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Original article

Synthesis of some novel pyrazolo[3,4-*b*]pyridine and pyrazolo[3,4-*d*]pyrimidine derivatives bearing 5,6-diphenyl-1,2,4-triazine moiety as potential antimicrobial agents

Tarik El-Sayed Ali

Department of Chemistry, Faculty of Education, Ain Shams University, Roxy, 11711 Cairo, Egypt

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ABSTRACT

The reaction of 5,6-diphenyl-3-hydrazino-1,2,4-triazine (**1**) with bis(methylthio)methylene]malononitrile (**2**) afforded 5-amino-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-3-(methylthio)-1*H*-pyrazole-4-carbonitrile (**3**). Compound **3** reacted with thiourea to give 3,4-diaminopyrazolo[3,4-*d*]pyrimidine **5**, which was treated with benzoyl chloride to give pyrazolo[5,4,3-*kl*]pyrimido[4,3-*d*]pyrimidine **6**. Treatment of **3** with acetic anhydride produced 3-methylthio-pyrazolo[3,4-*d*]pyrimidine derivative **7**, which was allowed to react with hydrazine hydrate to give the corresponding hydrazino derivative **8**. Heterocyclization of **8** with benzoyl chloride and sodium pyruvate afforded the polyfused heterocycles **9** and **10**, respectively. Reaction of **3** with benzoylacetone yielded pyrazolo[3,4-*b*]pyridine **12**, which was allowed to react with malononitrile and acetanilide to get heterocyclic systems **13** and **14**, respectively. Interaction of **3** with cyanoacetone gave pyrazolo[3,4-*b*]pyridine **15**, which was refluxed in formic acid to yield pyrazolo[4′,3′:5,6]pyrido[4,3-*d*]pyrimidine **16**. Reaction of **3** with **2** afforded the triazinylpyrazole derivative **17**, which was reacted with hydrazine hydrate to give dipyrazolo[1,5-a:3′,4′-*d*]pyrimidine **19**. Furthermore, treatment of the latter compound with methyl anthranilate furnished tetraheterocyclic compound **21**. Structures of the products have been determined by elemental analysis and spectral studies. All compounds have been screened for their antibacterial and antifungal activities. Compounds **9**, **10**, **13**, **19** and **21** showed maximum activity comparable to the standard drugs with lower toxicity in the case of **9** and **10**.

1. Introduction

Pyrazole, pyridine and pyrimidine derivatives attracted organic chemists very much due to their biological and chemotherapeutic importance. Pyrazolopyridine, pyrazolopyrimidine and related fused heterocycles are of interest as potential bioactive molecules. They are known to exhibit pharmacological activities such as CNS depressant [1,2], neuroleptic [3] and turberculostatic [4]. Pyrazolo[3,4-*b*]pyridines were reported as antimicrobial agents [5], inhibitors of glycogen synthase kinase-3 (GSK-3) [6] and potent antitumor agents [7]. Also, pyrazolo[3,4-*d*]pyrimidines were identified as a general class of adenosine receptors [8–10]. In the literature, we have found that replacement of 1*H* of pyrazole of pyrazolo[3,4-*b*]pyridine and pyrazolo[3,4-*d*]pyrimidine systems by some other bioactive moieties drastically alter their pharmacological properties.

Moreover, in recent years, 1,2,4-triazine compounds have been reported to possess biological activities as anti-AIDS [11], anticancer [12] and antimicrobial activities [13–16]. Prompted by the varied biological activities of pyrazolo[3,4-*b*]pyridine, pyrazolo[3,4-*d*]pyrimidine derivatives and 1,2,4-triazine compounds, we envisioned our approach toward the synthesis of novel pyrazolo[3,4-*b*]pyridine and pyrazolo[3,4-*d*]pyrimidine that are fused with nitrogen heterocycles and bearing 5,6-diphenyl-1,2,4-triazine moiety to study the biological activity.

2. Results and discussions

The starting material 5-amino-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-3-(methylthio)-1*H*-pyrazole-4-carbonitrile (**3**) was prepared in good yield by the reaction of 5,6-diphenyl-3-hydrazino-1,2,4-triazine (**1**) and [bis(methylthio)methylene]malononitrile (**2**) in boiling methanol (Scheme 1). The ^{1}H NMR spectrum of the product **3** showed SCH_3 protons as a singlet signal around δ 3.17 ppm, in addition to NH_2 protons as a broad signal near δ 3.58 ppm. Also, its

E-mail address: tarik_elsayed1975@yahoo.com

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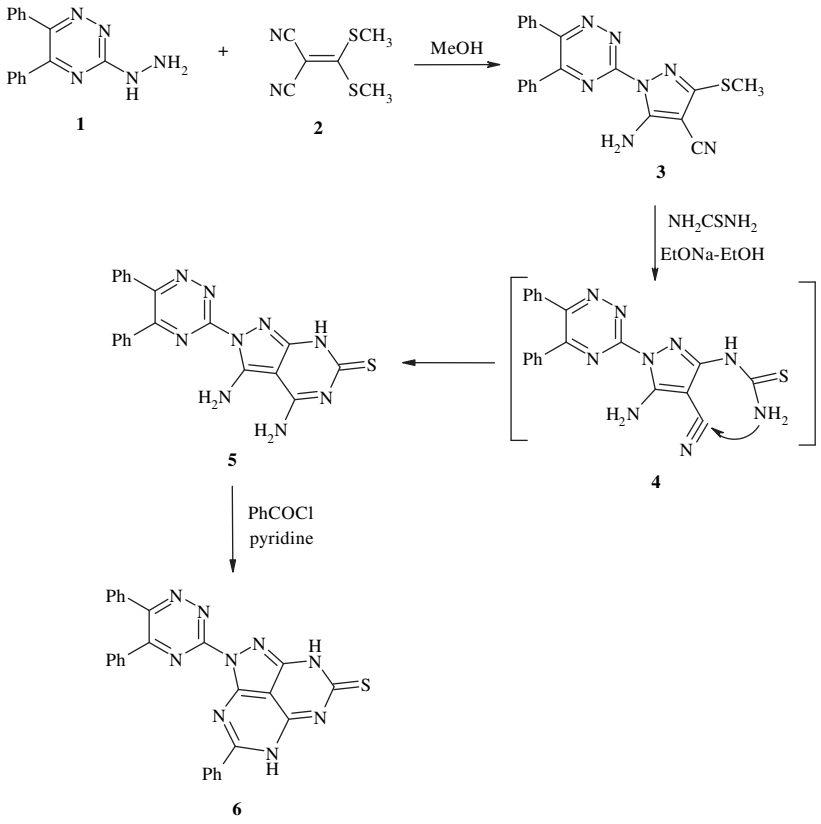


Scheme 1.

IR spectrum revealed absorptions bands due to NH_2 and C≡N groups near 3172 and 2216 cm^{-1}, respectively.

When compound **3** was treated with thiourea in ethanolic sodium ethoxide, it furnished a single product identified as 3,4-diamino-2-(5,6-diphenyl-1,2,4-triazin-3-yl)-2,7-dihydro-6*H*-pyrazolo[3,4-*d*] pyrimidine-6-thione (**5**) (Scheme 1), which was established on the basis of its elemental analysis and spectral data. For example, its IR spectrum showed absorptions bands at 3483–3219 cm^{-1} for NH_2 groups, beside disappearance of C≡N group band of compound **3**. Treatment of the diamino derivative **5** with benzoyl chloride in pyridine resulted in the formation of the fused triheterocyclic derivative **6** (Scheme 1). The ^{1}H NMR spectrum of **6** exhibited broad signals at δ 8.91 and 10.68 ppm due to NH protons [17–19].

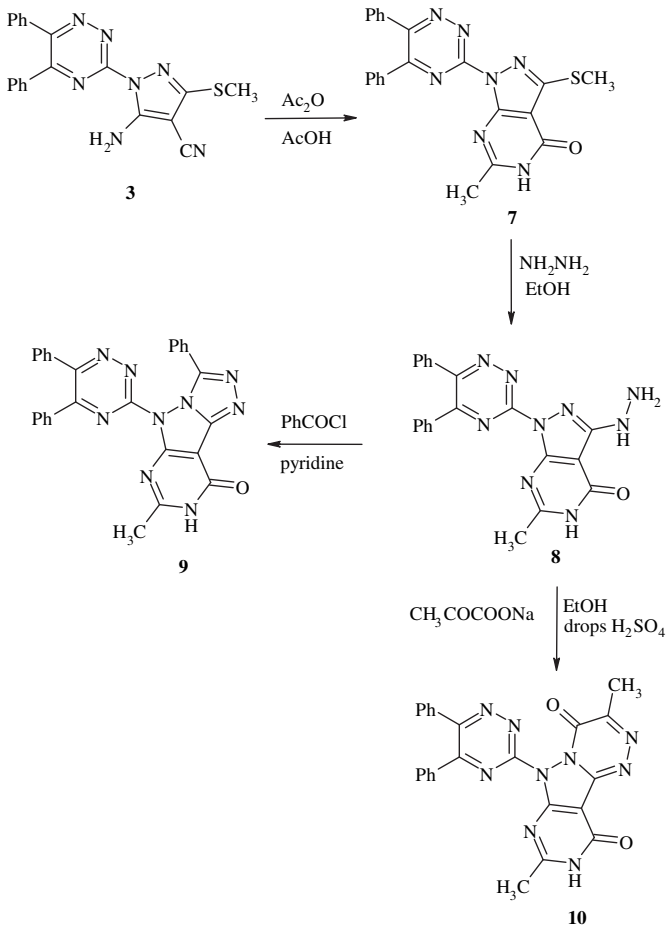
Refluxing of compound **3** in acetic anhydride in the presence of acetic acid afforded a pale brown product that was identified as 1-(5,6-diphenyl-1,2,4-triazin-3-yl)-6-methyl-3-(methylthio)-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one (**7**) (Scheme 2). Its IR spectrum showed disappearance of NH_2 and C≡N groups and appearance of new band at 1668 cm^{-1} for C=O group. Moreover, its ^{1}H NMR spectrum revealed two characteristic singlet signals at δ 2.50 and 13.59 ppm due to methyl protons at *C*-2 of pyrimidine moiety and NH proton, respectively [20]. Hydrazinolysis of compound **7** in boiling ethanol afforded the corresponding hydrazino derivative **8** (Scheme 2). The ^{1}H NMR of compound **8** showed hydrazino protons at δ 4.78 (NH_2) and 10.45 (NH) ppm.

The 3-hydrazinopyrazolo[3,4-*d*]pyrimidine **8** could be cyclized under different conditions to form a variety of triheterocyclic compounds. Thus, cyclocondensation of **8** with benzoyl chloride in pyridine and/or sodium pyruvate in ethanol containing few drops of concentrated sulfuric acid yielded 1,2,4-triazolo[4′,3′:1,5] pyrazolo[3,4-*d*]pyrimidine **9** and pyrimido[4′,5′:3,4]pyrazolo[5,1-*c*][1,2,4]triazine **10**, respectively (Scheme 2). The structures of **9** and **10** were established on the basis of their elemental analysis and spectral data. Their IR and ^{1}H NMR spectra proved disappearance of hydrazino group (see Experimental section).

On the other hand, treatment of compound **3** with benzoylacetone in basic medium gave the corresponding pyrazolo[3,4-*b*]pyridine derivative **12** (Scheme 3). The IR spectrum of the isolated product showed absorption bands at 3180 and 1673 cm^{-1} corresponding to NH_2 and C=O groups, respectively. Its ^{1}H NMR spectrum showed a singlet signal at δ 2.17 ppm for CH_3CO protons and a broad signal at δ 7.08 ppm for NH_2 protons. The formation of **12** indicated that the benzoyl group condensed with amino group to give the corresponding nonisolable arylidene **11**, which underwent cyclization *via* addition of active CH_2CO on the nitrile group.

Incorporation of various functionally-substituted nitrogen heterocyclic ring into pyrazolo[3,4-*b*]pyridine structure, was achieved by treating **12** with different reagents. Thus, treatment of **12** with malononitrile and acetanilide under basic conditions afforded the pyrazolo[3,4-*h*][1,6]naphthyridine derivative **13** and pyrazolo[5,4,3-*kl*]pyrido[4,3-*d*]pyrimidine derivative **14**, respectively (Scheme 3). In IR spectrum of compound **13**, the absorptions bands at 3182 and 2209 cm^{-1} correspond to NH_2 and C≡N groups, respectively, were observed. Besides, the normal signals that correspond to the different protons of aromatic moieties in ^{1}H NMR spectrum, there are three signals at δ 2.71, 3.17 and 6.80 ppm correspond to CH_3, SCH_3 and NH_2 protons, respectively. Also, ^{1}H NMR spectrum of **14** confirmed participation of SCH_3 and NH_2 groups in cyclization, beside it showed two singlet signals at δ 2.38 and 2.68 ppm assigned to CH_3CO and CH_3 protons, respectively.

On the other hand, interaction of compound **3** with cyanoacetone in dry DMF gave the corresponding pyrazolo[3,4-*b*]pyridine derivative **15** (Scheme 3). Bands of NH_2, C≡N and C=N groups of compound **15** appeared in IR spectrum, while its ^{1}H NMR spectrum confirmed the presence of CH_3, SCH_3 and NH_2 functionalities at δ 2.36, 3.41 and 5.05 ppm, respectively [18]. Cyclization of **15** upon



Scheme 2.

refluxing in formic acid afforded the pyrazolo[4′,3′:5,6]pyrido[4,3-*d*]pyrimidine derivative **16** (Scheme 3). IR spectrum of **16** revealed appearance of new band characteristic for C═O group at 1672 cm^{-1}, while its ^1H NMR spectrum exhibited a broad signal at δ 11.59 ppm assigned to NH proton.

Equimolar ratio of compounds **3** and **2** were refluxed in ethanol to afford the corresponding pyrazole derivative **17** in 76% yield (Scheme 4). The formation of **17** was supported by IR spectrum which showed absorption vibrations at 3123 (NH) and 2269–2211 (3 C≡N) cm^{-1}. Also, its ^1H NMR spectrum exhibited appearance of two singlet signals due to two SCH_3 groups at δ 3.06 and 3.07 ppm.

Interestingly, compound **17** seemed to be a useful candidate for further chemical transformations. Thus, hydrazinolysis of **17** in DMF gave the nonisolable intermediate **18**, which underwent an intramolecular cyclization *via* addition of NH functional group of pyrazole moiety at C≡N group to give the pyrazolo[1,5-*a*:3′,4′-*d*] pyrimidine derivative **19** as the final product (Scheme 4). The IR spectrum of **19** exhibited many characteristic absorption bands at 3300–3127, 2210 and 1598 cm^{-1} that corresponding to NH_2, NH, C≡N and C═N groups, respectively. Besides, ^1H NMR spectrum of **19** revealed a singlet signal at δ 3.08 ppm correspond to SCH_3 protons and two doublet signals at δ 8.84 and 8.95 ppm assigned to two NH_2 groups of pyrazolo[1,5-*a*]pyrimidine moiety [21].

Finally, 2,13-diamino-5-(5,6-diphenyl-1,2,4-trazin-3-yl)-7-oxo-6,14-dihydro pyrazolo[1,5-*a*][quinazolino[3′,2′-5,1](3-pyrzolino)[3,4-*d*]pyrimidine-3-carbonitrile (**21**) was achieved from refluxing compound **19** with methyl anthranilate in boiling DMF containing a catalytic amount of piperidine (Scheme 4). The reaction mechanism was postulated to proceed through nucleophilic attack of NH_2 group of methyl anthranilate at the methylthio moiety to give the intermediate **20**, which underwent an intramolecular cyclization with elimination of methanol molecule. Both elemental analysis and spectral data of **21** were consistent with the assigned structure. Thus, its IR spectrum revealed appearance of C═O group at 1686 cm^{-1}. Furthermore, its ^1H NMR spectrum confirmed disappearance of SCH_3 protons and showed two broad signals δ 8.87 and 9.82 ppm assigned to two NH_2 groups of pyrazolo[1,5-*a*]pyrimidine moiety.

3. Biological activity

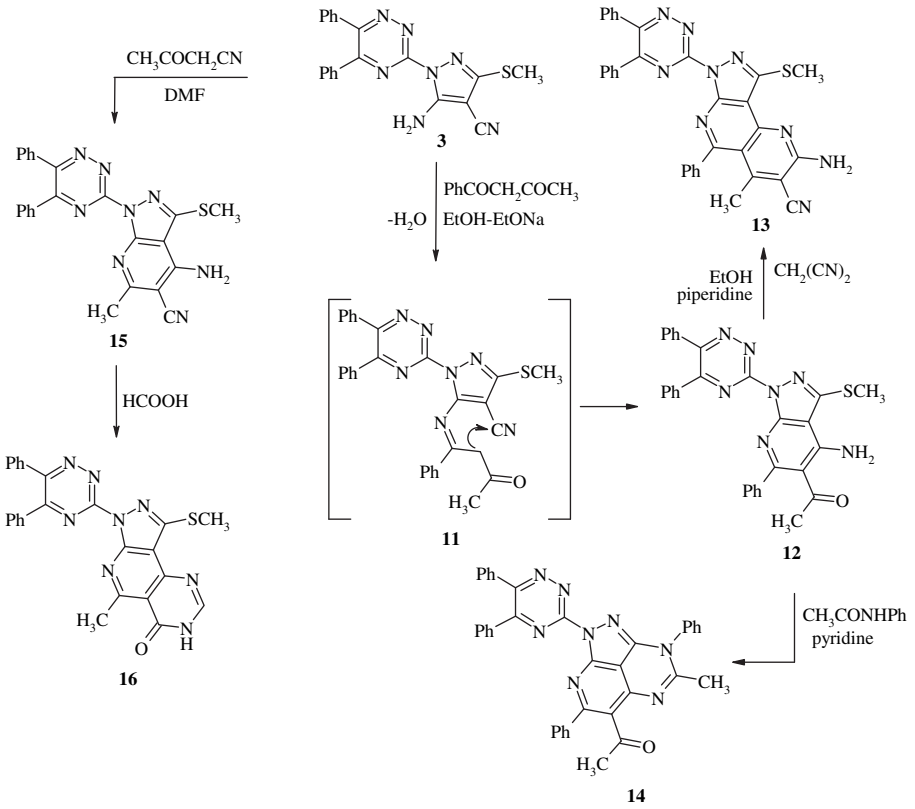
3.1. Antimicrobial activity

All the newly synthesized compounds were evaluated in *vitro* for their antimicrobial activity. The antimicrobial activities are carried out against three bacterial strains, *Staphylococcus aureus* (MTCCB 737), *Staphylococcus epidermidis* (MTCCB 1824) and *Escherichia coli* (MTCCB 1652) and three fungal strains, namely *Aspergillus fumigatus*, *Aspergillus niger* and *Alternaria alternata*. The preliminary screening results indicated that the most synthesized compounds showed antimicrobial activity from moderate to good. From the inhibition zone diameter data analysis (Table 1):

1) It can be noted that the most synthesized compounds showed a greater inhibitory effect against both the bacterial and fungal strains compared to the starting material **3** which confirmed improving biological properties by building of the fused nitrogen heterocycles on triazinylpyrazole moiety.
2) Compounds **6**, **7** and **8** showed a moderate inhibition against *S. aureus* (MTCCB 737) and lower inhibition against *S. epidermidis* (MTCCB 1824), *E. coli* (MTCCB 1652) and the three species of fungal strains.
3) Also, compounds **12** and **14**–**17** showed in general good inhibitions against *S. aureus* (MTCCB 737) and *S. epidermidis* (MTCCB 1824). However, they showed lower inhibitions against the three species of fungal strains which suggest that they have more comprehensive bacteria inhibitory prosperities than fungicidal activity due to the presence of pyrazolo[3,4-*b*]pyridine moiety in compounds **12**, **14**, **15**, **16** and methylthio and nitrile groups in compound **17**.
4) Compounds **9**, **10**, **13**, **19** and **21** showed in general good inhibitions against all species of bacterial and fungal strains with respect to the standard drugs. Compounds **9** and **10** are good antibacterial agents due to presence of pyrazolo[3,4-*b*]pyrimidine fused with bioactive heterocycles as 1,2,4-triazole and 1,2,4-triazine moieties while **19** and **21** are good antifungal agents due to presence of dipyrazolopyrimidine system.
5) We can conclude from preliminary antimicrobial screening that the pyrazolo[3,4-*b*]pyridine and pyrazolo[3,4-*d*]pyrimidine systems bearing 5,6-diphenyl-1,2,4-triazin-3-yl moiety enhance the biological properties especially when these systems are fused with some certain bioactive moieties as pyrazole, 1,2,4-triazole and 1,2,4-triazine.

3.2. The minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC, μg/mL) of the most active compounds **9**, **10**, **13**, **19** and **21** against two species of bacteria (*S. epidermidis* (MTCCB 1824) and *E. coli* (MTCCB 1652) and also two species of fungi (*A. niger* and *A. alternata*)) were determined (Table 2). Compounds **9** and **10** demonstrated good inhibitions against the selected bacterial and fungal strains.



Scheme 3.

3.3. Cytotoxicity activity

The LC_{50} values of the tested compounds **9**, **10**, **13**, **19** and **21** were found to be 3.54, 6.49, 0.58, 2.31 and 1.39 μg/mL, respectively (Table 3). The standard drug Bleomycin has LC_{50} value at 0.41 μg/mL. The lowest LC_{50} value was found in the case of compound **13**, indicating its higher cytotoxicity than the other compounds. Compounds **9** and **10** showed potent biocidal activity against brine shrimp due to their lower cytotoxicity that agreement with preliminary antimicrobial screening and the minimum inhibitory concentration (MIC). So, we can conclude that the pyrazolo[3,4-*d*]pyrimidine system bearing 5,6-diphenyl-1,2,4-triazin-3-yl moiety at 1*H* of pyrazole moiety that are fused with 6-methyl-1,2,4-triazin-5-one moiety is most active potent antibacterial and antifungal agent with lower toxicity in host cells.

4. Conclusion

We have successfully synthesized a series of novel pyrazolo [3,4-*b*]pyridines and pyrazolo[3,4-*d*]pyrimidines fused with nitrogen heterocycles and bearing 5,6-diphenyl-1,2,4-triazine moiety. The antimicrobial activity data of the prepared compounds showed that some fused heteropolycyclic rings showed good antimicrobial activity with lower toxicity. In our study, we have replaced the 1*H*-atom of the pyrazole of pyrazolo[3,4-*b*]pyridine and pyrazolo[3,4-*d*]pyrimidine by 5,6-diphenyl-1,2,4-triazine moiety as bioactive moiety. These structural changes made increasing activity against the tested organisms.

5. Experimental

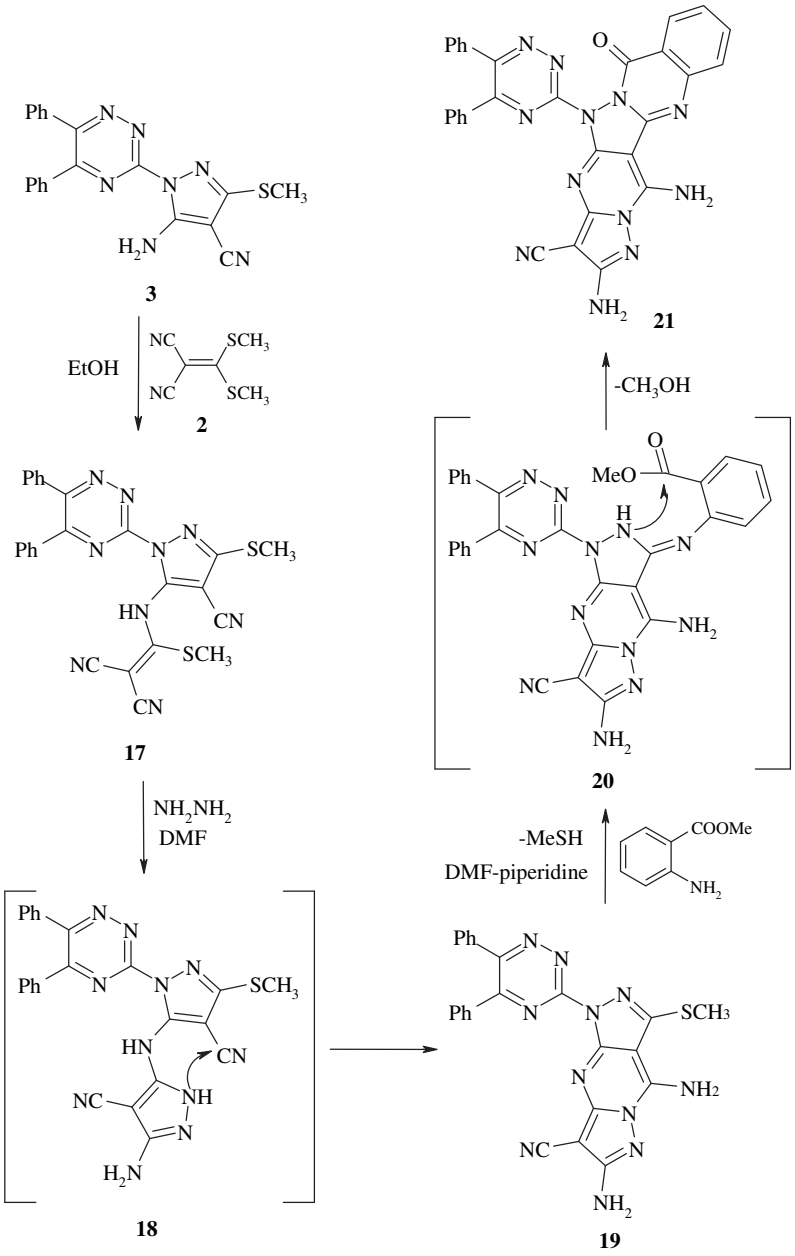
Melting points were determined on a digital Stuart SMP-3 apparatus. Infrared spectra were measured on Perkin–Elmer 293 spectrophotometer (cm^{-1}), using KBr disks.^{1}H NMR spectra were measured on Gemini-200 spectrometer (200 MHz), using DMSO-d_6 as a solvent and TMS (δ) as the internal standard. Elemental microanalyses were performed at microanalysis center in Bulgarian Academy of Science, Sofia, Bulgaria. The purity of the synthesized compounds was checked by thin layer chromatography (TLC). 5,6-Diphenyl-3-hydrazino-1,2,4-triazine (**1**) [22] and [bis(methylthio)methylene]malononitrile (**2**) [23] were prepared by published methods in literature.

5.1. Synthesis of 5-amino-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-3-(methylthio)-1H-pyrazole-4-carbonitrile (*3*)

A mixture of 5,6-diphenyl-3-hydrazino-1,2,4-triazine (**1**) (0.005 mol, 1.31 g) and [bis(methylthio)methylene]malononitrile (**2**) (0.005 mol, 0.85 g) in methanol (30 mL) was refluxed for 4 h. After cooling, the resulting precipitate which separated out was collected and crystallized from methanol to afford pale yellow crystals. Yield 72%; mp 212–214 °C. IR (KBr), ν (cm^{-1}): 3172 (NH_2), 3025 (C–H_{arom}), 2945 (C–H_{aliph}), 2216 (C≡N), 1607 (C=N), 1444 (NH_2 def). ^{1}H NMR (DMSO-d_6), δ: 3.17 (s, 3H, SCH_3), 3.58 (br, 2H, NH_2), 7.35–7.49 (m, 10H, aromatic protons). Anal. Calcd for $C_{20}H_{15}N_7S$ (385.44): C, 62.32; H, 3.92; N, 25.44; Found: C, 61.93; H, 3.70; N, 24.98.

5.2. Synthesis of 3,4-diamino-2-(5,6-diphenyl-1,2,4-triazin-3-yl)-2,7-dihydro-6H-pyrazolo[3,4-d]pyrimidine-6-thione (*5*)

A mixture of compound **3** (0.005 mol, 1.92 g) and thiourea (0.005 mol, 0.38 g) in ethanolic sodium ethoxide solution [(prepared by dissolving sodium metal (0.005 mol, 0.12 g) in absolute ethanol (30 mL))], was refluxed for 4 h. After cooling, the resulting precipitate which separated out was collected and



Scheme 4.

crystallized from ethanol to afford pale green crystals. Yield 74%; mp 168–170 °C. IR (KBr), ν (cm^{-1}): 3483, 3321 (NH_2), 3245, 3219 (NH_2), 3195 (NH), 3052 (C–H_{arom}), 1633 (C=N), 1427 (NH_2 def), 1223 (C=S). ^{1}H NMR (DMSO-d_6), δ: 4.16 (s, 2H, NH_2), 7.10–7.55 (m, 10H, aromatic protons), 9.50 (s, 2H, NH_2), 10.71 (s, 1H, NH). Anal. Calcd for $C_{20}H_{15}N_9S$ (413.45): C, 58.10; H, 3.66; N, 30.49; Found: C, 57.78; H, 3.41; N, 29.98.

5.3. Synthesis of 1-(5,6-diphenyl-1,2,4-triazin-3-yl)-7-phenyl-pyrazolo[5,4,3-kl]pyrimido[4,3-d]pyrimidine-4(2H)-thione (**6**)

A mixture of compound **5** (0.005 mol, 1.84 g) and benzoyl chloride (0.005 mol, 1 g) in pyridine (30 mL) was refluxed for 8 h. The reaction mixture was cooled at room temperature, poured into ice water, and neutralized with dilute HCl. The isolated solid was filtered off and crystallized from ethanol to afford buff crystals. Yield 95%; mp 186–188 °C. IR (KBr), ν (cm^{-1}): 3422, 3183 (NH, NH), 3057 (C–H_{arom}), 1627 (C=N), 1254 (C=S). ^{1}H NMR (DMSO-d_6), δ: 7.21–7.93 (m, 15H, aromatic protons), 8.91 (br, 1H, NH), 10.68 (br, 1H, NH). Anal. Calcd for $C_{27}H_{17}N_9S$ (499.54): C, 64.92; H, 3.43; N, 25.23; Found: C, 64.69; H, 3.22; N, 24.95.

5.4. Synthesis of 1-(5,6-diphenyl-1,2,4-triazin-3-yl)-6-methyl-3-(methylthio)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**7**)

A solution of compound **3** (0.005 mol, 1.92 g) in glacial acetic acid (10 mL) and acetic anhydride (20 mL) was heated for 6 h. The solvent was evaporated under reduced pressure to give solid, which was crystallized from diluted methanol to afford pale brown crystals. Yield 47%; mp 145–147 °C. IR (KBr), ν (cm^{-1}): 3192 (NH), 3054 (C–H_{arom}), 2980 (C–H_{aliph}), 1668 (C=O), 1590 (C=N). ^{1}H NMR (DMSO-d_6), δ: 2.50 (s, 3H, CH_3), 3.37 (s, 3H, SCH_3), 7.28–7.43 (m,

Table 1
The antimicrobial activity of the synthesized compounds at 100 μg/mL concentration.

Compd. No.	Diameter of the inhibition zone[a] (mm)					
	Bacteria			Fungi		
	Staphylococcus aureus MTCCB 737	*Staphylococcus epidermidis* MTCCB 1824	*Escherichia coli* MTCCB 1652	*Aspergillus fumigatus*	*Aspergillus niger*	*Alternaria alternata*
3	12	7	3	8	6	0
5	9	11	13	11	7	2
6	16	13	9	9	11	5
7	14	11	5	12	9	10
8	18	10	8	10	11	13
9	27	27	26	17	12	8
10	28	26	29	19	15	16
12	16	18	13	10	4	5
13	25	22	19	16	11	9
14	18	13	10	3	7	9
15	19	16	12	6	7	5
16	17	17	10	11	10	12
17	17	21	11	9	6	8
19	24	22	17	22	18	16
21	26	23	20	21	19	19
Tetracycline[b]	30	25	28	–	–	–
Ketoconazole[b]	–	–	–	18	20	21

[a] 12 mm or less: resistant or no inhibition1, 13–17 mm: moderate inhibition, 18 mm or more: maximum inhibition.
[b] The concentration of used standard drugs was 30 μg/mL.

10H, aromatic protons), 13.59 (s, 1H, NH). Anal. Calcd for $C_{22}H_{17}N_7OS$ (427.48): C, 61.81; H, 4.01; N, 22.94; Found: C, 61.42; H, 3.79; N, 22.63.

5.5. Synthesis of 1-(5,6-diphenyl-1,2,4-triazin-3-yl)-3-hydrazino-6-methyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**8**)

A mixture of compound **7** (0.005 mol, 2.13 g) and hydrazine hydrate (0.007 mol, 0.35 mL) in ethanol (30 mL) was refluxed for 4 h. After cooling, the resulting precipitate was filtered off and crystallized from ethanol to afford pale yellow crystals. Yield 83%; mp 170–172 °C. IR (KBr), ν (cm^{-1}): 3341, 3266 (NH_2), 3216 (NH), 3056 (C–H_{arom}), 2930 (C–H_{aliph}), 1676 (C=O), 1604 (C=N). ^1H NMR (DMSO-d_6), δ: 2.78 (s, 3H, CH_3), 4.78 (d, 2H, $J = 5.6$ Hz, NH_2), 7.18–7.84 (m, 10H, aromatic protons), 10.45 (s, 1H, NH), 10.74 (s, 1H, NH). Anal. Calcd for $C_{21}H_{17}N_9O$ (411.41): C, 61.31; H, 4.16; N, 30.64; Found: C, 60.99; H, 3.79; N, 30.38.

5.6. Synthesis of 5-(5,6-diphenyl-1,2,4-triazin-3-yl)-7-methyl-3-phenyl-5H-[1,2,4]triazolo[4′,3′: 1,5]pyrazolo[3,4-d]pyrimidin-9-(8H)-one (**9**)

A mixture of compound **8** (0.005 mol, 2.05 g) and benzoyl chloride (0.005 mol, 0.70 g) in pyridine (30 mL) was refluxed for 8 h. The reaction mixture was cooled at room temperature, poured into ice water, and neutralized with dilute HCl. The isolated solid was filtered off and crystallized from ethanol to afford yellow crystals. Yield 54%; mp 208–210 °C. IR (KBr), ν (cm^{-1}): 3194 (NH), 3055 (C–H_{arom}), 2986 (C–H_{aliph}), 1667 (C=O), 1584 (C=N). ^1H NMR (DMSO-d_6), δ: 2.50 (s, 3H, CH_3), 7.28–7.53 (m, 15H, aromatic protons), 13.45 (br, 1H, NH). Anal. Calcd for $C_{28}H_{19}N_9O$ (497.51): C, 67.60; H, 3.85; N, 25.34; Found: C, 67.31; H, 3.58; N, 25.02.

5.7. Synthesis of 6-(5,6-diphenyl-1,2,4-triazin-3-yl)-3,8-dimethylpyrimido[4′,5′:3,4]pyrazolo[5,1-c][1,2,4]triazine-4,10-(6H,9H)-dione (**10**)

A mixture of compound **8** (0.005 mol, 2.05 g) and sodium pyruvate (0.005 mol, 0.55 g) in ethanol (30 mL) containing concentrated H_2SO_4 (0.5 mL) was refluxed for 6 h. After cooling, the obtained solid was filtered off and crystallized from ethanol to afford pale brown crystals. Yield 66%; mp 256–258 °C. IR (KBr), ν (cm^{-1}): 3166 (NH), 3037 (C–H_{arom}), 2935 (C–H_{aliph}), 1686 (C=$O_{pyrimidinone}$), 1650 (C=$O_{triazinone}$), 1605 (C=N). ^1H NMR (DMSO-d_6), δ: 2.49 (s, 3H, CH_3), 2.89 (s, 3H, CH_3), 7.11–8.12 (m, 10H, aromatic protons), 11.23 (br, 1H, NH). Anal. Calcd for $C_{24}H_{17}N_9O_2$ (463.45): C, 62.20; H, 3.70; N, 27.20; Found: C, 61.84; H, 3.41; N, 26.83.

5.8. Synthesis of 5-acetyl-4-amino-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-6-phenyl-3-(methylthio)-1H-pyrazolo[3,4-b]pyridine (**12**)

A solution of compound **3** (0.005 mol, 1.92 g) and benzoylacetone (0.005 mol, 0.81 g) in ethanolic sodium ethoxide solution [(prepared by dissolving sodium metal (0.005 mol, 0.12 g) in absolute ethanol (30 mL))], was refluxed for 8 h. After cooling, add some water

Table 2
The minimum inhibitory concentration (MIC, μg/mL) of the synthesized compounds **9**, **10**, **13**, **19** and **21**.

The selected organisms	The minimum inhibitory concentration (MIC)					
	9	**10**	**13**	**19**	**21**	Standard[a]
Staphylococcus aureus (MTCCB 737)	50	25	50	50	50	6.25
Escherichia coli (MTCCB 1652)	25	12.5	25	>100	>100	12.5
Aspergillus niger	50	25	>100	25	>100	6.25
Alternaria alternata	50	50	>100	25	50	6.25

[a] Tetracycline and ketoconazole were used as standard drugs against bacterial and fungal strains, respectively.

Table 3
Cytotoxicity activity of the synthesized compounds **9**, **10**, **13**, **19** and **21**.

Samples	95% confidence limit ppm			Regression equation	X^2 (df)
	LC_{50}	Lower	Upper		
9	3.54	2.08	6.02	$y = 3.98 + 1.85x$	3.38(2)
10	6.49	4.15	10.15	$y = 3.17 + 2.27x$	0.35(2)
13	0.58	0.19	1.77	$y = 4.08 + 1.22x$	0.20(2)
19	2.31	1.30	4.10	$y = 4.36 + 1.78x$	0.32(2)
21	1.39	0.69	2.82	$y = 3.54 + 1.29x$	0.41(2)
Bleomycin[a]	0.41	0.27	0.62	$y = 3.16 + 2.98x$	0.62(2)
Gallic acid[a]	4.53	3.33	6.15	$y = 3.93 + 1.62x$	1.25(2)

[a] Bleomycin and gallic acid were used as standard drugs in cytotoxicity activity.

(10 mL), the resulting precipitate was filtered off and crystallized from diluted ethanol to afford yellow crystals. Yield 67%; mp 188–191 °C. IR (KBr), ν (cm^{-1}): 3180 (br, NH_2), 3050 (C–H_{arom}), 2910 (C–H_{aliph}), 1673 (C═O), 1603 (C═N). ^1H NMR (DMSO-d_6), δ: 2.17 (s, 3H, $COCH_3$), 3.30 (s, 3H, SCH_3), 7.08 (br, 2H, NH_2), 7.30–7.48 (m, 15H, aromatic protons). Anal. Calcd for $C_{30}H_{23}N_7OS$ (529.61): C, 68.03; H, 4.38; N, 18.51; Found: C, 67.79; H, 4.09; N, 18.19.

5.9. Synthesis of 2-amino-7-(5,6-diphenyl-1,2,4-triazin-3-yl)-4-methyl-9-(methylthio)-5-phenyl-7H-pyrazolo[3,4-h][1,6]naphthyridine-3-carbonitrile (**13**)

A mixture of compound **12** (0.005 mol, 2.65 g) and malononitrile (0.005 mol, 0.33 g) in ethanol (30 mL) containing few drops of piperidine was refluxed for 10 h. The obtained solid was filtered off and crystallized from DMF/ethanol to afford brown crystals. Yield 81%; mp 210–211 °C. IR (KBr), ν (cm^{-1}): 3182 (br, NH_2), 3057 (C–H_{arom}), 2928 (C–H_{aliph}), 2209 (C≡N), 1625 (C═N), 1443 (NH_2 def). ^1H NMR (DMSO-d_6), δ: 2.71 (s, 3H, CH_3), 3.17 (s, 3H, SCH_3), 6.80 (br, 2H, NH_2), 7.35–7.52 (m, 15H, aromatic protons). Anal. Calcd for $C_{33}H_{23}N_9S$ (577.66): C, 68.61; H, 4.01; N, 21.82; Found: C, 68.31; H, 3.83; N, 21.51.

5.10. Synthesis of 6-acetyl-3,7diphenyl-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-4-methyl-pyrazolo[5,4,3-kl]pyrido[4,3-d] pyrimidine (**14**)

A mixture of compound **12** (0.005 mol, 2.65 g) and acetanilide (0.005 mol, 0.67 g) in pyridine (30 mL) was refluxed for 12 h. The reaction mixture was cooled at room temperature, poured into ice water, and neutralized with dilute HCl. The isolated solid was filtered off and crystallized from diluted DMF to afford yellow crystals. Yield 69%; mp 234–237 °C. IR (KBr), ν (cm^{-1}): 3056 (C–H_{arom}), 2919 (C–H_{aliph}), 1683 (C═O), 1620 (C═N). ^1H NMR (DMSO-d_6), δ: 2.38 (s, 3H, $COCH_3$), 2.68 (s, 3H, CH_3), 7.27–7.54 (m, 20H, aromatic protons). Anal. Calcd for $C_{37}H_{26}N_8O$ (598.65): C, 74.23; H, 4.38; N, 18.72; Found: C, 73.91; H, 4.08; N, 18.39.

5.11. Synthesis of 4-amino-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-6-methyl-3-(methylthio)-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile (**15**)

A mixture of chloroacetone (0.005 mol, 0.42 g) and potassium cyanide (0.005 mol, 0.33 g) in dry DMF (10 mL), was heated for 10 min. A solution of compound **3** (0.005 mol, 1.92 g) in DMF (20 mL) was added to the above mixture and refluxed for 10 h. The reaction mixture was cooled at room temperature, poured into ice water to isolate the solid, which was filtered off and crystallized from diluted ethanol to afford yellow crystals. Yield 59%; mp 176–178 °C. IR (KBr), ν (cm^{-1}): 3381, 3124 (NH_2), 3010 (C–H_{arom}), 2815 (C–H_{aliph}), 2119 (C≡N), 1621 (C═N). ^1H NMR (DMSO-d_6), δ: 2.36 (s, 3H, CH_3), 3.41 (br, 3H, SCH_3), 5.05 (s, 2H, NH_2), 7.43–7.90 (m, 10H, aromatic protons). Anal. Calcd for $C_{24}H_{18}N_8S$ (450.51): C, 63.98; H, 4.03; N, 24.87; Found: C, 63.69; H, 3.86; N, 24.51.

5.12. Synthesis of 7-(5,6-diphenyl-1,2,4-triazin-3-yl)-5-methyl-9-(methylthio)-3,7-dihydro-4H-pyrazolo[4′,3′: 5,6]pyrido[4,3-d]pyrimidin-4-one (**16**)

A solution of compound **15** (0.005 mol, 2.25 g) in formic acid (20 mL), was heated for 4 h. The reaction mixture was cooled at room temperature, poured into ice water and neutralized with NaOH solution (5%) to isolate the solid, which was filtered off and crystallized from DMF/ethanol to afford pale brown crystals. Yield 58%; mp 199–201 °C. IR (KBr), ν (cm^{-1}): 3182 (br, NH), 3057 (C–H_{arom}), 2925 (C–H_{aliph}), 1672 (C═O), 1599 (C═N). ^1H NMR (DMSO-d_6), δ: 2.33 (s, 3H, CH_3), 3.39 (br, 3H, SCH_3), 7.38–7.95 (m, 10H, aromatic protons), 8.51 (br, 1H, C_2–$H_{pyrimidine}$), 11.59 (brs, 1H, NH). Anal. Calcd for $C_{25}H_{18}N_8OS$ (478.52): C, 62.75; H, 3.79; N, 23.42; Found: C, 62.43; H, 3.52; N, 23.08.

5.13. Synthesis of [{[4-cyano-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-3-(methylthio)-1H-pyrazol-5-yl]amino}(methylthio) methylene]malononitrile (**17**)

A mixture of compound **3** (0.005 mol, 1.92 g) and **2** (0.005 mol, 0.85 g) in ethanol (30 mL) was refluxed for 4 h. The reaction mixture was cooled at room temperature to isolate the solid, which was filtered off and crystallized from ethanol to afford yellow crystals. Yield 76%; mp 175–178 °C. IR (KBr), ν (cm^{-1}): 3123 (NH), 3014 (C–H_{arom}), 2971, 2898 (C–H_{aliph}), 2269–2211 (3 C≡N), 1623 (C═N), 1597 (C═C). ^1H NMR (DMSO-d_6), δ: 3.06 (s, 3H, SCH_3), 3.07 (s, 3H, SCH_3), 4.15 (s, 1H, NH), 7.44–7.71 (m, 10H, aromatic protons). Anal. Calcd for $C_{25}H_{17}N_9S_2$ (507.59): C, 59.16; H, 3.38; N, 24.83; Found: C, 58.89; H, 3.19; N, 24.59.

5.14. Synthesis of 4,7-diamino-1-(5,6-diphenyl-1,2,4-triazin-3-yl)-3-(methylthio)-1H-dipyrazolo[1,5-a:3′,4′-d]pyrimidine-8-carbonitrile (**19**)

A mixture of compound **17** (0.005 mol, 2.53 g) and hydrazine hydrate (0.005 mol, 0.25 g) in DMF (30 mL) was refluxed for 8 h. The reaction mixture was cooled at room temperature, poured into ice water to isolate the solid, which was filtered off and crystallized from diluted ethanol to afford yellow crystals. Yield 71%; mp 212–214 °C. IR (KBr), ν (cm^{-1}): 3300, 3127 (NH_2, NH), 3062 (C–H_{arom}), 2940 (C–H_{aliph}), 2210 (C≡N), 1598 (C═N), 1446 (NH_2 def). ^1H NMR (DMSO-d_6), δ: 3.08 (s, 3H, CH_3), 7.22–7.77 (m, 10H, aromatic protons), 8.84 (d, 2H, $NH_{2pyrazole}$), 8.95 (d, 2H, $NH_{2pyrimidine}$). Anal. Calcd for $C_{24}H_{17}N_{11}S$ (491.53): C, 58.64; H, 3.49; N, 31.35; Found: C, 58.41; H, 3.22; N, 31.09.

5.15. Synthesis of 2,13-diamino-5-(5,6-diphenyl-1,2,4-trazin-3-yl)-7-oxo-6,14-dihydropyrazolo[1,5-a]quinazolino[3′,2′-5,1](3-pyrzolino)[3,4-d]pyrimidine-3-carbonitrile (**21**)

A mixture of compound **19** (0.005 mol, 2.45 g) and methyl anthranilate (0.005 mol, 0.76 g) in DMF (30 mL) containing few drops of piperidine, was refluxed for 12 h. The reaction mixture was cooled at room temperature, poured into ice water to isolate the solid, which was filtered off and crystallized from diluted DMF to afford pale brown crystals. Yield 53%; mp 286–288 °C. IR (KBr), ν (cm^{-1}): 3376, 3314, 3181 (NH_2, NH), 3050 (C–H_{arom}), 2273 (C≡N), 1686 (C═O), 1629, 1602 (C═N). ^1H NMR (DMSO-d_6), δ: 7.34–8.14 (m, 14H, aromatic protons), 8.87 (br, 2H, $NH_{2pyrazole}$), 9.82 (br, 2H, $NH_{2pyrimidine}$). Anal. Calcd for $C_{30}H_{18}N_{12}O$ (562.54): C, 64.05; H, 3.23; N, 29.88; Found: C, 63.81; H, 3.11; N, 29.61.

5.16. Antimicrobial screening

All the newly synthesized compounds were evaluated in *vitro* for their antimicrobial activity. The antimicrobial activities are carried out against three bacterial strains, *S. aureus* (MTCCB 737), *S. epidermidis* (MTCCB 1824) and *E. coli* (MTCCB 1652) and three fungal strains, *A. fumigatus*, *A. niger* and *A. alternata* employing the nutrient agar disc diffusion method [24,25] at 100 μg/mL concentration. DMSO was used as blank exhibited no activity against any of the used organisms. The antimicrobial activity was determined by measuring of the inhibition zone (Table 1), after 16–20 h of incubation at 37 °C for bacterial strains and 3–4 days at 37 °C for fungal strains. Tetracycline and ketoconazole were used as standard

drugs against bacterial and fungal strains, respectively at 30 μg/mL concentration.

5.17. The minimum inhibitory concentration (MIC)

A current definition of the minimum inhibitory concentration MIC is "the lowest concentration which resulted in maintenance or reduction of inoculum viability". The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of antimicrobial agent needed to prevent microbial growth. The method displays tubes of growth broth containing a test level of preservatives, into which an inoculum of microbes was added. The end result of the test was the minimum concentration of antimicrobial. The serial dilution technique [26] was applied for the determination of MIC of the tested compounds **9**, **10**, **13**, **19** and **21** against two species of bacterial strains (*S. aureus* MTCCB 737 and *E. coli* MTCCB 1652) and two species of fungal strains (*A. niger* and *A. alternata*). Dilution series were set up with 6.25, 12.5, 25, 50 and 100 μg/mL of nutrient broth medium to each tube, 100 μL of standardized suspension of the test microbes (10^7 cells/mL) were added and incubated at 37 °C for 24 h (Table 2).

5.18. Cytotoxicity bioassay

Brine shrimp lethality bioassay [27,28] is a recent development in the assay which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. antimicrobial, anticancer, antiviral, insecticidal, pesticidal, AIDS, etc). In this method, the eggs of the brine shrimp, *Artemia salina* leach, were hatched for 48 h to mature shrimp. 38 g of sea salt was weighed, dissolved in one liter of distilled water, filtered off and was kept in a small tank. The eggs were then added to the divided tank. Constant oxygen supply was provided and temperature 37 °C was maintained for 48 h to hatch and mature the shrimp called as nauplii (Larvae). The solutions of compounds **9**, **10**, **13**, **19** and **21** were prepared by dissolving 10 mg of each compound in 2 mL of DMSO. From this stock, a series of solutions 5, 10, 20, 40 and 80 μg/mL were transferred to fifteen vials (three for each dilutions were used for each test sample and LC_{50} is the mean of three values) and one vial was kept as control having 2 mL of DMSO. Then about 10 brine shrimp nauplii were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 h was counted. The resulting data were transformed to the probit analysis [29] for the determination of LC_{50} values for the five tested compounds (Table 3).

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