Full Paper

Synthesis of Some Triazolophthalazine Derivatives for Their Anti-Inflammatory and Antimicrobial Activities

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Several novel series of triazolophthalazine derivatives namely; pyrazolylethenyltriazolophthalazinones (**4a–d**), styryltriazolophthalazinones (**5a,b**), aryloxopropenyltriazolophthalazinones (**7a,b**), pyrazolinyl- (**8a,b**), (**9a,b**) and (**10a–f**), pyrazolyl- (**11a–d**), (1,2-oxazol-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-ones (**14a,b**), triazolo[3,4-a]phthalazin-3-yl-pyridine-3-carbonitriles (**12a,b**), triazolo[3,4-a]phthalazin-3-yl)-ethylthioacetic acids (**13a,b**) and 2-aryl-5-arylamino-1*H*,5*H*-pyrazolo[2″,3″-1′,5′]imidazo[3′,4′-1,5]-1,2,4-triazolo[3,4-a]phthalazin-12(13*H*)-ones (**15a–c**) have been synthesized. The anti-inflammatory activity of representative compounds has been studied. Compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** showed anti-inflammatory activities comparable to that of the reference standard, indomethacin. They exhibit also minimal ulcerogenic effect relevant to the reference standard and were found to be non-toxic up to 120 mg/kg orally or up to 75 mg/kg through parenteral route. Concerning the antimicrobial activity; compounds **12b** and **13b** were found to be equipotent to ampicillin against *Staphylococcus aureus*, while compounds **10a** and **10f** were found to be as potent as ampicillin against *E. coli*, whereas compound **14b** exhibited equipotency to clotrimazole against *Candida albicans.* Compounds **8b**, **10f**, **11b**, **12a**, and **13b** exhibited, besides their antimicrobial activity, moderate to potent anti-inflammatory profiles. This represents a fruitful matrix for the development of a new class of dual non-acidic anti-inflammatory/antimicrobial agents.

Keywords: Acute toxicity / Anti-inflammatory/antimicrobial activities / Triazolophthalazines / Ulcerogenic effect

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Introduction

Several triazolophthalazines were found to exhibit antifungal and antibacterial activities [1–3]. In addition, phthalazines and triazolophthalazines were found to exhibit anti-inflammatory profiles [4–7]. Among the already marketed COX-2 inhibitors that comprise pyrazole nucleus, celecoxib (4-[5-(4-tolyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide), 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 as selective COX-2 enzyme inhibitor [8]. The discovery that the naturally occurring pyrazole C-nucleoside, pyrazofurin (4-hydroxy-3-β-D-ribofuranosyl-1*H*-pyrazole-5-carboxamide), possesses broad spectra of antimicrobial and antiviral activities in addition of being active against several tumor cell lines [9, 10] has enlightened the way for many pyrazole derivatives to be screened for comparable activities [11–15]. Motivated by the afore-mentioned findings, and as a continuation of our ongoing program in the field of anti-inflammatory/antimicrobial agents [16–21], it was designed to synthesize novel series of pyrazole derivatives that would have dual function with minimal GI disorders and high safety margin. For this reason, in addition of subjecting the target compounds to anti-inflammatory and antimicrobial screening, the ulcerogenic and acute toxicity profiles were also determined. Some substituted isoxazole derivatives

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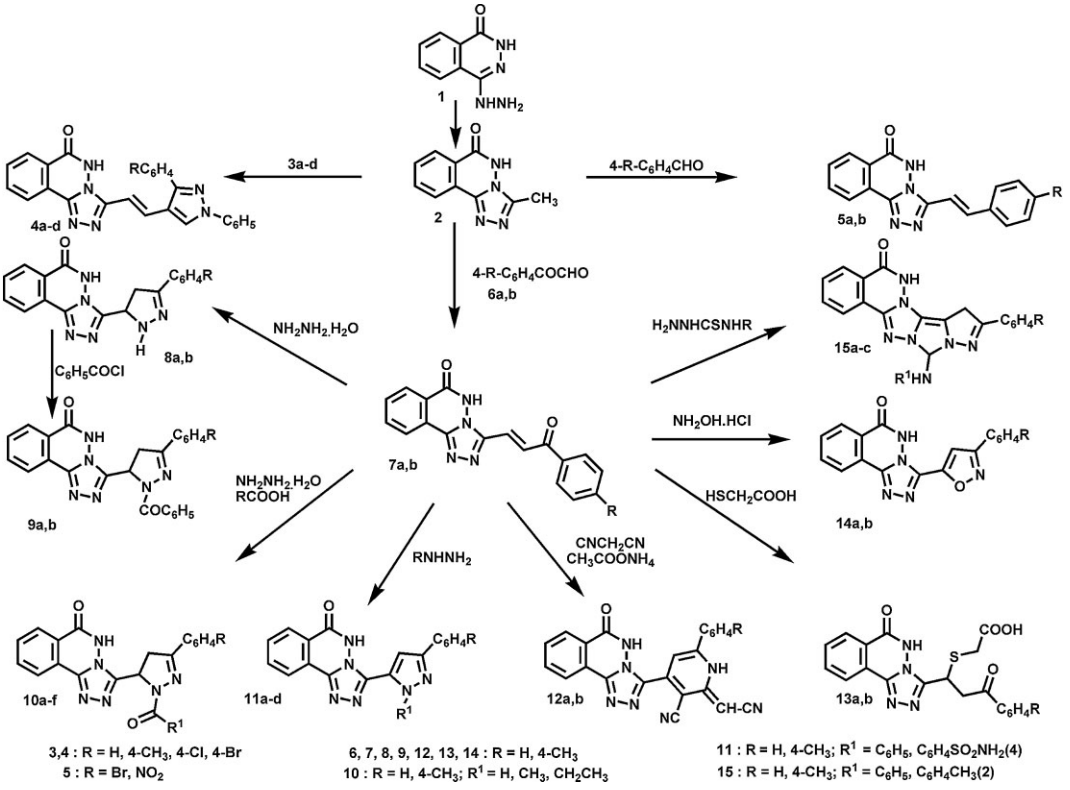
were found to possess anti-inflammatory [22, 23] and antimicrobial [24, 25] activities. This prompted us to synthesize compounds having both triazolophthalazine and isoxazole ring systems hoping to obtain compounds with dual anti-inflammatory and antimicrobial activities. Moreover, design to synthesize several pyridyltriazolophthalazinones was rationalized on the fact that some pyridine derivatives were reported to possess anti-inflammatory [26, 27] and antimicrobial activities [1, 28]. Furthermore, arylacetic acid derivatives as indomethacin and ibuprofen are receiving intensive interest as anti-inflammatory agents [29]. Therefore, it became of interest to synthesize triazolophthalazylthioacetic acid derivatives hoping to go a step forward in the field of anti-inflammatory agents.

Results and discussion

Chemistry

To achieve the synthesis of the target compounds, the steps outlined in Scheme 1 were adopted. Thus, reaction of 1-hydrazinophthalazinone **1** with acetic anhydride afforded the key starting material, 3-methyl-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-one **2** [30], which was reacted with 1,3-diarylpyrazole-4-carbaldehydes **3a-d** yielding 3-[2-(1,3-diaryl-1*H*-pyrazol-4-yl)ethenyl]-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-ones (**4a–d**). Refluxing **2** with 4-substituted benzaldehydes afforded 3-(4-substituted styryl)-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-ones (**5a,b**), whereas refluxing **2** with 4-substituted arylglyoxals afforded 3-(3-aryl-3-oxopropenyl)-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-ones (**7a,b**). The latter compounds were subjected to cyclization using hydrazine hydrate to yield 3-(3-aryl-2-pyrazolin-5-yl)-1,2,4-triazolo[3,4-*a*]phthalazin-6(5*H*)-ones (**8a,b**). These were subjected to benzoylation giving rise to 3-(3-aryl-1-benzoyl-2-pyrazolin-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-ones (**9a,b**). Refluxing **7a,b** with hydrazine hydrate in excess carboxylic acids afforded 3-(1-formyl or acyl-3-aryl-2-pyrazolin-5-yl)-1,2,4-triazolo[3,4-*a*]phthalazin-6(5*H*)-ones (**10a–f**). In addition, refluxing **7a,b** with substituted hydrazines yielded 3-(1-substituted 3-aryl-1*H*-pyrazol-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-ones (**11a–d**), with malononitrile/ammonium acetate afforded 6-aryl-2-cyanomethylidene-4-(6-oxo-5,6-dihydro-1,2,4-triazolo[3,4-a]phthalazin-3-yl)-1,2-dihydropyridine-



Scheme 1. Synthetic pathway.

3-carbonitriles (**12a,b**), with hydroxylamine 3-(3-aryl-1,2-oxazol-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5*H*)-ones (**14a,b**) were obtained whereas, with substituted thiosemicarbazides yielded 2-aryl-5-arylamino-1*H*,5*H*-pyrazolo[2″,3″-1′,5′]imidazo[3′,4′-1,5]-1,2,4-triazolo[3,4-a]phthalazin-12(13*H*)-ones (**15a–c**). Moreover, the prepared chalcones **7a,b** were allowed to react with thioglycolic acid to give 2-(4-substituted benzoyl)-1-(6-oxo-5,6-dihydro-1,2,4-triazolo[3,4-*a*]phthalazin-3-yl)ethylthioacetic acids (**13a,b**). The structures of the newly synthesized compounds were confirmed by IR, ^{1}H-NMR, microanalyses as well as mass spectra for some representative examples.

^{1}H-NMR of compound **4d** was characterized by the presence of two doublets at 7.31 and 7.84 (J = 16.8 Hz) assigned for CH=CH, indicating E configuration.

^{1}H-NMR of **5a** showed two doublets at 7.38 and 7.46 ppm due to CH = CH. ^{1}H-NMR of **7a** showed two triplets and a doublet assigned for phenyl protons, two doublets at 8.25 and 8.52 ppm characteristic for CH=CH.

^{1}H-NMR of **8b** showed a singlet at 2.33 assigned for CH_3 protons and two double doublets at 3.48 and 3.80 ppm assigned for pyrazoline-C_4 protons and another double doublet at 5.34 ppm characteristic for pyrazoline-C_5-H.

IR spectra of **9a**,**b** showed additional C=O absorption band at 1694-1692 cm^{-1} not found in their precursors due to the benzoyl C=O. ^{1}H-NMR of **9a** showed two double doublets at 3.57 and 3.95 characteristic for pyrazoline-C_4 protons and another double doublet at 6.24 assigned for pyrazoline-C_5-H, a triplet at 7.52, a multiplet between 7.71–7.74 and a doublet at 7.85 ppm assigned for benzoyl protons.

IR spectra of **10a–f** showed additional C=O absorption band or the two C=O absorption bands overlapped together and appeared as broad band. ^{1}H-NMR of **10a** showed a singlet at 8.10 ppm for CHO, ^{1}H-NMR of **10b** and **10e** showed additional singlet-not found in their precursors-assigned for $COCH_3$, whereas ^{1}H-NMR of **10f** showed a triplet at 1.00 and a quartet at 2.69 ppm characteristic for $COCH_2CH_3$.

^{1}H-NMR of **11a** showed two triplets and a doublet assigned for *N*-phenyl protons, a multiplet assigned for the second phenyl protons and a singlet at 7.63 ppm characteristic for pyrazole-C_4-H. ^{1}H-NMR of the aminosulphonyl derivative **11b** showed two multiplets assigned for phenyl protons, two doublets at 7.58 and 7.88 ppm assigned for $C_6H_4SO_2$- and a deuterium exchangeable singlet assigned for NH_2.

IR spectra of **12a,b** showed two C≡N at 2259-2255 and 2213-2208 cm^{-1} absorption bands in addition to NH, C=O and C=N absorption bands. ^{1}H-NMR spectra of **12a** and **12b** showed two singlets at 6.92–7.02 and 7.32–7.40 ppm assigned for =CH and pyridine C_5 protons, respectively. MS of **12a** and **12b** showed the molecular ion peaks at *m*/*z* 403 and 417, respectively, which are also the base peaks.

IR spectra of **13a,b** revealed OH broad absorption band between 3100 and 2300 and acid C=O band at 1718–1715 cm^{-1}. Ketone C=O and phthalazinone C=O overlapped together and appeared as one band at 1686-1683 cm^{-1}. ^{1}H-NMR of **13b** showed a singlet at 3.51 ppm assigned for SCH_2 proton, and three double doublets; two assigned for CH_2CO and the third assigned for CH proton. MS of **13a** did not show the molecular ion peak (M^+) at *m*/*z* 408 and showed the base peak at *m*/*z* 287. FAB mass spectrum of **13a** showed a peak at *m*/*z* 409 corresponding to (M^+ + H).

IR spectra of **14a,b** showed several stretching C–O–C vibration bands due to isoxazole in addition to the band due to NH, C=O and C=N. ^{1}H-NMR of **14b** showed a singlet at 8.02 ppm assigned for isoxazole C_4-H.

^{1}H-NMR of **15b** showed two singlets at 2.23 and 4.19 ppm assigned for CH_3 and CH_2 protons and two deuterium exchangeable singlets at 12.57 and 12.95 assigned to two NH protons, while that of **15c** revealed three singlets at 2.25, 2.32 and 4.19 ppm assigned for two CH_3 groups and CH_2 protons, respectively. MS of **15a**, **15b**, and **15c** showed the molecular ion peaks at *m*/*z* 433, 447 and 461 corresponding to $C_{25}H_{19}N_7O$, $C_{26}H_{21}N_7O$ and $C_{27}H_{23}N_7O$, respectively, which are also the base peaks.

Biology

Anti-inflammatory activity

The anti-inflammatory activity of twenty two representatives of the synthesized compounds **2**, **7b**, **8a,b**, **10a–f**, **11a–d**, **12a,b**, **13b**, **14a,b**, and **15a–c** was 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 synthesized compounds altering the course of inflammation. This was assessed by determining their effects on the inflammatory exudate parameters: Exudate volume, total leukocyte cell count (TLC), differential leukocyte cell count (DLC), reduced cytochrome C and interleukin-1 beta (IL-1β) levels (Tables 1 and 2).

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.

Effect on exudate volume

Results presented in Table 1 revealed that pre-treatment with indomethacin significantly reduced the exudate volume in

Table 1. Effect of inflammation induced by sponge implantation, in drug-pretreated rats, on non-immunological parameters

Compd.	Exudate volume[a] X (± SE)	TLC[b] X (± SE)	DLC (neutrophil %)[c] X (± SE)
Control	0.349 (± 0.016)	261.82 (± 17.24)	89.32 (± 3.37)
Indomethacin	0.221 (± 0.012)	122.38 (± 13.21)	47.16 (± 2.16)
2	0.319 (± 0.015)	231.17 (± 16.28)	76.39 (± 3.37
7b	0.316 (± 0.015)	251.24 (± 16.28)	81.56 (± 3.21)
8a	0.308 (± 0.014)	247.29 (± 17.21)	77.26 (± 3.16)
8b	0.256 (± 0.012)*	139.42 (± 12.28)*	48.27 (± 2.24)*
10a	0.310 (± 0.016)	241.56 (± 15.27)	84.16 (± 3.56)
10b	0.299 (± 0.014)	237.26 (± 16.51)	72.16 (± 3.39)
10c	0.248 (± 0.013)*	143.18 (± 13.16)*	51.23 (± 2.21)*
10d	0.332 (± 0.017)	199.26 (± 15.37)	76.37 (± 4.10)
10e	0.298 (± 0.015)	233.63 (± 16.27)	79.36 (± 3.57)
10f	0.236 (± 0.013)*	143.25 (± 14.21)*	52.39 (± 2.63)*
11a	0.329 (± 0.014)	228.36 (± 16.28)	75.36 (± 3.27)
11b	0.234 (± 0.011)*	137.52 (± 14.23)*	49.28 (± 2.37)*
11c	0.315 (± 0.017)	205.33 (± 16.37)	80.33 (± 3.58)
11d	0.356 (± 0.016)	238.11 (± 17.28)	72.27 (± 3.36)
12a	0.264 (± 0.013)*	139.22 (± 13.16)*	53.18 (± 3.39)*
12b	0.297 (± 0.014)	209.28 (± 17.16)	71.25 (± 4.16)
13b	0.248 (± 0.013)*	147.19 (± 12.71)*	46.17 (± 3.51)*
14a	0.328 (± 0.015)	216.24 (± 16.28)	70.16 (± 3.96)
14b	0.305 (± 0.016)	213.51 (± 17.67)	78.21 (± 4.28)
15a	0.229 (± 0.011)*	129.36 (± 14.11)*	52.31 (± 2.28)*
15b	0.307 (± 0.017)	213.29 (± 17.51)	74.36 (± 3.36)
15c	0.332 (± 0.018)	213.26 (± 17.86)	72.18 (± 3.91)

$P > 0.05$ unless otherwise stated; *$P < 0.001$. [a] Exudate Volume (expressed in mL). [b] Total leukocytic count (TLC) (expressed as cell/cm^3). [c] Differential leukocytic count (DLC) (expressed as exudate neutrophil %).

comparison to the inflammatory control group ($P < 0.001$). The mean values were 0.221 ± 0.012 and 0.349 ± 0.016 mL respectively.

Pre-treatment with compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** significantly reduced the exudate volume as compared to the inflammatory control group ($P < 0.001$). The mean values were 0.256 ± 0.012, 0.248 ± 0.013, 0.236 ± 0.013, 0.234 ± 0.011, 0.264 ± 0.013, 0.248 ± 0.013, and 0.229 ± 0.011 mL, respectively, compared to a control mean value of 0.349 ± 0.016 mL.

Effect on total leukocyte cell count (TLC)

From Table 1 it is clearly shown that pre-treatment with indomethacin significantly reduced the exudate TLC (122.38 ± 13.21) as compared to the inflammatory control group (261.82 ± 17.24) cell/cm^3 ($P < 0.001$). Pre-treatment with compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** significantly reduced the exudate TLC as compared to the inflammatory control group ($P < 0.001$). The mean values were 139.42 ± 12.28, 143.18 ± 13.16, 143.25 ± 14.21, 137.52 ± 14.23, 139.22 ± 13.16, 147.19 ± 12.71, and 129.36 ± 14.11 cell/cm^3, respectively, compared to a control mean value of 261.82 ± 17.24 cell/cm^3.

Effect on differential leukocyte cell count (DLC)

Table 1 clearly shows that pre-treatment with indomethacin significantly reduced the exudate neutrophil % (47.16 ± 2.16%) as compared to the inflammatory control group (89.32 ± 3.37%) ($P < 0.001$). Pre-treatment with compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** significantly reduced the exudate neutrophil %. The mean values were 48.27 ± 2.24, 51.23 ± 2.21, 52.39 ± 2.63, 49.28 ± 2.37, 53.18 ± 3.39, 46.17 ± 3.51, and 52.31 ± 2.28%, compared to a control mean value of 89.32 ± 3.37%.

Effect on neutrophil phagocyte function

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) was estimated. Results are expressed as nmol O^{2-}/(2×10^6 PMN h). Table 2 revealed that pre-treatment with indomethacin significantly reduced the unstimulated reduced cytochrome C level (0.711 ± 0.012) as compared to the inflammatory control group (1.416 ± 0.019) ($P < 0.001$). Results showed that pre-treatment with the investigated compounds **8b**, **10c**, **10f**, **11b**,

Table 2. Effect of inflammation induced by sponge implantation, in drug-pretreated rats, on immunological parameters[a,b]

Compd.	Unstimulated reduced cytochrome C levels *X* (± SE)	Stimulated reduced cytochrome C levels *X* (± SE)	Unstimulated IL-1β levels *X* (± SE)	LPS stimulated IL-1β levels *X* (± SE)
Control	1.416 (± 0.019)	1.516 (± 0.026)	194.36 (± 14.21)	281.21 (± 17.31)
Indomethacin	0.711 (± 0.012)	0.821 (± 0.015)	106.21 (± 6.29)	141.29 (± 7.28)
2	1.173 (± 0.019)	1.282 (± 0.022)	175.24 (± 13.31)	263.41 (± 16.31)
7b	1.316 (± 0.018)	1.392 (± 0.021)	173.27 (± 13.51)	255.17 (± 16.53)
8a	1.256 (± 0.019)	1.361 (± 0.024)	181.21 (± 13.57)	259.24 (± 16.27)
8b	0.781 (± 0.011)*	0.856 (± 0.015)*	120.36 (± 5.38)*	164.18 (± 7.36)*
10a	1.217 (± 0.018)	1.311 (± 0.025)	176.17 (± 14.59)	236.39 (± 17.27)
10b	1.196 (± 0.017)	1.286 (± 0.026)	169.28 (± 13.36)	206.26 (± 16.27)
10c	0.796 (± 0.013)*	0.854 (± 0.015)*	119.37 (± 7.35)*	163.62 (± 6.28)*
10d	1.186 (± 0.016)	1.256 (± 0.024)	166.36 (± 12.56)	232.41 (± 16.21)
10e	1.213 (± 0.019)	1.326 (± 0.025)	174.27 (± 13.81)	263.72 (± 16.69)
10f	0.786 (± 0.013)*	0.868 (± 0.017)*	124.36 (± 7.23)*	171.39 (± 8.19)*
11a	1.136 (± 0.017)	1.246 (± 0.023)	168.45 (± 13.28)	227.26 (± 15.21)
11b	0.786 (± 0.014)*	0.912 (± 0.016)*	122.76 (± 7.26)*	176.34 (± 8.16)*
11c	1.263 (± 0.017)	1.326 (± 0.023)	175.86 (± 13.52)	251.36 (± 16.36)
11d	1.181 (± 0.014)	1.287 (± 0.022)	176.2 (± 14.21)	244.27 (± 16.21)
12a	0.756 (± 0.011)*	0.846 (± 0.016)*	119.36 (± 7.36)*	162.31 (± 8.16)*
12b	1.271 (± 0.018)	1.381 (± 0.023)	170.18 (± 12.56)	261.23 (± 15.22)
13b	0.781 (± 0.013)*	0.876 (± 0.014)*	118.31 (± 6.16)*	171.23 (± 18.24)*
14a	1.296 (± 0.019)	1.376 (± 0.023)	187.36 (± 13.91)	235.17 (± 15.28)
14b	1.196 (± 0.018)	1.273 (± 0.022)	194.36 (± 14.21)	246.23 (± 14.37)
15a	0.806 (± 0.013)*	0.891 (± 0.016)*	175.24 (± 13.31)*	186.22 (± 8.18)*
15b	1.168 (± 0.017)	1.251 (± 0.021)	173.27 (± 13.51)	256.24 (± 17.11)
15c	1.127 (± 0.018)	1.234 (± 0.023)	176.17 (± 14.59)	266.16 (± 16.18)

$P > 0.05$ unless otherwise stated; $^{*}P < 0.001$. [a] Neutrophil Phagocyte Function (expressed in nmol $O^{-2}/2X10^{6}$ 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.

12a, **13b**, and **15a** significantly reduced the cytochrome C level as compared to the inflammatory control group ($P < 0.001$). The mean values were 0.781 ± 0.011, 0.796 ± 0.013, 0.786 ± 0.013, 0.786 ± 0.014, 0.756 ± 0.011, 0.781 ± 0.013, and 0.806 ± 0.013, respectively, compared to a control value of 1.416 ± 0.019.

Furthermore, results clearly show that pre-treatment with indomethacin significantly reduced the stimulated cytochrome C level (0.821 ± 0.015) as compared to the inflammatory control group (1.516 ± 0.026) ($P < 0.001$). Pre-treatment with compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** significantly decreased the stimulated reduced cytochrome C level in comparison to the inflammatory control group ($P < 0.001$). Their mean value were 0.856 ± 0.015, 0.854 ± 0.015, 0.868 ± 0.017, 0.912 ± 0.016, 0.846 ± 0.016, 0.876 ± 0.014, and 0.891 ± 0.016, respectively, compared to a control mean value of 1.516 ± 0.026.

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.

Results, presented in Table 2, revealed that pre-treatment with indomethacin significantly decreased the unstimulated IL-1β level (106.21 ± 6.29) as compared to the inflammatory control group (194.36 ± 14.21) ($P < 0.001$). Pre-treatment with compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** significantly decreased the unstimulated IL-1β levels in comparison to the inflammatory control group ($P < 0.001$). The mean values were 120.36 ± 5.38, 119.37 ± 7.35, 124.36 ± 7.23, 122.76 ± 7.26, 119.36 ± 7.36, 118.31 ± 6.16, and 175.24 ± 13.31, respectively, compared to a control mean value of 194.36 ± 14.21. Results presented in Table 2 also showed that pre-treatment with indomethacin significantly decreased the LPS-stimulated IL-1β levels (141.29 ± 7.28) as compared to the inflammatory control group (281.21 ± 17.31) ($P < 0.001$). Pre-treatment with compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** significantly decreased the LPS-stimulated IL-1β levels in comparison to the inflammatory control group ($P < 0.001$). The mean values were 164.18 ± 7.36,

163.62 ± 6.28, 171.39 ± 8.19, 176.34 ± 8.16, 162.31 ± 8.16, 171.23 ± 8.24, and 186.22 ± 8.18, respectively, compared to a control mean value of 281.21 ± 17.31.

It can be concluded 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. Results revealed that pre-treatment with the investigated compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** significantly altered the inflammatory markers, not only, the non-immunological parameters (exudate volume, TLC and DLC), but also the immunological ones (reduced cytochrome C and IL-1β levels). Therefore, the indicated compounds are considered capable of modulating the inflammatory response and are assumed to have *in vivo* anti-inflammatory activity similar to that of indomethacin.

Ulcerogenic effects

Compounds which exhibited moderate to potent anti-inflammatory profiles in the pre-mentioned animal models (**8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a**) were evaluated for their ulcerogenic potential in rats [31]. Phenylbutazone and indomethacin were tested as reference drugs. It was found that the incidence of ulcers with these compounds was 30%, 30%, 20%, 40%, 10%, 20%, and 30%, respectively, compared with phenylbutazone (60% ulceration) or indomethacin (100% ulceration).

Acute toxicity

Compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** which showed promising anti-inflammatory activities were further investigated for their oral as well as parenteral route acute toxicity in male mice [32, 33]. All of the tested compounds proved to be non-toxic up to 120 mg/kg when given orally. In addition, the same compounds proved to be non-toxic up to 75 mg/kg when administered parenterally.

It is clear that compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** under test were found to exhibit minimum ulcerogenic activity as compared to phenylbutazone and indomethacin. They are well tolerated by experimental animals and showed high safety margin as revealed from their LD_{50} 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.

It is to be noted down that the tolylpyrazoline derivative **8b** showed anti-inflammatory activity whereas its phenyl analogue **8a** was devoid of activity. Similarly, for the tolylthioacetic acid derivative **13b** against its phenyl analogue **13a**. On the other hand, its phenylpyridyl derivative **12a** was found to elicit anti-inflammatory activity whereas its tolyl analogue **12b** was not. Among the pyrazole derivatives **11a–d** only **11b** which contains a sulphone moiety was active anti-inflammatory agent. Among the acylpyrazoline series **10a–f**, the propanoyl derivatives **10c** and **10f** were active whereas their formyl and acetyl derivatives were not.

Antimicrobial activity

Twenty representative compounds were selected for antimicrobial screening by measuring their minimal inhibitory concentration (MIC) against *Staphylococcus aureus* as Gram-positive bacteria, *Escherichia coli* as Gram-negative bacteria and *Candida albicans* as a fungal strain (Table 3) [34].

It deserves mentioning that the tolylpyridyl derivative **12b** was found to be as potent as ampicillin against *S. aureus* (MIC 12.5 μg/mL), whereas its phenyl analogue **12a** was less active. Moreover, both **12a** and **12b** exhibited half the potency of the reference against *E. coli*. Among the acylpyrazoline series **10a–f**, the formylpyrazoline **10a** and **10d** exhibited half the potency of the reference standard against *S. aureus*, However, the formyl derivative **10a** and propanoyl derivative **10f** were found to be as potent as ampicillin against *E. coli* and the acetyl analogue **10e** exhibited half the potency of the reference against *E. coli*. Among the pyrazole series **11a–d**, **11c** exhibited half the potency of the reference standard *S. aureus*,

Table 3. Minimum inhibitory concentrations (MIC) of representative compounds.

Compd.	MIC (μg/mL)		
	E. coli	***S. aureus***	***C. albicans***
7b	100	>200	>200
8b	50	>200	>200
10a	25	25	100
10b	>200	100	50
10c	>200	100	100
10d	>200	25	>200
10e	50	100	100
10f	25	100	>200
11a	>200	>200	50
11b	50	>200	100
11c	>200	25	>200
11d	>200	50	>200
12a	50	50	>200
12b	50	12.5	>200
13b	50	12.5	>200
14a	>200	>200	25
14b	>200	>200	12.5
15a	100	>200	50
15b	>200	50	>200
15c	50	50	>200
Ampicillin	25	12.5	–
Clotrimazole	–	–	12.5

whereas **11b** exhibited half the potency of the reference standard against *E. coli.*

The tolylthioacetic acid derivative **13b** was found to be as potent as ampicillin against *S. aureus* but it showed half its potency against *E. coli*. The most active compound against *C. albicans* was the isoxazolyl derivative **14b** which was found to be equipotent to clotrimazole, whereas the 3-phenyl analogue **14a** showed half its potency. The pyrazoline **8b** exhibited half the potency of ampicillin against *E. coli*. Moreover, only the tolylpyrazoloimidazotriazolophthalazinone **15c** showed half the potency of the reference against *E. coli*.

It can be concluded that compounds **8b**, **10f**, **11b**, **12a**, and **13b** possess dual anti-inflammatory/antimicrobial activities. These compounds would represent a fruitful matrix for the development of a new class of dual non-acidic anti-inflammatory/antimicrobial agents.

Experimental

Chemistry

Melting points were performed on a Gallen Kamp apparatus and are uncorrected. IR spectra (KBr) were measured on a Perkin-Elmer 1430 Spectrophotometer. ^{1}H-NMR spectra were measured on a Varian Gemini 200 spectrometer, 200 MHz with TMS as internal standard. The chemical shifts are given in δ (ppm). Microanalyses were performed at the Microanalytical Unit, Faculty of Science, Assiut University, Egypt. All the values of C, H, N, and S were within $\pm$0.4% of the calculated data.

3-[2-(1,3-Diaryl-1H-pyrazol-4-yl)ethenyl]1,2,4-triazolo[3,4-a]phthalazin-6(5H)-ones ***4a–d***

A mixture of **2** (0.4 g, 2 mmol) and the appropriate **3** (2 mmol) in Ac_2O (5 mL) was heated under reflux for 5 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried, and crystallized from gl. HOAc (Table 4). IR (KBr, cm^{-1}): 3413–

Table 4. Physicochemical data of compounds **4–10**

Compd.	R	R^1	Yield (%)	M.p. (°C) Cryst. solv.	Mol. formula (Mol. wt.)
4a	H	–	51	>300	$C_{26}H_{18}N_6O$ (430.47)
4b	CH_3	–	53	>300	$C_{27}H_{20}N_6O \cdot {}^1/_2\ H_2O$ (453.51)
4c	Cl	–	63	>300	$C_{26}H_{17}ClN_6O \cdot {}^1/_2\ H_2O$ (473.93)
4d	Br	–	70	>300	$C_{26}H_{17}\ BrN_6O \cdot {}^1/_2\ H_2O$ (518.38)
5a	Br	–	63	>300	$C_{17}H_{11}BrN_4O$ (367.21)
5b	NO_2	–	87	>300	$C_{17}H_{11}N_5O_3$ (333.31)
7a	H	–	70	>300	$C_{18}H_{12}N_4O_2$ (316.32)
7b	CH_3	–	70	>300	$C_{19}H_{14}N_4O_2$ (330.35)
8a	H	–	91	>300	$C_{18}H_{14}N_6O$ (330.35)
8b	CH_3	–	90	>300	$C_{19}H_{16}N_6O$ (344.38)
9a	H	–	48	>300	$C_{25}H_{18}N_6O_2$ (434.46)
9b	CH_3	–	56	>300	$C_{26}H_{20}N_6O_2$ (448.49)
10a	H	H	75	286–288 DMF	$C_{19}H_{14}N_6O_2$ (358.36)
10b	H	CH_3	90, 91*	297–299 DMF	$C_{20}H_{16}N_6O_2$ (372.39)
10c	H	CH_2CH_3	90	188–190 DMF/H_2O	$C_{21}H_{18}N_6O_2 \cdot 2\ H_2O$ (422.45)
10d	CH_3	H	67	>300 DMF/H_2O	$C_{20}H_{16}N_6O_2 \cdot {}^1/_2\ H_2O$ (381.40)
10e	CH_3	CH_3	95, 97*	201–203 HOAc/H_2O	$C_{21}H_{18}N_6O_2 \cdot H_2O$ (404.43)
10f	CH_3	CH_2CH_3	98	188–190 EtOH/H_2O	$C_{22}H_{20}N_6O_2 \cdot 2\ H_2O$ (436.47)

* Yield according to Method B.

3127 (NH); 1663–1661 (C=O); 1638–1626 (C=N); 1599–1595, 1503–1501 (C=C); 1537–1519 (δ NH); 1415–1411, 1349–1333 (C–N lactam). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **4d**: 7.31 (d, J = 16.8 Hz, 1H, CH = CH); 7.35 (t, J = 7.6 Hz, 1H, phenyl-C_4-H); 7.53 (t, J = 7.6 Hz, 2H, phenyl-$C_{3,5}$-H); 7.67, 7.72 (two d, J = 8.4 Hz, each 2H, bromophenyl-H); 7.84 (d, J = 16.8 Hz, 1H, CH=CH); 7.85 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_8-H); 7.94 (d, J = 7.6 Hz, 2H, phenyl-$C_{2,6}$-H); 7.99 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_9-H); 8.17 (d, J = 8.4 Hz, 1H, triazolophthalazine-C_{10}-H); 8.43 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_7-H); 9.15 (s, 1H, pyrazole-C_5-H); 13.23 (s, 1H, NH, D_2O exchangeable).

3-(4-Substituted styryl)-1,2,4-triazolo[3,4-a]phthalazin-6(5H)-ones ***5a,b***

A mixture of **2** (0.4 g, 2 mmol) and *p*-bromo- or *p*-nitrobenzaldehyde (2 mmol) in Ac_2O (5 mL) was heated under reflux for 5 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried, and crystallized from the proper solvent (Table 4). IR (KBr, cm^{-1}): 3391–3327 (NH); 1660 (C=O); 1642, 1624–1618 (C=N); 1595–1594, 1515–1513, 1488 (C=C); 1554 (δ NH); 1461, 1334 (C–N lactam). For **5a**, 700 (C-Br), for **5b**, 1554, 1343 (NO_2). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Varian Gemini 200 MHz) of **5a**: 7.38, 7.46 (two d, J = 10 Hz, each 1H, CH=CH); 7.6–7.8 (m, 4H, bromophenyl-H); 7.88 (t, J = 7.8 Hz, 1H, triazolophthalazine-C_8-H); 8.02 (t, J = 7.8 Hz, 1H, triazolophthalazine-C_9-H); 8.21(d, J = 8 Hz, 1H, triazolophthalazine-C_{10}-H); 8.47 (d, J = 7.9 Hz, 1H, triazolophthalazine-C_7-H); 13.01 (s, 1H, NH, D_2O exchangeable).

3-(3-Aryl-3-oxopropenyl)-1,2,4-triazolo[3,4-a]phthalazin-6(5H)-ones ***7a,b***

A mixture of **2** (2 g, 10 mmol) and phenyl or *p*-tolylglyoxal **6a** or **b** (11 mmol) in Ac_2O (10 mL) was heated under reflux for 1 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, and crystallized from dioxane. IR (KBr, cm^{-1}): 3375–3332 (NH); 1668–1665 (C=O); 1647, 1616 (C=N); 1599–1594, 1516–1512 (C=C); 1570–1553 (δ NH); 1411, 1341–1337 (C–N lactam). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Varian Gemini 200 MHz) of **7a**: 7.62 (t, J = 7.63 Hz, 2H, phenyl-$C_{3,5}$-H); 7.72 (d, J = 7.63 Hz, 2H, phenyl-$C_{2,6}$-H); 7.85 (t, J = 7.63 Hz, 1H, phenyl-C_4-H); 7.93 (t, J = 7.6 Hz, 1H, triazolophthalazine-C_8-H); 8.07 (t, J = 7.6 Hz, 1H, triazolophthalazine-C_9-H); 8.16 (t, J = 8 Hz, 1H, triazolophthalazine-C_{10}-H); 8.25 (d, J = 7.68 Hz, 1H, CH=CH-C=O); 8.48 (t, J = 8.3 Hz, 1H, triazolophthalazine-C_7-H); 8.52 (d, J = 7.68 Hz, 1H, CH=CH-C=O); 13.3 (s, 1H, NH, D_2O exchangeable). MS, m/z (%) of (**7a**): 316 (16) (M^+), 287 (100).

3-(3-Aryl-2-pyrazolin-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5H)-ones ***8a,b***

To a suspension of the appropriate **7** (1 mmol) in abs. EtOH (5 mL), $NH_2NH_2 \cdot H_2O$ (0.2 g, 0.19 mL, 4 mmol) was added. The reaction mixture was heated under reflux for 30 min then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried, and crystallized from DMF/EtOH, 1:2 (Table 4). IR (KBr, cm^{-1}): 3269–3260, 3149 (NH); 1677–1663 (C=O); 1647, 1627–1626 (C=N); 1596–1595, 1519–1481 (C=C); 1532–1530 (δ NH); 1419–1412, 1333–1308 (C–N lactam). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Varian 300 MHz) of **8b**: 2.33 (s, 3H, CH_3); 3.48 (dd, J = 16.5, 10.2 Hz, 1H, pyrazoline-C_4-H, under DMSO); 3.80 (dd, J = 16.5, 6 Hz, 1H, pyrazoline-C_4-H); 5.34 (dd, J = 10.2, 6 Hz, 1H, pyrazoline-C_5-H,); 7.23, 7.59 (two d, J = 8.1 Hz, each 2H, tolyl-H); 7.84–8.24 (m, 2H, triazolophthalazine-$C_{8,9}$-H); 8.43 (d, J = 7.8 Hz, 1H, triazolophthalazine-C_{10}-H); 8.52 (d, J = 8.1 Hz, 1H, triazolophthalazine-C_7-H); 13.2 (s, 1H, NH, D_2O exchangeable).

3-(3-Aryl-1-benzoyl-2-pyrazolin-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5H)-ones ***9a,b***

To a suspension of the appropriate **8** (1 mmol) in gl. HOAc (3 mL), benzoyl chloride (0.15 g, 0.13 mL, 1.1 mmol) was added. The reaction mixture was heated under reflux while stirring for 1 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH, dried, and crystallized from DMF/EtOH (Table 4). IR (KBr, cm^{-1}): 3307 (NH); 1694–1692 (COC_6H_5); 1663 (C=O); 1644, 1631–1627 (C=N); 1599–1593, 1501 (C=C); 1533 (δ NH); 1427–1411, 1341–1313 (C–N lactam). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **9a**: 3.57 (dd, J = 17.57, 5.35 Hz, 1H, pyrazoline-C_4-H); 3.95 (dd, J = 17.57, 12.2 Hz, 1H, pyrazoline-C_4-H); 6.24 (dd, J = 12.2, 5.35 Hz, 1H, pyrazoline-C_5-H); 7.43–7.5 (m, 5H, C_6H_5); 7.52 (t, J = 6.9 Hz, 1H, benzoyl-C_4-H); 7.71–7.74 (m, 2H, benzoyl-$C_{3,5}$-H); 7.85 (d, J = 7.65 Hz, 2H, benzoyl-$C_{2,6}$-H); 7.87 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_8-H); 7.99 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_9-H); 8.19 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_{10}-H); 8.41 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_7-H); 13.28 (s, 1H, NH, D_2O exchangeable).

5.1.6. 3-(1-Formyl or acyl-3-aryl-2-pyrazolin-5-yl)-1,2,4-triazolo[3,4-a]-phthalazin-6(5H)-ones ***10a–f***

Method A:

A mixture of the appropriate **7** (1 mmol), $NH_2NH_2 \cdot H_2O$ (0.2 g, 0.19 mL, 4 mmol) and the appropriate carboxylic acid (10 mL) was heated under reflux for 8–12 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH and crystallized from the proper solvent (Table 4).

Method B:

A suspension of the appropriate **8** (1 mmol) in Ac_2O (3 mL) was heated under reflux for 1 h during which dissolution and reprecipitation occurred. The reaction mixture was allowed to cool. The obtained precipitate was filtered, washed with EtOH, and crystallized from the proper solvent. IR (KBr, cm^{-1}): 3414–3175 (NH); 1694–1691, 1660 (C=O); 1643–1621 (C=N); 1609–1595, 1520–1500 (C=C); 1555–1533 (δ NH); 1439–1412, 1336–1311 (C–N lactam). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **10a**: 2.85 (dd, J = 17.98, 6.5 Hz, 1H, pyrazoline-C_4-H); 3.20 (dd, J = 17.98, 11.85 Hz, 1H, pyrazoline-C_4-H); 5.35 (dd, J = 11.85, 6.5 Hz, 1H, pyrazoline-C_5-H); 6.61–6.7 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.01–7.10 (m, 3H, phenyl-$C_{2,6}$-H and triazolophthalazine-C_8-H); 7.16 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_9-H); 7.49 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_{10}-H); 7.65 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_7-H); 8.10 (s, 1H, CHO). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Varian Gemini 200 MHz) of **10b**: 2.33 (s, 3H, CH_3); 3.55 (dd, J = 20, 4.7 Hz, 1H, pyrazoline-C_4-H); 3.95 (dd, J = 20, 11 Hz, 1H, pyrazoline-C_4-H); 6.04 (dd, J = 11, 4.7 Hz, 1H, pyrazoline-C_5-H); 7.45–7.56 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.78–7.86 (m, 2H, phenyl-$C_{2,6}$-H); 7.88 (t, J = 7.7 Hz, 1H, triazolophthalazine-C_8-H); 8.02 (t, J = 7.7 Hz, 1H, triazolophthalazine-C_9-H); 8.21 (d, J = 7.94 Hz, 1H, triazolophthalazine-C_{10}-H); 8.44 (d, J = 7.88 Hz, 1H, triazolophthalazine-C_7-H); 13.22 (s, 1H, NH, D_2O exchangeable). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **10e**: 2.26 (s, 3H, CH_3); 2.32 (s, 3H, CH_3); 3.48 (dd, J = 18, 5.35 Hz, 1H, pyrazoline-C_4-H); 3.87 (dd, J = 18, 12.2 Hz, 1H, pyrazoline-C_4-H); 5.97 (dd, J = 12.2, 5.35 Hz, 1H, pyrazoline-C_5-H); 7.27, 7.66 (two d, J = 8 Hz, each 2H, tolyl-H); 7.85 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_8-H);

7.98 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_9-H); 8.17 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_{10}-H); 8.39 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_7-H); 13.75 (s, 1H, NH, D_2O exchangeable). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **10f**: 1.00 (t, J = 7.65 Hz, 3H, CH_2CH_3); 2.32 (s, 3H, CH_3); 2.69 (q, J = 7.65 Hz, 2H, CH_2CH_3); 3.48 (dd, J = 17.55, 5.35 Hz, 1H, pyrazoline-C_4-H); 3.86 (dd, J = 17.55, 12.2 Hz, 1H, pyrazoline-C_4-H); 5.99 (dd, J = 12.2, 5.35 Hz, 1H, pyrazoline-C_5-H); 7.26, 7.66 (two d, J = 8 Hz, each 2H, tolyl-H); 7.84 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_8-H); 7.98 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_9-H); 8.17 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_{10}-H); 8.39 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_7-H); 13.26 (s, 1H, NH, D_2O exchangeable). MS, m/z (%) of **10f**: 400 (20) (M^+), 158 (100).

*3-(1-Substituted 3-aryl-1H-pyrazol-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5H)-ones **11a–d***

A mixture of the appropriate **7** (1 mmol), phenyl hydrazine · HCl or 4-hydrazinobenzenesulphonamide · HCl (1.1 mmol) and NaOAc (0.09 g, 1.1 mmol) in abs. EtOH (10 mL) or abs. EtOH containing few drops gl. HOAc was heated under reflux for 10 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH then with H_2O, dried, and crystallized from dioxane/H_2O (Table 5). IR (KBr, cm^{-1}): 3398–3263 (NH); 1663–1661 (C=O); 1644–1643, 1627–1615 (C=N); 1599–1594, 1513–1500 (C=C); 1570–1532 (δ NH); 1411–1405, 1342–1332 (C–N lactam); for **11b** and **11d** 1386–1383, 1162–1160 (SO_2). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **11a**: 7.31 (t, J = 6.9 Hz, 1H, N-phenyl-C_4-H); 7.34–7.42 (m, 5H, phenyl-H); 7.47 (t, J = 7.6 Hz, 2H, N-phenyl-$C_{3,5}$-H); 7.63 (s, 1H, pyrazole-C_4-H); 7.86 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_8-H); 7.95 (d, J = 7.6 Hz, 2H, *N*-phenyl-$C_{2,6}$-H); 8.01 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_9-H); 8.15 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_{10}-H); 8.44 (d, J = 7.65 Hz, 1H, triazolophthalazine-C_7-H); 13.16 (s, 1H, NH, D_2O exchangeable). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **11b**: 7.32–7.4 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.43 (s, 2H, NH_2, D_2O exchangeable); 7.58, 7.88 (two d, J = 8.4 Hz, each 2H, $C_6H_4SO_2$-); 7.76 (s, 1H, pyrazole-C_4-H); 7.82–7.84 (m, 2H, phenyl-$C_{2,6}$-H); 7.85 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_8-H); 7.98 (t, J = 7.65 Hz, 1H, triazolophthalazine-C_9-H); 8.23 (d, J = 8.4 Hz, 1H, triazolophthalazine-C_{10}-H); 8.41 (d, J = 8.4 Hz, 1H, triazolophthalazine-C_7-H).

*6-Aryl-2-cyanomethylidene-4-(6-oxo-5,6-dihydro-1,2,4-triazolo[3,4-a]-phthalazin-3-yl)-1,2-dihydropyridine-3-carbonitriles **12a,b***

A mixture of the appropriate **7** (1 mmol), malononitrile (0.07 g, 1.1 mmol), and NH_4OAc (0.31 g, 4 mmol) in abs. EtOH (10 mL) was heated under reflux for 6 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH then with H_2O, dried, and crystallized from DMF/EtOH (Table 5). IR (KBr, cm^{-1}): 3380, 3235–3234, 3113–3111 (NH); 2259–2255, 2213–2208 (C≡N); 1662–1660 (C=O); 1642, 1626 (C=N); 1587, 1520, 1484 (C=C); 1551 (δ NH); 1416, 1321 (C–N lactam). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Varian 300 MHz) of **12a**: 7.02 (s, 1H, =CH); 7.40 (s, 1H, pyridine-C_5-H); 7.52–7.6 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.66–7.72 (m, 2H, phenyl-$C_{2,6}$-H); 7.93 (t, J = 7.6 Hz, 1H, triazolophthalazine-C_8-H); 8.08 (t, J = 7.6 Hz, 1H, triazolophthalazine-C_9-H); 8.22 (d, J = 7.6 Hz,

Table 5. Physicochemical data of compounds **11–15**.

Compd.	R	R[1]	Yield (%)	M.p. (°C) Cryst. solv.	Mol. formula (Mol. wt.)
11a	H	C_6H_5	75	292–294	$C_{24}H_{16}N_6O \cdot H_2O$ (422.45)
11b	H	4-$C_6H_4SO_2NH_2$	80	263–265	$C_{24}H_{17}N_7O_3S \cdot {}^1/_2\,H_2O$ (492.52)
11c	CH_3	C_6H_5	89	294–295	$C_{25}H_{18}N_6O$ (418.46)
11d	CH_3	4-$C_6H_4SO_2NH_2$	87	>300	$C_{25}H_{19}N_7O_3S \cdot 1{}^1/_2\,H_2O$ (524.56)
12a	H	–	45	>300	$C_{23}H_{13}N_7O \cdot H_2O$ (421.42)
12b	CH_3	–	47	>300	$C_{24}H_{15}N_7O \cdot {}^1/_2\,H_2O$ (426.44)
13a	H	–	59	284–286	$C_{20}H_{16}N_4O_4S$ (408.44)
13b	CH_3	–	69	276–278	$C_{21}H_{18}N_4O_4S$ (422.47)
14a	H	–	69	284–286	$C_{18}H_{11}N_5O_2 \cdot H_2O$ (347.34)
14b	CH_3	–	87	>300	$C_{19}H_{13}N_5O_2$ (343.35)
15a	H	C_6H_5	93	266–268 DMF/H_2O	$C_{25}H_{19}N_7O \cdot H_2O$ (451.49)
15b	H	$C_6H_4CH_3$(2)	95	224–226 EtOH/H_2O	$C_{26}H_{21}N_7O \cdot H_2O$ (465.52)
15c	CH_3	$C_6H_4CH_3$(2)	94	218–220 EtOH/H_2O	$C_{27}H_{23}N_7O \cdot H_2O$ (479.55)

1H, triazolophthalazine-C_{10}-H); 8.55 (d, $J = 7.6$ Hz, 1H, triazolophthalazine-C_7-H); 13.2 (s, 1H, NH, D_2O exchangeable). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Varian 300 MHz) of **12b**: 2.39 (s, 3H, CH_3); 6.92 (s, 1H, =CH); 7.14, 13.18 (two s, each 1H, 2-NH); 7.32 (s, 1H, pyridine-C_5-H); 7.37, 8.06 (two d, $J = 7.2$ Hz, each 2H, tolyl-H); 7.58 (t, $J = 7.8$ Hz, 1H, triazolophthalazine-C_8-H); 7.92 (t, $J = 7.8$ Hz, 1H, triazolophthalazine-C_9-H); 8.20 (d, $J = 8.1$ Hz, 1H, triazolophthalazine-C_{10}-H); 8.52 (d, $J = 7.5$ Hz, 1H, triazolophthalazine-C_7-H). MS, *m/z* (%) of (**12a**): 404 (28) ($M^+ + 1$), 403 (100) (M^+). MS, *m/z* (%) of (**12b**): 418 (29) ($M^+ + 1$), 417 (100) (M^+).

2-(4-Substituted benzoyl)-1-(6-oxo-5,6-dihydro-1,2,4-triazolo[3,4-a]phthalazin-3-yl)ethylthioacetic acids ***13a,b***

To a suspension of the appropriate **7** (1 mmol) in dry dioxane or gl. HOAc (10 mL), thioglycolic acid (0.18 g, 0.14 mL, 2 mmol) was added. The reaction mixture was under reflux for 12 h then filtered while hot. The obtained precipitate was washed with dioxane, dried, and crystallized from DMF/EtOH (Table 5). IR (KBr, cm^{-1}): br 3100–2300 (OH); 3100 (NH); 1718–1715 (C=O acid); 1686–1683 (C=O ketone, phthalazinone); 1625 (C=N); 1595, 1515–1514 (C=C); 1551–1532 (δ NH); 1412–1409, 1333–1331 (C–N lactam); 1279–1267, 1061–1057 (C–S–C); 1120 (C–O). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **13b**: 2.34 (s, 3H, CH_3); 3.51 (s, 2H, SCH_2); 3.86 (dd, $J = 19.15, 5.35$ Hz, 1H, CH_2CO); 4.29 (dd, $J = 19.15, 9.15$ Hz, 1H, CH_2CO); 5.02 (dd, $J = 9.15, 5.35$ Hz, 1H, CH); 7.31, 7.86 (two d, $J = 8.4$ Hz, each 2H, tolyl-H); 7.84 (t, $J = 7.7$ Hz, 1H, triazolophthalazine-C_8-H); 7.95 (t, $J = 7.7$ Hz, 1H, triazolophthalazine-C_9-H); 8.16 (d, $J = 7.7$ Hz, 1H, triazolophthalazine-C_{10}-H); 8.37 (d, $J = 7.7$ Hz, 1H, triazolophthalazine-C_7-H); 12.99 (s, 1H, NH, D_2O exchangeable). MS, *m/z* (%) of **13a**: 408 (absent), 344 (4), 317 (10), 316 (20), 288 (25), 287 (100), 272 (12), 271 (20), 213 (10), 195 (13), 140 (10), 130 (26), 129 (10), 128 (10), 105 (13), 104 (6), 103 (16), 102 (21), 77 (22), 76 (11), 51 (7). FAB mass spectrum of **13a**: $m/z = 409$ ($M^+ + H$).

3-(3-Aryl-1,2-oxazol-5-yl)-1,2,4-triazolo[3,4-a]phthalazin-6(5H)-ones ***14a,b***

A mixture of the appropriate **7** (1 mmol), $NH_2OH \cdot HCl$ (0.1 g, 1.5 mmol), and NaOH (0.08 g, 2 mmol) in *n*-butanol (5 mL) was heated under reflux for 8 h then allowed to cool. The obtained precipitate was filtered, washed with EtOH then with H_2O, dried, and crystallized from dioxane/H_2O (Table 5). IR (KBr, cm^{-1}): 3266–3213, 3188–3134 (NH); 1687–1660 (C=O); 1644, 1627 (C=N); 1596–1591, 1507–1482 (C=C); 1552–1525 (δ NH); 1407, 1340–1333 (C–N lactam); 1265–1227, 1240, 1189–1163, 1140–1104, 1086–1083 (C–O–C). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **14b**: 2.34 (s, 3H, CH_3); 7.33, 7.83 (two d, $J = 7.65$ Hz, each 2H, tolyl-H); 7.91 (t, $J = 7.6$ Hz, 1H, triazolophthalazine-C_8-H); 8.02 (s, 1H, isoxazole-C_4-H); 8.04 (t, $J = 7.6$ Hz, 1H, triazolophthalazine-C_9-H); 8.22 (d, $J = 7.6$ Hz, 1H, triazolophthalazine-C_{10}-H); 8.49 (d, $J = 7.6$ Hz, 1H, triazolophthalazine-C_7-H); 13.43 (s, 1H, NH, D_2O exchangeable). MS, *m/z* (%) of **14a**: 329 (4) (M^+), 213 (100).

2-Aryl-5-arylamino-1H,5H-pyrazolo[2′,3′-1′,5′]imidazo[3′,4′-1,5]-1,2,4-triazolo[3,4-a]phthalazin-12(13H)-ones ***15a–c***

To a mixture of **7** (1 mmol) and the appropriate thiosemicarbazide (1.1 mmol) in abs. EtOH (5 mL), a few drops conc. H_2SO_4 were added. The reaction mixture was heated under reflux for 6 h then filtered while hot. The filtrate was neutralized with $NaHCO_3$ solution. The obtained precipitate was filtered, washed with H_2O, dried, and crystallized from the proper solvent (Table 5). IR (KBr, cm^{-1}): 3378–3326, 3212–3149 (NH); 1661–1660 (C=O); 1644–1642, 1625–1617 (C=N); 1595–1593, 1517–1503 (C=C); 1551–1536 (δ NH); 1414–1403, 1338–1333 (C–N lactam). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Jeol 500 MHz) of **15b**: 2.23 (s, 3H, CH_3); 4.19 (s, 2H, CH_2); 6.44 (t, $J = 7.65$ Hz, 1H, tolyl-C_4-H); 6.81 (t, $J = 7.65$ Hz, 1H, tolyl-C_5-H); 6.92 (d, $J = 6.1$ Hz, 1H, tolyl-C_6-H); 7.36 (t, $J = 6.9$ Hz, 1H, phenyl-C_4-H); 7.4–7.51 (m, 4H, phenyl-$C_{3,5}$-H, tolyl-C_3-H and pyrazoloimidazotriazolophthalazine-C_5-H); 7.69 (d, $J = 6.9$ Hz, 2H, phenyl-$C_{2,6}$-H); 7.81 (t, $J = 7.65$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_{10}-H); 7.95 (t, $J = 7.65$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_9-H); 8.13 (d, $J = 7.65$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_8-H); 8.37 (d, $J = 7.65$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_{11}-H); 12.57, 12.95 (two s, each 1H, 2-NH, D_2O exchangeable). ^{1}H-NMR (DMSO-d_6), (δ ppm), (Varian 300 MHz) of **15c**: 2.25, 2.32 (two s, each 3H, 2 CH_3); 4.21 (s, 2H, CH_2); 6.49 (t, $J = 6.9$ Hz, 1H, *o*-tolyl-C_4-H); 6.85 (t, $J = 6.9$ Hz, 1H, *o*-tolyl-C_5-H); 6.95 (d, $J = 7.2$ Hz, 1H, *o*-tolyl-C_6-H); 7.28 (d, $J = 8$ Hz, 3H, *p*-tolyl-$C_{3,5}$-H and *o*-tolyl-C_3-H); 7.40 (m, 1H, pyrazoloimidazotriazolophthalazine-C_5-H); 7.57 (d, $J = 8$ Hz, 2H, *p*-tolyl-$C_{2,6}$-H); 7.85 (t, $J = 7.5$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_{10}-H); 7.99 (t, $J = 7.5$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_9-H); 8.17 (d, $J = 7.5$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_8-H); 8.41 (d, $J = 7.5$ Hz, 1H, pyrazoloimidazotriazolophthalazine-C_{11}-H). MS, m/z (%) of **15a**: 434 (55) ($M^+ + 1$), 433 (100) (M^+). MS, *m/z* (%) of **15b**: 448 (56) ($M^+ + 1$), 447 (100) (M^+). MS, *m/z* (%) of **15c**: 462 (41) ($M^+ + 1$), 461 (100) (M^+).

Biology

Anti-inflammatory Screening

The anti-inflammatory activity of twenty two representatives of the synthesized compounds **2**, **7b**, **8a,b**, **10a–f**, **11a–d**, **12a,b**, **13b**, **14a,b**, and **15a–c** was evaluated *in vivo* using the sponge implantation model of inflammation in rats with indomethacin as a standard.

Materials and methods

One hundred and forty-four 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 twenty four groups each of six rats as follows:

Group I (control group): Received 1% 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-Parco Pharmaceuticals, Egypt) suspended in 1% gum acacia, in a dose of 10 mg/kg body weight per day orally, divided into two equal doses, for three successive days [34]. On the third day, the first dose was administrated 30 min before sponge implantation and the second dose one hour before its removal.

Group III to XXIV (pre-treated inflammatory groups): each being treated with the appropriate synthesized compound. Each compound was suspended in 1% gum acacia, and was given in a dose of 10 mg/kg body weight per day, orally, divided into two equal doses, for three successive days, as indomethacin.

Induction of inflammation

Inflammation was induced by subcutaneous implantation of dry polyester sponge (type E_{41}-blue-kay Modeler, UK) [35]. 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 (Cereal), and were then dried in an oven (Galena, Poland) at 100°C for 1.5 h.

For implantation, 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 salon in 70% (v/v) ethanol/water [36–38]. 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 (Merrill 3/0-sterile silk suture, Ethic on, UK) [36] and the rats were kept in their cages for 6 h.

Collection of exudates

After 6 h, animals were reanesthetized with ether. The ventral incisions were quickly opened and the sponges were carefully dissected out to avoid local bleeding. Both sponges were gently squeezed with a 5-mL syringe plunger and the total exudates were collected in polyethylene centrifuge tubes containing 0.1 mL of 1.5% EDTA [36]. This was used to determine non-immunological markers, namely: Exudates volume, total and differential leukocyte cell counts, in addition to immunological markers, namely: Cytochrome C reduction test and interleukin-1 beta levels.

Cell isolation and preparations

Granulocyte cell suspension was prepared using a modification of the method of Lehrer and Cline [39].

Neutrophil phagocyte function estimation [39–43]

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 by evaluation of the cytochrome C reduction test as detected spectrophotometrically.

Interleukin-1 beta level estimation (IL-1β) [44]

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.

Stimulation of granulocytes by lipopolysaccharides (LPS) as a mitogen (lyophilized E coli, Sigma, USA) [45] 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 pipetted 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 kit.

Statistical methods

Data are expressed as means with their corresponding standard errors. Data were evaluated by the one way analysis of variance and were subjected to the least significant difference 'LSD' test [46].

Ulcerogenic effects

Compounds which exhibited moderate to potent anti-inflammatory profiles in the pre-mentioned animal models (**8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a**) were evaluated for their ulcerogenic potential in rats [31]. 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 per day orally.

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

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

The compounds were administrated orally in two equal doses at 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 X magnifying lens for any evidence for hyperemia and hemorrhage, definite hemorrhagic erosion or ulcer. The percentage ulceration for each group was calculated as follows:

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

It was found that the incidence of ulcers with these compounds was 30%, 30%, 20%, 40%, 10%, 20%, and 30%, respectively, compared with phenylbutazone (60% ulceration) or indomethacin (100% ulceration).

Acute toxicity

The promising compounds **8b**, **10c**, **10f**, **11b**, **12a**, **13b**, and **15a** were further investigated for their oral acute toxicity 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, 40, 80, 100, and 120 mg/kg, respectively. Twentyfour hours later, the % mortality in each group and for each compound was recorded.

Moreover, these compounds were tested for their toxicity through parenteral route. Groups of mice each consisting of six animals were used. The compounds or their vehicle propylene glycol (control) were given intraperitoneal injection in doses 10, 25, 50, and 75 mg/kg, respectively. Survival was followed up to 7 days.

All of the tested compounds proved to be non-toxic up to 120 mg/kg when given orally. In addition, the same compounds proved to be non-toxic up to 75 mg/kg when administered parenterally.

Antimicrobial Screening

Twenty compounds, constituting representatives of the present investigation were selected for antimicrobial screening by measuring their minimum inhibitory concentration (MIC) against *Staphylococcus aureus* as Gram-positive bacteria and *Escherichia coli* as Gram-negative bacteria as well as *Candida albicans* as a fungal strain [34].

Preparation of stock solutions

All compounds, ampicillin trihydrate and clotrimazole, were first dissolved in DMF in a stock concentration of 1 mg/mL and kept at −20°C until use.

Minimum inhibitory concentration (MIC) determination

1- For each compound four sets of two-fold dilutions of the compound in a sterile broth were prepared. In each set, the concentration gradient starts from 800 μg/mL to 1.5 μg/mL (i.e.10 tubes/set).
2- Each tube of the first set of dilution was inoculated with one drop (0.02 mL) of a diluted overnight broth culture of *Escherichia coli* (ATCC 25922) (i.e., a drop containing 10^6 colony forming unit, CFU). The second and the third sets were similarly inoculated with reference strains *Staphylococcus aureus* (ATCC 19433) and *Candida albicans*, respectively. The fourth set received no test organisms and served as broth sterility control (control **a**).
3- For each run three controls were also included testing the following:
   - The activity of ampicillin trihydrate against the reference strain of *Escherichia coli* (control **b**).
   - The activity of ampicillin trihydrate against the reference strain of *Staphylococcus aureus* (control **c**).
   - The activity of clotrimazole against the reference strain of *Candida albicans* (control **d**).
4- The tubes were incubated for 18–24 h, except for *C. albicans* where incubation was left for at least 48 h, at 37°C and examined for turbidity due to bacterial growth. Control sets were examined first. Control **a** was used as indicator of broth sterility (clear tubes) and control sets (**b–d**) were used as indicators of reproducibility of the results.
5- Tubes containing the lowest concentration of the test compound that prevents visible growth of the respective organism were judged to contain the MIC of that organism (Table 3).

The authors have declared no conflict of interest.

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