10a**

(MIC 50 μg mL^{-1}) were moderately active against the same organism (Table 4). The rest of the tested compounds were weakly active against both organisms with MIC values ranging between 100 and >200 μg mL^{-1} (Table 4). All the tested compounds showed weak antifungal activity against *C. albicans* (MIC values of 50–>200 μg mL^{-1}) when compared with the reference antifungal agent clotrimazole (Cansten^{R}, Bayer) (MIC 12.5 μg mL^{-1}).

Collectively, compounds **5b**, **9a**, **10a** and **12a** possessed a broad spectrum of antibacterial activities against both Gram positive and -negative bacteria but with insignificant antifungal activity.

4. Conclusion

The collective results clearly showed that compounds derived from series **B**; 3-aryl-2-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidenehydrazono]-5-substituted-2,3-dihydrothiazolo-[4,5-*d*]pyrimidin-7(6*H*)-ones exhibited better biological activities than their structurally related analogues **A**; 3-aryl-6-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylideneamino]-5-substituted-2-thioxo-2,3-dihydrothiazolo-[4,5-*d*]pyrimidin-7(6*H*)-ones (Fig. 1). Compounds **5a**, **9a**, **9b**, **10b** and **12a** revealed remarkable anti-inflammatory activity comparable to that of indomethacin in both local and systemic in vivo bioassays at the same dose level. Meanwhile, they displayed more distinct in vitro inhibitory activity against COX-2 than COX-1 when compared to indomethacin, except for compound **10b**. These findings were supported by the super GI safety profile of such compounds (0–10% ulceration). In addition, they are well tolerated by the experimental animals and showed high safety margin as revealed from its LD_{50} value (>500 mg kg^{-1}). These findings substantiate the idea that the anti-inflammatory activity of such type of compounds might be attributed to the selective inhibition of the COX-2 rather than the COX-1 enzyme. On the other hand, **9a**; 4-amino-2-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidene-hydrazono]-3-phenyl-2,3-dihydrothiazole-5-carboxamide and **12a**; 2-[3-(4-methylphenyl)-1-phenyl-1*H*-pyrazol-4-methylidenehydrazono]-3-phenyl-5-thioxo-2,3-dihydro-thiazolo[4,5-*d*]-pyrimidin-7(6*H*)-one, showed pronounced antibacterial activities comparable to ampicillin against Gram positive and -negative bacteria. Therefore, such 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 derivatisation.

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 were uncorrected. The infrared (IR) spectra were recorded on 470-Shimadzu IR 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.

5.1.1. *N-[3-(4-Methylphenyl)-1-phenyl-1H-pyrazol-4-methylidene]-cyanoacetic acid hydrazide (**2**)*

An equimolar mixture of 3-(4-methylphenyl)-1-phenyl-1*H*-pyrazole-4-carboxaldehyde (**1**) [23] (3.33 g, 10 mmol) and cyanoacetic hydrazide [24] (1 g, 10 mmol) in absolute EtOH (30 mL) was heated under reflux for 6 h. The precipitate formed after cooling was filtered, washed with EtOH, dried and recrystallised from EtOH. Yield: 4.05 g (93%); m.p. 199–200 °C. IR (cm^{-1}): 3462–3420 (NH), 2255 (CN), 1700 (C=O), 1625 (C=N). ^{1}H-NMR ($CDCl_3$): δ 2.36 (s, 3H, CH_3), 3.82 (s, 2H, CH_2–CN), 7.36–7.62 (m, 9H, Ar–*H*), 8.12 (s, 1H, pyrazole C-5–*H*), 8.71 (s, 1H, C*H*=N), 9.64 (s, 1H, N*H*-exchangeable). Anal. Calc. for $C_{20}H_{17}N_5O$: 343.39.

5.1.2. *4-Amino-3-aryl-5-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylidenehydrazinocarbonyl]thiazole-2(3H)-thiones (**3a,b**)*

To a stirred solution of **2** (3.43 g, 10 mmol), finely divided sulphur (0.32 g, 10 mmol) and Et_3N (1.25 mL) in a mixture of DMF (10 mL) and EtOH (10 mL), the appropriate aryl isothiocyanate (10 mmol) was added. The reaction mixture was heated under reflux for 4 h, during which a solid product was partially crystallised out. The reaction mixture was allowed to attain room temperature (r.t.) and the product was filtered, washed with EtOH and recrystallised from aq. DMF. IR (cm^{-1}): 3454–3286 (NH), 1681–1677 (C=O), 1635–1630 (C=N). Physical and analytical data of compound **3a**; yield: 3.9 g (76.5%); m.p. 242–243 °C. Anal. Calc. for $C_{27}H_{22}N_6OS_2$: 510.64. ^{1}H-NMR ($CDCl_3$): δ 2.35 (s,

3H, C*H*$_3$), 6.28 (s, 2H, N*H*$_2$), 7.34–7.69 (m, 14H, Ar–*H*), 7.96 (s, 1H, pyrazole C-5–*H*), 8.64 (s, 1H, C*H*=N), 8.79 (s, 1H, N*H*-exchangeable). Physical and analytical data of compound **3b**; yield: 4.3 g (82%); m.p. 234–236 °C. Anal. Calc. for $C_{28}H_{24}N_6OS_2$: 524.67. ^{1}H-NMR ($CDCl_3$): δ 2.37 (s, 3H, C*H*$_3$), 2.43 (s, 3H, C*H*$_3$), 6.31 (s, 2H, N*H*$_2$), 7.31–7.72 (m, 13H, Ar–*H*), 7.94 (s, 1H, pyrazole C-5–*H*), 8.67 (s, 1H, C*H*=N), 8.75 (s, 1H, N*H*-exchangeable).

5.1.3. 3-Aryl-6-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylidene-amino]-2-thioxo-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones (**4a**,**b**)

A solution of the appropriate **3a**,**b** (2 mmol) in a mixture of triethyl orthoformate (2.5 mL) and Ac_2O (2.5 mL) was heated under reflux for 3 h, during which the product was partially crystallised out. The reaction mixture was allowed to cool and the separated solid product was filtered, washed with EtOH, dried and recrystallised from aq. DMF. Physical and analytical data of the products are recorded in Table 5. IR (cm^{-1}): 1685–1679 (C=O), 1635–1632 (C=N). ^{1}H-NMR ($CDCl_3$) for compound **4a**: δ 2.33 (s, 3H, C*H*$_3$), 7.37–7.72 (m, 14H, Ar–*H*), 7.94 (s, 1H, pyrazole C-5–*H*), 8.62 (s, 1H, C*H*=N), 8.76 (s, 1H, thia-C$_5$–*H*). ^{1}H-NMR ($CDCl_3$) for compound **4b**: δ 2.35 (s, 3H, C*H*$_3$), 2.44 (s, 3H, C*H*$_3$), 7.33–7.75 (m, 13H, Ar–*H*), 7.92 (s, 1H, pyrazole C-5–*H*), 8.63 (s, 1H, C*H*=N), 8.78 (s, 1H, thia-C$_5$–*H*).

5.1.4. 3-Aryl-5-methyl-6-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylideneamino]-2-thioxo-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones (**5a**,**b**)

A solution of the proper **3a**,**b** (2 mmol) in Ac_2O (5 mL) was heated under reflux for 4 h. The reaction mixture was allowed to cool and the solid product separated was filtered, washed with EtOH, dried and recrystallised from aq. DMF. Physical and analytical data of the products are recorded in Table 5. IR (cm^{-1}): 1682–1680 (C=O), 1630–1628 (C=N). ^{1}H-NMR ($CDCl_3$) for compound **5a**: δ 2.32 (s, 3H, C*H*$_3$), 2.58 (s, 3H, thia-C$_5$–C*H*$_3$), 7.36–7.74 (m, 14H, Ar–*H*), 7.95 (s, 1H, pyrazole C-5–*H*), 8.61 (s, 1H, C*H*=N). ^{1}H-NMR ($CDCl_3$) for compound **5b**: δ 2.32 (s, 3H, C*H*$_3$), 2.45 (s, 3H, C*H*$_3$), 2.59 (s, 3H, thia-C$_5$–C*H*$_3$), 7.34–7.78 (m, 13H, Ar–*H*), 7.93 (s, 1H, pyrazole C-5–*H*), 8.64 (s, 1H, C*H*=N).

5.1.5. 3-Aryl-6-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylideneamino]-2,5-dithioxo-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones (**6a**,**b**)

To a stirred solution of the appropriate **3a**,**b** (5 mmol) in DMF (10 mL), carbon disulphide (0.3 mL, 5 mmol) and NaOH (0.2 g, 5 mmol) were added. The reaction mixture was stirred at r.t. for 6 h then diluted with an equal volume of water and treated with dilute HCl (pH 4). The separated solid product was filtered, washed thoroughly with water, dried and recrystallised from aq. DMF. Physical and analytical data of the products are recorded in Table 5. IR (cm^{-1}): 3445–3210 (NH), 1685–1680 (C=O), 1635–1630 (C=N). ^{1}H-NMR ($CDCl_3$) for compound **6a**: δ 2.33 (s, 3H, C*H*$_3$), 7.35–7.74 (m, 14H, Ar–*H*), 7.96 (s, 1H, pyrazole C-5–*H*), 8.65 (s, 1H, C*H*=N). ^{1}H-NMR ($CDCl_3$) for compound **6b**: δ 2.34 (s, 3H, C*H*$_3$), 2.43 (s, 3H, C*H*$_3$), 7.36–7.77 (m, 13H, Ar–*H*), 7.92 (s, 1H, pyrazole C-5–*H*), 8.66 (s, 1H, C*H*=N).

5.1.6. 4-Amino-3-aryl-2-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylidenehydrazono]-2,3-dihydrothiazole-5-carboxamides (**9a**,**b**)

A mixture of the appropriate 4-amino-2-hydrazono-3-aryl-2,3-dihydro-thiazole-4-carboxamides (**8a**,**b**) [26] (10 mmol) and the pyrazole aldehyde **1** (3.33 g, 10 mmol) in absolute EtOH (20 mL) was heated under reflux for 8 h during which a solid product was partially crystallised out. The reaction mixture was allowed to cool, the solid product was filtered and recystallised from EtOH. IR

Table 5
Physical and analytical data of compounds **4**–**6** and **10**–**12**

Compound no.	R	Yield (%)	M.p. (°C)	Molecular formula [a] (molecular weight)
4a	H	85	254–256	$C_{28}H_{20}N_6OS_2$ (520.64)
4b	CH_3	82	262–263	$C_{29}H_{22}N_6OS_2$ (534.67)
5a	H	73	252–253	$C_{29}H_{22}N_6OS_2$ (534.67)
5b	CH_3	79	271–273	$C_{30}H_{24}N_6OS_2$ (548.69)
6a	H	76	278–280	$C_{28}H_{20}N_6OS_3$ (552.70)
6b	CH_3	78	284–286	$C_{29}H_{22}N_6OS_3$ (566.73)
10a	H	85	265–267	$C_{28}H_{21}N_7OS$ (503.59)
10b	CH_3	84	258–260	$C_{29}H_{23}N_7OS$ (517.62)
11a	H	90	265–267	$C_{29}H_{23}N_7OS$ (517.62)
11b	CH_3	87	271–273	$C_{30}H_{25}N_7OS$ (531.64)
12a	H	82	283–284	$C_{28}H_{21}N_7OS_2$ (535.65)
12b	CH_3	86	276–278	$C_{29}H_{23}N_7OS_2$ (549.68)

[a] Analysed for C, H, N, S; results are within 0.4% of the theoretical values for the formulae given.

(cm^{-1}): 3450–3195 (NH), 1682–1680 (C=O), 1638–1635 (C=N). Physical and analytical data for compound **9a**; yield: 4.7 g (95.2%); m.p. 234–236 °C. Anal. Calc. for $C_{27}H_{23}N_7OS$: 493.59. ^{1}H-NMR (CF_3COOH): δ 2.33 (s, 3H, C*H*$_3$), 6.82–7.54 (m, 14H, Ar–*H*), 7.94 (s, 1H, pyrazole C-5–*H*), 8.85 (s, 1H, C*H*=N). Physical and analytical data for compound **9b**; yield: 4.95 g (97.6%), m.p. 242–243 °C. Anal. Calc. for $C_{28}H_{25}N_7OS$: 507.62. ^{1}H-NMR (CF_3COOH): δ 2.32 (s, 3H, C*H*$_3$), 2.45 (s, 3H, C*H*$_3$), 6.84–7.58 (m, 13H, Ar–*H*), 7.96 (s, 1H, pyrazole C-5–*H*), 8.89 (s, 1H, C*H*=N).

*5.1.7. 3-Aryl-2-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylidenehydrazono]-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones (**10a,b**)*

A solution of the appropriate **9a**,**b** (2 mmol) in a mixture of triethyl orthoformate (2.5 mL) and Ac_2O (2.5 mL) was heated under reflux for 3 h. The reaction mixture was worked up as described under compounds **4a**,**b**. The products were recrystallised from aq. DMF. Physical and analytical data of the products are recorded in Table 5. IR (cm^{-1}): 3150–5145 (NH), 1660–1658 (C=O), 1638–1636 (C=N). ^{1}H-NMR ($CDCl_3$) for compound **10a**: δ 2.34 (s, 3H, C*H*$_3$), 7.32–7.74 (m, 15H, Ar–*H*, N*H*), 7.93 (s, 1H, pyrazole C-5–*H*), 8.63 (s, 1H, C*H*=N), 8.77 (s, 1H, thia-C_5–*H*). ^{1}H-NMR ($CDCl_3$) for compound **10b**: δ 2.33 (s, 3H, C*H*$_3$), 2.44 (s, 3H, C*H*$_3$), 7.30–7.78 (m, 14H, Ar–*H*, N*H*), 7.94 (s, 1H, pyrazole C-5–*H*), 8.65 (s, 1H, C*H*=N), 8.77 (s, 1H, thia-C_5–*H*).

*5.1.8. 3-Aryl-5-methyl-2-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylidenehydrazono]-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones (**11a,b**)*

A solution of the proper **9a**,**b** (2 mmol) in Ac_2O (5 mL) was heated under reflux for 4 h. The reaction mixture was worked up as described under compounds **5a**,**b**. The products were recrystallised from aq. DMF. Physical and analytical data of the products are recorded in Table 5. IR (cm^{-1}): 3145–3143 (NH), 1660–1657 (C=O), 1636–1634 (C=N). ^{1}H-NMR ($CDCl_3$) for compound **11a**: δ 2.32 (s, 3H, C*H*$_3$), 2.56 (s, 3H, thia-C_5–C*H*$_3$), 7.31–7.76 (m, 15H, Ar–*H*, N*H*), 7.96 (s, 1H, pyrazole C-5–*H*), 8.64 (s, 1H, C*H*=N). ^{1}H-NMR ($CDCl_3$) for compound **11b**: δ 2.34 (s, 3H, C*H*$_3$), 2.46 (s, 3H, C*H*$_3$), 2.58 (s, 3H, thia-C_5–C*H*$_3$), 7.32–7.79 (m, 14H, Ar–*H*, *NH*), 7.94 (s, 1H, pyrazole C-5–*H*), 8.63 (s, 1H, C*H*=N).

*5.1.9. 3-Aryl-2-[3-(4-methylphenyl)-1-phenyl-1H-pyrazol-4-methylidenehydrazono]-5-thioxo-2,3-dihydrothiazolo[4,5-d]pyrimidin-7(6H)-ones (**12a,b**)*

To a stirred solution of the appropriate **9a**,**b** (5 mmol) in DMF (10 mL), carbon disulphide (0.3 mL, 5 mmol) and NaOH (0.2 g, 5 mmol) were added. Working up of the reaction mixture was performed as described under compounds **6a**,**b**. The products were recrystallised from aq. DMF. Physical and analytical data of the products are recorded in Table 5. IR (cm^{-1}): 3395, 3240, 3185 (NH), 1655–1653 (C=O), 1640–1638 (C=N). ^{1}H-NMR ($CDCl_3$) for compound **12a**: δ 2.35 (s, 3H, C*H*$_3$), 7.32–7.75 (m, 15H, Ar–*H*, N*H*), 7.95 (s, 1H, pyrazole C-5–*H*), 8.67 (s, 1H, C*H*=N). ^{1}H-NMR ($CDCl_3$) for compound **12b**: δ 2.35 (s, 3H, C*H*$_3$), 2.46 (s, 3H, C*H*$_3$), 7.35–7.79 (m, 14H, Ar–*H*, *NH*), 7.95 (s, 1H, pyrazole C-5–*H*), 8.69 (s, 1H, C*H*=N).

5.2. Anti-inflammatory activity

5.2.1. Cotton pellet-induced granuloma bioassay

Adult male Sprague–Dawley rats (120–140 g) were used. 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 (six rats each). Cotton pellet (35 ± 1 mg) cut from dental rolls were impregnated with 0.2 mL (containing 10 μmol) of a solution of the test compound in $CHCl_3$ or C_3H_6O 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^{-1}). 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 sacrified 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).

5.2.2. Carrageenan-induced paw edema in rats

Male albino rats weighing 120–150 g were used throughout the work. They were kept in the animal house under standard condition of light and temperature with free access to food and water. The animals were randomly divided into groups each of six rats. The paw edema was induced by subplanter injection of 50 μL of 2% carrageenan solution in saline (0.9%). Indomethacin and the test compounds were dissolved in DMSO and were injected subcutaneously in a dose of 10 μmol kg^{-1} body weight, 1 h prior to carrageenan injection. Dimethylsulphoxide was injected to the control group. The volume of paw edema (mL) was determined by means of water plethysmometer immediately after

injection of carrageenan and 4 h later. The increase in paw volume between time 0 and +4 h was measured. The percentage protection against inflammation was calculated. The anti-inflammatory activity of the test compounds relative to that of indomethacin was also determined.

5.2.3. 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. [29]. 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 μmol) 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 μmol) 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 μl of a stop solution composed of Et_2O–MeOH–1 M citric acid (30:4:1, v/v). After centrifugation of the mixture at $1700 \times g$ for 5 min at 4 °C, 50 μl 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 PGH_2 and its decomposition products, using a radiometric photographic system. The concentration of the compound causing 50% inhibition (IC_{50}) was calculated.

5.2.4. Ulcerogenic effects

Compounds **4b**, **6b**, **9a**, **9b**, **10b**, **11a**, **11b** and **12a** that exhibited moderate to potent anti-inflammatory profiles in the pre-mentioned animal models were evaluated for their ulcerogenic potential in rats [31]. Indomethacin was used as reference standard. Male albino rats (100–120 g) were fasted for 12 h prior to the administration of the compounds. Water was given ad libitum. The animals were divided into groups, each of six animals. Control group received 1% gum acacia orally. Other groups received indomethacin or the test compounds orally in two equal doses at 0 and 12 h for three successive days at a dose of 30 μM kg^{-1} per day. Animals were sacrificed by Et_2O 6 h after the last dose and the stomach was removed. An opening at the greater curvature was made and the stomach was cleaned by washing with cooled saline and inspected with a 3× magnifying lens for any evidence of hyperemia, hemorrhage, definite hemorrhagic erosion or ulcer. An arbitrary scale was used to calculate the ulcer index which indicates the severity of the stomach lesions [28] (Table 1). The percentage ulceration for each group was calculated as follows:

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

5.2.5. Acute toxicity

The same biologically significant compounds were further investigated for their approximate LD_{50} in male mice [32,33]. 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^{-1}, respectively. Twenty-four hours later, the percentage 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 [33].

5.3. 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 [34]. The utilised test organisms were: *E. coli* ATCC 25922 as an example of Gram negative bacteria, *S. aureus* ATCC 19433 as an example of Gram positive bacteria and *C. albicans* as yeast like fungi. Ampicillin trihydrate and clotrimazole were used as standards antibacterial and antifungal agents, respectively. Solutions of the test compounds, ampicillin trihydrate and clotrimazole were prepared in DMSO at concentration of 1600 μg mL^{-1}. The twofold dilution of the compounds were prepared (800, 400, ..., 6.25 μg mL^{-1}). The microorganism suspensions at 10^6 CFU mL^{-1} (Colony Forming Unit mL^{-1}) concentration were inoculated to the corresponding wells. Plates were incubated at 36 °C for 24–48 h. The incubation chamber was kept sufficiently humid. At the end of the incubation period, the MICs were determined controls for the DMSO microorganisms and media microorganisms were also done (Table 4).

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