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Original article

Synthesis and antibacterial activity of some new thiadiaza/triazaphospholes, thiadiaza/triaza/tetrazaphosphinines and thiadiaza/tetrazaphosphepines containing 1,2,4-triazinone moiety

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ABSTRACT

Some new thiadiaza/triazaphospholes, thiadiaza/triaza/tetrazaphosphinines and thiadiaza/tetrazaphosphepines fused with 6-methyl-1,2,4-triazin-5-one moiety were synthesized *via* reactions of α,β-bifunctional compounds derived from 4-amino-3-mercapto-6-methyl-1,2,4-triazin-5(4*H*)-one (**1**) with various phosphorus reagents. The *in vitro* antibacterial activities of the synthesized compounds were evaluated against some bacterial strains. Compounds **16** and **21** exhibited good inhibitory activities against most the tested organisms with MIC values in the range 6.25–12.5 μg/mL and lower cytotoxicity in comparison with the reference drugs.

1. Introduction

Various substituted 1,2,4-triazin-5-one derivatives have a great importance as biological agents in medicinal and agricultural fields [1–5]. Recently, significant activities have been directed toward this class of compounds, in particular, 4-amino-1,2,4-triazin-5(2*H*)-one derivatives which have considerable interest because of their herbicidal [6,7], antimicrobial [8–10], anti-HIV [11] and anticancer activities [12,13]. On the other hand, heterocyclic systems containing phosphorus atoms have considerable attention due to a large variety of interesting pharmacological and biological activities, such as herbicidal [14–16], insecticidal [17], antimicrobial [18] and anticancer properties [19].

In view of the above observations, new phosphorus heterocyclic systems containing 1,2,4-triazinone moiety were synthesized *via* heterocyclization reactions of α,β-bifunctional 1,2,4-triazinone derivatives with phosphorus reagents and their antibacterial activities were investigated.

2. Results and discussion

6-Methyl-2-oxido-2-phenyl-1,2-dihydro-7*H*-[1,3,4,2]thiadiazaphospholo[5,4-*c*][1,2,4]triazin-5-one (**2**) was synthesized by the reaction of 4-amino-3-mercapto-6-methyl-1,2,4-triazin-5(4*H*)-one (**1**) with phenylphosphonic dichloride in THF containing 2 equiv amounts of triethylamine to remove hydrogen chloride (Scheme 1). The molecular structure of **2** was deduced from its IR spectrum, which showed the absorption bands of NH, C=O and P=O groups at 3158, 1681 and 1225 cm^{-1}, respectively. Also, the ^1H NMR spectrum of **2** recorded a signal of NH proton as a doublet at δ 8.25 ppm ($J_{NH,P} = 3.3$ Hz) while its ^{31}P NMR spectrum showed a signal at δ +55.1 ppm [20]. Furthermore, its mass spectrum showed the molecular ion peak at *m/e* 280 (3.99%) and base peak at *m/e* 186.

Reaction of compound **1** with acetyl triphenylphosphonium chloride and phenacyl triphenylphosphonium bromide in boiling DMF containing a catalytic amount of piperidine afforded 1,2,4-triazino[4,3-*e*][1,4,5,2]thiadiazaphosphinine **4** and 1,2,4-triazino[4,3-*f*][1,5,6,2]thiadiazaphosphepine **6** derivatives, respectively (Scheme 1). Formation of compounds **4** and **6** may occur through the attack of electrons of the lone pair of the SH group on phosphorus atom of the phosphonium salt to remove hydrogen halide which may afford the intermediates **3** and **5**, respectively, followed by an intramolecular nucleophilic attack of the amino group on carbonyl group with elimination of water to give **4** and **6**, respectively. The IR and ^1H NMR spectra of products **4** and **6** showed the absence of the SH and NH_2 groups, which confirmed the cyclized state. Moreover, the ^{13}C NMR spectrum of the former **4** recorded two singlet signals at δ 16.3 and 17.0 ppm due to the presence of two methyl groups, while the latter compound **6**

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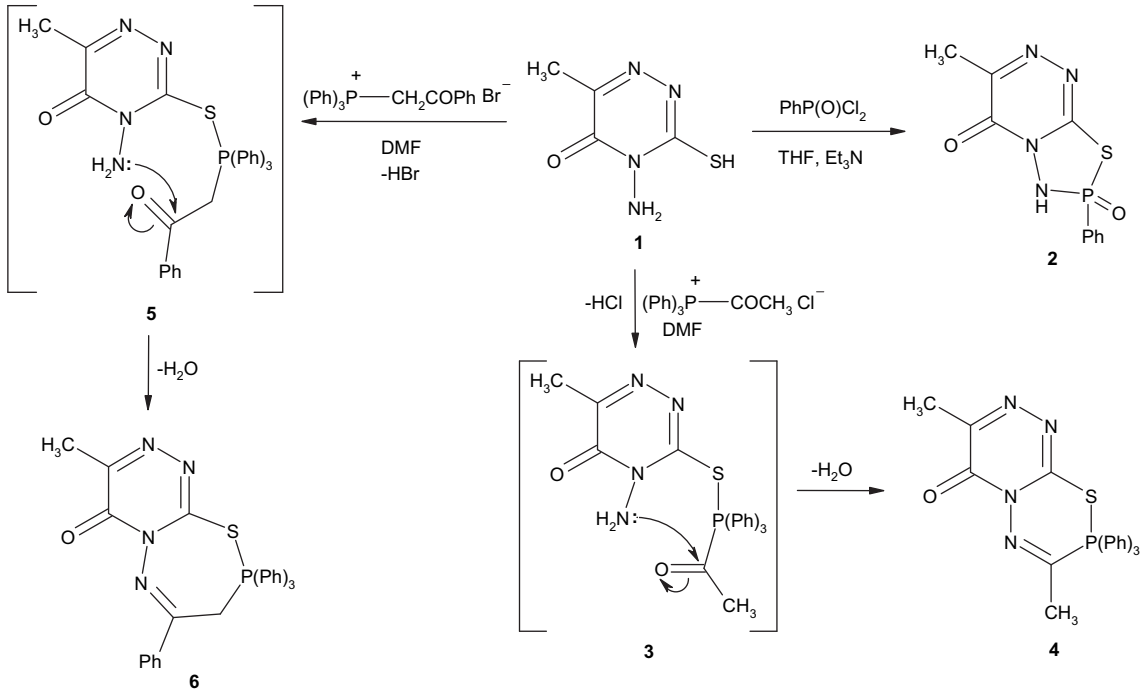


Scheme 1.

recorded one signal at δ 41.5 ppm ($J_{C,P} = 55.8$ Hz) which is assigned to CH_2–P group. Additionally, the ^{31}P NMR spectrum showed one singlet signal for each at upfield δ −11.2 and −18.4 ppm for compounds **4** and **6**, respectively, which are in accordance with phosphorus (V) derivatives [21].

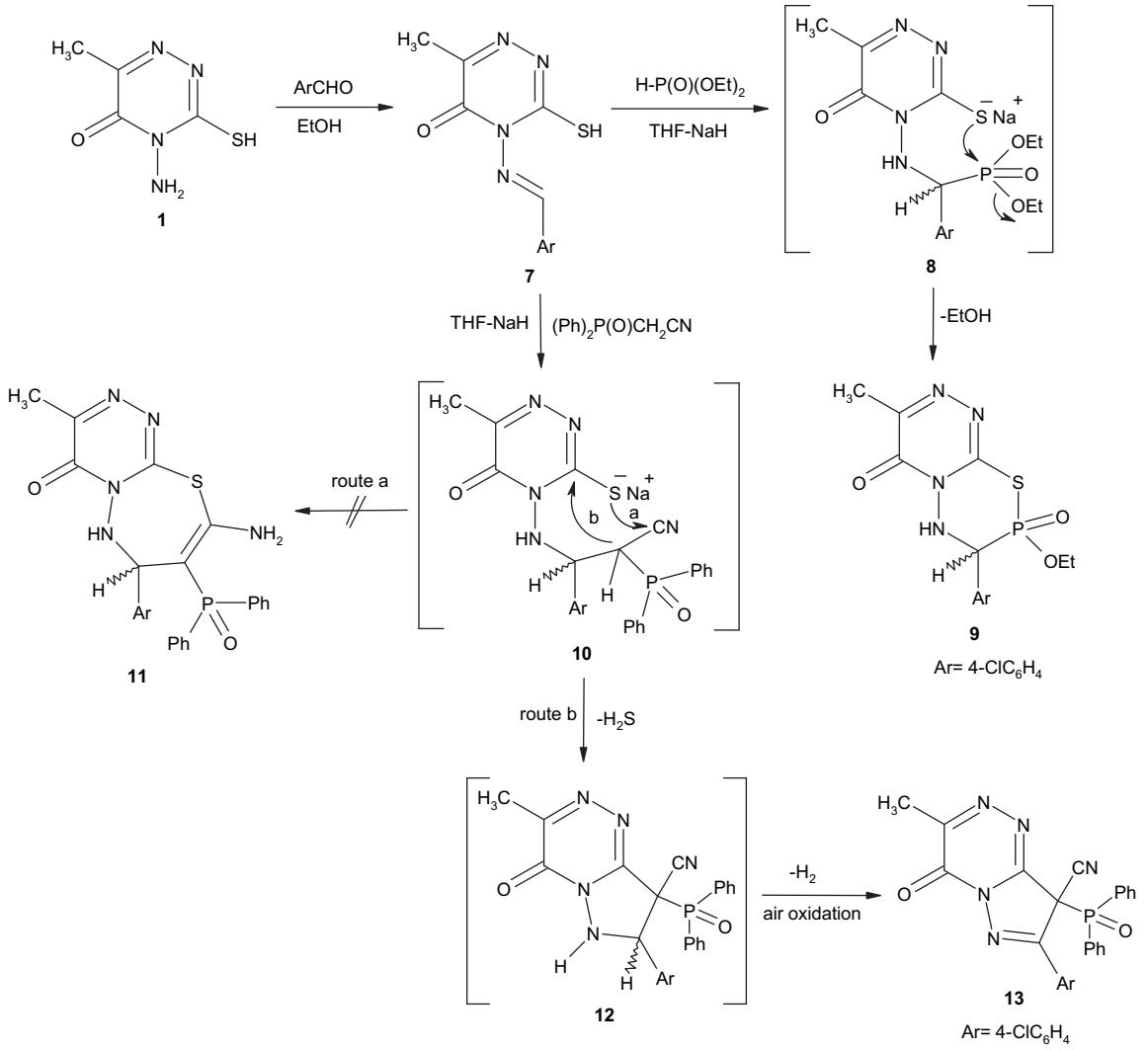
Condensation of **1** with 4-chlorobenzaldehyde resulted in hydrazone **7**. The Pudovik reaction of hydrazone **7** using diethyl phosphite in boiling THF containing a catalytic amount of sodium hydride produced a cyclic α-aminophosphonate ester **9** as only one isomer (Scheme 2). The IR spectrum of **9** revealed a significant band for P═O group at 1217 cm^{-1}. The ^{13}C NMR spectrum of **9** showed three signals at δ 16.7, 59.8 and 48.8 ppm ($J_{C,P} = 150$ Hz) which are due to the carbon atoms of CH_3CH_2O and CH–P groups, respectively, while its ^1H NMR spectrum showed a broad signal at δ 5.41 ppm which is assigned to CH–P. Also, the ^{31}P NMR spectrum showed a signal at δ +38.9 ppm which confirms that the phosphorus atom is in a cyclic thiadiazaphosphinine system. Moreover, the mass spectrum of **9** recorded the molecular ion peak at *m*/*e* 373 (2.34%) and base peak at *m*/*e* 124.

Consequently, compound **7** was heated under reflux with diphenylphosphoryl acetonitrile for 12 h in THF in the presence of sodium hydride as a catalyst to afford 7-(4-chlorophenyl)-8-(diphenylphosphoryl)-3-methyl-4-oxo-4,8-dihydropyrazolo[5,1-*c*][1,2,4]triazine-8-carbonitrile (**13**) and not 1,3,4-thiadiazaphosphepine derivative **11** (Scheme 2). This reaction pathway proceeds *via* *C*-nucleophilic attack by the reactive methylene of diphenylphosphoryl acetonitrile on the N–N═CH–Ar moiety to give the intermediate **10** which underwent cyclization by elimination of one molecule of H_2S followed by an air oxidation process (route b). The structure of **13** was deduced from its IR absorption spectrum which showed the C≡N group at 2226 cm^{-1}, while its ^{13}C NMR spectrum displayed a signal at δ 110.1 ppm due to the presence of a nitrile group.

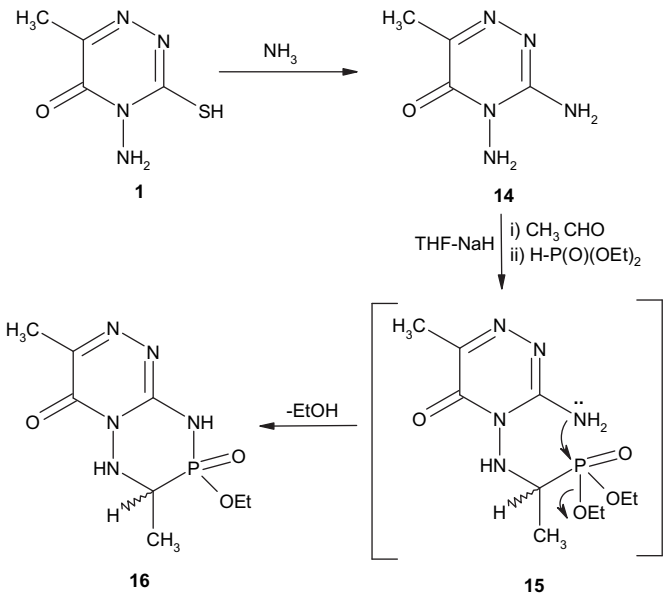
The Kabachnik–Fields reaction [22] using 3,4-diamino-6-methyl-1,2,4-triazin-5(4*H*)-one (**14**), acetaldehyde and diethyl phosphite in THF in the presence of sodium hydride as a catalyst led to only one isomer of 1,2,4-triazino[4,3-*b*][1,2,4,5]triazaphosphinine derivative **16** (Scheme 3). The ^1H NMR spectrum of **16** exhibited two signals at δ 1.17, 3.77 ppm which are characteristic of ethoxy protons, a broad signal at δ 5.38 ppm for CH–P proton and two signals for the two NH protons at δ 6.49 and 8.24 ppm. The ^{13}C NMR spectrum of **16** revealed the expected signals at δ 47.5 ppm ($J_{C,P} = 147.1$ Hz) corresponding to CH–P and δ 16.4 and 62.5 ppm due to ethoxy carbons, while its ^{31}P NMR spectrum showed a signal at δ +21.5 ppm [23]. Also, the mass spectrum of **16** showed the molecular ion peak at *m*/*e* 259 (3.82%) and base peak at *m*/*e* 213.

On the other hand, 4-amino-3-hydrazino-1,2,4-triazin-5(4*H*)-one (**17**) was used as starting material for the preparation of novel phosphorus heterocycles. Thus, the one-pot, three components reaction of compound **17** with acetaldehyde and phenylphosphonic dichloride in boiling THF containing a catalytic amount of triethylamine led to the formation of the 1,2,4,3-triazaphospholo[5,1-*c*][1,2,4]triazinone derivative **18** (Scheme 4). Analytical and spectral data for compound **18** were in agreement with the assigned structure. The bands of NH, C═N and P═O groups appeared in the IR spectrum, while ^1H and ^{13}C NMR spectra confirmed the presence of –N═CH–CH_3 and Ph–P═O functionalities (See Experimental Section). In addition, its ^{31}P NMR spectrum showed a signal at δ +26.4 ppm [24].

The one-pot Kabachnik–Fields reaction of compound **17**, acetaldehyde and diethyl phosphite in THF containing sodium hydride as a catalyst produced one isomer of 1,2,4-triazino[3,2-*c*][1,2,4,5]triazaphosphinine **21**, likely through the nonisolable intermediate **19**, which spontaneously cyclized through *N*-2 of the triazine ring and not the exocyclic *N*-amino, with elimination of one molecule of ethanol (route b, Scheme 4). The IR spectrum of **21** exhibited absorption bands at 3420, 3250, 1624 and 1210 cm^{-1} due to the presence of the NH_2, NH, C═N and P═O functional groups, respectively. The ^1H NMR spectrum of compound **21** showed two signals at δ 1.17 and 3.72 ppm which are due to ethoxy protons. Also, the resonance frequencies associated with NH_2 and NH protons were measured at δ 6.49 and 7.70 ppm, respectively. The ^{13}C NMR spectrum of **21** recorded characteristic signals at δ 16.2, 16.7, 49.5 ($J_{C,P} = 145.2$ Hz) and 60.5 ppm due to CH_3CH_2O–P(O)–CH–CH_3



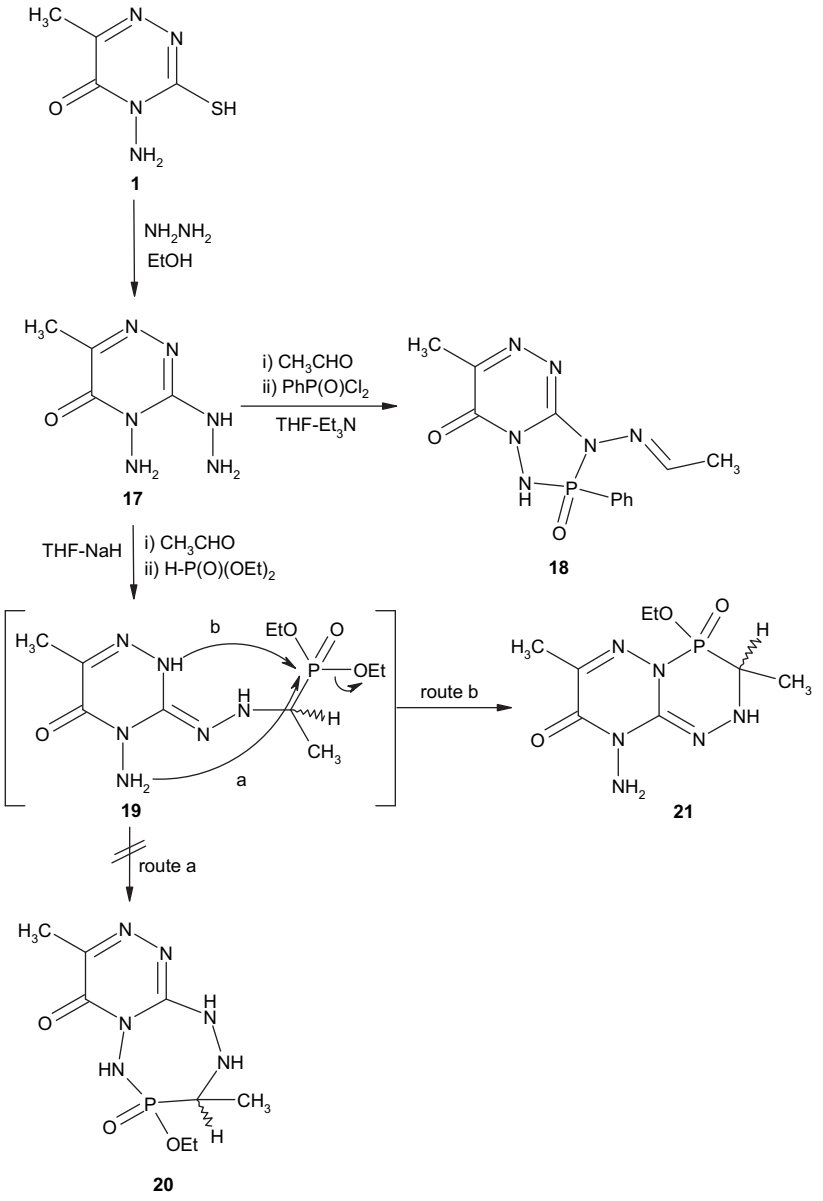
Scheme 2.



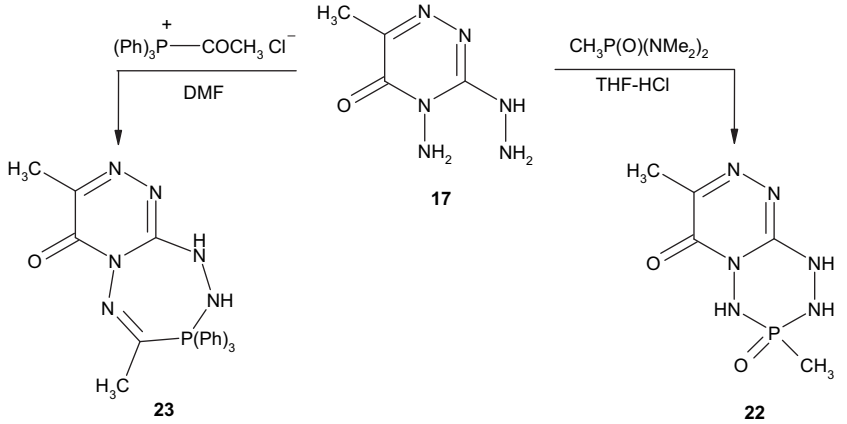
Scheme 3.

fragment while its ^{31}P NMR spectrum showed a signal at δ +22.3 ppm. The mass spectrum of **21** showed the molecular ion peak at *m*/*e* 274 (2.45%) and the base peak at *m*/*e* 211.

Finally, novel six and seven-membered phosphorus heterocycles, namely 2,7-dimethyl-2-oxido-1,2,3,4-tetrahydro-8*H*-[1,2,4]triazino[4,3-*e*][1,2,4,5,3]tetrazaphosphinin-8-one (**22**) and 4,8-dimethyl-3,3,3-triphenyl-2,3-dihydro-3-λ^5-[1,2,4]triazino[4,3-*e*][1,2,5,6,3]tetrazaphosphepin-7(1*H*)-one (**23**) were obtained by cyclocondensation of compound **17** with bis(dimethylamino)methylphosphonate in THF in the presence of few drops of hydrochloric acid and acetyl triphenylphosphonium chloride in DMF containing few drops of piperidine, respectively (Scheme 5). The structures of **22** and **23** were established on the basis of their elemental analysis and spectral data (IR, ^1H, ^{13}C, ^{31}P NMR and MS). The ^{13}C NMR spectrum of **22** was characterized by signals for two methyl carbons at δ 17.0 and 16.6 ppm for $CH_{3,triazine}$ and CH_3–P=O, respectively, while compound **23** showed signals at δ 17.0 ppm for $CH_{3,triazine}$, 16.0 ppm for CH_3–C–P and at δ 145.9 ppm due to N=C–P ($J_{C,P}$ = 62.5 Hz). Also, the ^{31}P NMR spectra showed one singlet signal for each at δ +27.1 and −22.3 ppm for **22** and **23**, respectively. The mass spectrum of compound **22** recorded the molecular ion peak at *m*/*e* 216 (1.09%) while the base peak was found at *m*/*e* 105.



Scheme 4.



Scheme 5.

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

Compd. no.	Diameter of the inhibition zone (in mm)[a]			
	Gram positive bacteria		Gram negative bacteria	
	Staphylococcus aureus (MTCCB 737)	*Staphylococcus epidermidis* (MTCCB 1824)	*Escherichia coli* (MTCCB 1652)	*Pseudomonas aeruginosa* (ATCC 27853)
1	13	14	14	15
2	20	17	21	23
4	20	19	19	22
6	21	19	16	19
9	27	23	15	20
13	14	18	17	18
14	16	15	14	13
16	27	24	25	29
17	17	15	15	15
18	20	17	14	16
21	25	23	21	28
22	19	16	15	20
23	19	16	22	22
Tetracycline[b]	30	25	28	29

[a] 12 mm or less: resistant or less inhibition, 13–17 mm: moderate inhibition, 18 mm or more: maximum inhibition.
[b] The concentration of used reference drug was 30 μg/mL.

3. Biological activity

3.1. Antibacterial activity

All the precursors and newly synthesized compounds were screened *in vitro* for their antibacterial activity. The antibacterial assays were carried out against four bacterial strains, *Staphylococcus aureus* (MTCCB 737), *Staphylococcus epidermidis* (MTCCB 1824), *Escherichia coli* (MTCCB 1652) and *Pseudomonas aeruginosa* (ATCC 27853) employing the nutrient agar disc diffusion method [25] at 100 μg/mL concentration. The antibacterial activity was determined by measuring the inhibition zone (Table 1). The minimum inhibitory concentration (MIC, μg/mL) of the tested compounds against all species of bacterial strains was also determined. The tube dilution technique [26] was applied for the determination of MIC of the tested compounds. Dilution series were set up with 3.12, 6.25, 12.5, 25 and 50 μg/mL (Table 2).

The investigation of antibacterial screening at MIC data revealed that:

(1) Compounds **1**, **13**, **17** and **18** showed inhibitions against most of the bacterial strains at 50 μg/mL.

Table 2
The minimum inhibitory concentration (MIC, μg/mL) of the synthesized compounds.

Compd. no.	The minimum inhibitory concentration (MIC)			
	Staphylococcus aureus (MTCCB 737)	*Staphylococcus epidermidis* (MTCCB 1824)	*Escherichia coli* (MTCCB 1652)	*Pseudomonas aeruginosa* (ATCC 27853)
1	50	50	50	25
2	25	25	12.5	12.5
4	25	25	12.5	12.5
6	25	12.5	50	50
9	6.25	6.25	50	25
13	50	25	50	50
14	25	25	50	50
16	6.25	6.25	6.25	6.25
17	50	50	25	50
18	25	50	50	50
21	6.25	6.25	12.5	6.25
22	25	25	50	25
23	25	25	12.5	12.5
Tetracycline	6.25	6.25	6.25	6.25

Table 3
Cytotoxicity data of some of the synthesized compounds.

Compd. no.	LC_{50} (μg/mL)
2	7.85
9	13.82
16	32.31
21	25.94
23	5.66
Bleomycin	0.41

(2) Compound **22** showed inhibition against all the bacterial strains at 25 μg/mL except *E. coli* which was inhibited at 50 μg/mL. Also, compound **6** showed inhibition against *E. coli* and *P. aeruginosa* at 50 μg/mL and against *S. aureus* and *S. epidermidis* at 25 and 12.5 μg/mL, respectively.
(3) Compounds **2**, **4** and **23** showed inhibitions against *S. aureus* and *S. epidermidis* at 25 μg/mL and against *E. coli* and *P. aeruginosa* at 12.5 μg/mL.
(4) Compound **9** showed inhibition against *S. aureus* and *S. epidermidis* at 6.25 μg/mL while compound **21** showed the same inhibition against all the bacterial strains except *E. coli*.
(5) Compound **16** showed a level of inhibition almost equivalent to the reference drug against all the bacterial strains at 6.25 μg/mL.

3.2. Cytotoxicity activity

The synthesized compounds **2**, **9**, **16**, **21** and **23** were screened for their cytotoxicity (brine shrimp bioassay). Their LC_{50} values were found to be 7.85, 13.82, 32.31, 25.94 and 5.66 μg/mL, respectively (Table 3). The standard drug Bleomycin has LC_{50} value at 0.41 μg/mL. Lower LC_{50} values were found in the case of compounds **2** and **23**, which suggested a comparatively high degree of toxicity for these compounds. On the other hand, the LC_{50} values of compounds **16** and **21** showed weak cytotoxic activity against brine shrimp, therefore they can be considered less cytotoxic antibacterial agents.

4. Conclusion

This article presented the synthesis and characterization of some novel phosphorus heterocycles containing 1,2,4-triazinone moiety based on the reaction of α,β-bifunctional 1,2,4-triazinone with some phosphorus reagents. The examination of their antibacterial activities provides the following trends:

(1) All the tested compounds showed moderate to good inhibition of bacterial growth, in particular with respect to the precursors **1**, **14** and **17** which confirms that combination of 6-methyl-1,2,4-triazin-5-(4*H*)one moiety with heterocycles containing phosphorus atom enhances the antibacterial properties.
(2) Seven-membered ring containing phosphorus atom resulted in weaker inhibitory activity than five- and six-membered rings.
(3) Compounds **16** and **21** exhibited good inhibitory activity against most the bacterial strains and lower cytotoxicity. The beneficial presence of the cyclic α-aminophosphonate monoester, previously noted for its impact on biological systems [27,28], on the bioactive 6-methyl-1,2,4-triazin-5-one moiety in these compounds may suggest a path forward for 6-methyl-1,2,4-triazin-5-one with improved antibacterial activity and reduced cytotoxicity.

5. Experimental

Melting points of the products were determined on a Kofler microscope and are uncorrected. The IR spectra were recorded on a Bruker IFS 1113 spectrophotometer or Elmer 293 spectrophotometer (γ in cm^{-1}), using KBr disks. The ^1H NMR spectra were recorded on a Bruker DRX (250 MHz) spectrometer or Gemini-200 spectrometer (200 MHz), using DMSO-d_6 as a solvent and TMS (δ, 0.0 ppm) as internal standard. All ^{13}C and ^{31}P NMR spectra were registered on a Varian Inova 500 MHz spectrometer at room temperature using DMSO-d_6 as a solvent and TMS as internal standard and 85% H_3PO_4 as external reference. The mass spectra were recorded on a JOEL JMS 300 mass spectrometer operating at 70 eV. The purity of the synthesized compounds was checked by thin layer chromatography (TLC), which was performed on Kieselgel 60 F254 plastics sheets (Merck Sigma Chemical Co., Germany). Elemental microanalyses were performed at microanalysis center in Bulgarian Academy of Science, Sofia, Bulgaria. 4-Amino-6-methyl-3-thioxo-3,4-dihydro-1,2,4-triazin-5(2*H*)-one (**1**) [29], 4-{[(4-chlorophenyl)methylene]amino}-6-methyl-3-thioxo-3,4-dihydro-1,2,4-triazin-5(2*H*)-one (**7**) [30], 3,4-diamino-6-methyl-1,2,4-triazin-5(4*H*)-one (**14**) [31] and 4-amino-3-hydrazino-6-methyl-1,2,4-triazin-5(4*H*)-one (**17**) [32] were prepared by the published methods.

5.1. Synthesis of 6-methyl-2-oxido-2-phenyl-1,2-dihydro-7H-[1,3,4,2]thiadiazaphospholo[5,4-c][1,2,4]triazin-5-one (2)

A mixture of 4-amino-3-mercapto-6-methyl-1,2,4-triazin-5(4*H*)-one (**1**) (0.005 mol, 0.79 g) and phenylphosphonic dichloride (0.005 mol, 0.975 g) in THF (40 mL) containing triethylamine (0.01 mol, 1.01 g), was heated under reflux for 3 h. The reaction mixture was evaporated under reduced pressure, giving the crude product which was crystallized from ethanol. Yellow crystals in 75% yield; mp 184–185 °C. IR (KBr), ν (cm^{-1}): 3158 (NH), 3058 (C–H_{arom}), 2900 (C–H_{aliph}), 1681 (C=$O_{triazinone}$), 1614 (C=N), 1225 (P=O). ^1H NMR (DMSO), δ: 2.08 (s, 3H, CH_3), 7.59–7.71 (m, 5H, aromatic protons), 8.25 (d, 1H, $J_{NH,P} = 3.3$ Hz, NH). ^{13}C NMR (DMSO), δ: 17.0 ($CH_{3,triazine}$), 128.0–130.0 (phenyl carbons), 150.1 (C=N), 169.9 (C=O), 172.2 (C–S). ^{31}P NMR (DMSO), δ: +55.1 ppm. MS (*m*/*e*, %): 283 (M + 3, 2.22%), 282 (M + 2, 9.09), 280 (M^+, 3.99), 265 (17.64), 243 (13.71), 224 (9.02), 186 (100), 128 (45.78), 85 (15.54), 77 (15.65), 66 (30.22). Calcd for $C_{10}H_9N_4O_2PS$ (280.24): C, 42.85; H, 3.23; N, 19.99; Found: C, 42.64; H, 3.10; N, 19.83.

5.2. Synthesis of 3,7-dimethyl-2,2,2-triphenyl-2H,6H-2λ⁵-[1,2,4]triazino[4,3-c][1,4,5,2] thiadiazaphosphinin-6-one (4) and 8-methyl-2,2,2,4-tetraphenyl-2,3-dihydro-7H-2λ⁵-[1,2,4]triazino[4,3-f][1,5,6,2]thiadiazaphosphepin-7-one (6)

A mixture of **1** (0.005 mol, 0.79 g) and acetyl triphenylphosphonium chloride (0.005 mol, 1.70 g) or phenacyl triphenylphosphonium bromide (0.005 mol, 2.30 g) in DMF (30 mL) containing few drops of piperidine, was heated under reflux for 12 h, cooled and then poured into ice. The obtained solids were filtered off and crystallized from ethanol to give **4** and **6**, respectively.

4: Brown crystals in 85% yield; mp 120–122 °C. IR (KBr), ν (cm^{-1}): 3049 (C–H_{arom}), 2929 (C–H_{aliph}), 1677 (C=$O_{triazinone}$), 1593 (C=N). ^1H NMR (DMSO), δ: 2.08 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 7.50–7.66 (m, 15H, aromatic protons). ^{13}C NMR (DMSO), δ: 16.3 ($CH_{3,thiadiazaphosphinine}$), 17.0 ($CH_{3,triazine}$), 127.6–131.0 (phenyl carbons), 147.0 (N=***C***–P, $J_{C,P} = 68$ Hz), 150.2 (C=N), 172.2 (C=O), 175.6 (C–S). ^{31}P NMR (DMSO), δ: −11.2 ppm. Calcd for: $C_{24}H_{21}N_4OPS$ (444.48): C, 64.85; H, 4.76; N, 12.60; Found: C, 64.66; H, 4.56; N, 12.37.

6: Yellow crystals in 65% yield; mp 122–124 °C. IR (KBr), ν (cm^{-1}): 3054 (C–H_{arom}), 2923 (C–H_{aliph}), 1678 (C=$O_{triazinone}$), 1592 (C=N). ^1H NMR (DMSO), δ: 2.51 (s, 3H, CH_3), 5.00 (br, CH_2, 2H), 7.03–7.93 (m, 20H, aromatic protons). ^{13}C NMR (DMSO), δ: 17.0 ($CH_{3,triazine}$), 41.5 (***CH_2***–P, $J_{C,P} = 55.8$ Hz), 128.0–131.1 (phenyl carbons), 150.0 (C=N), 153.3 (N=***C***–Ph), 170.9 (C=O), 171.8 (C–S). ^{31}P NMR (DMSO), δ: −18.4 ppm. Calcd for: $C_{30}H_{25}N_4OPS$ (520.58): C, 69.21; H, 4.84; N, 10.76; Found: C, 68.89; H, 4.72; N, 10.54.

5.3. Synthesis of 3-(4-chlorophenyl)-3,4-dihydro-2-ethoxy-2-oxido-7-methyl-2H,6H-[1,2,4]triazino[4,3-e][1,4,5,2]thiadiazaphosphinin-6-one (9)

A mixture of 4-{[(4-chlorophenyl)methylene]amino}-3-mercapto-6-methyl-3,4-dihydro-1,2,4-triazin-5(2*H*)-one (**7**) (0.005 mol, 1.40 g) and diethyl phosphite (0.007 mol, 0.966 g) in THF (30 mL) containing amount of sodium hydride (0.005 mol, 0.12 g) was heated under reflux for 12 h. The reaction mixture was evaporated under reduced pressure, giving crude solid which was crystallized from ethanol. Pale yellow crystals in 55% yield; mp 192–194 °C. IR (KBr), ν (cm^{-1}): 3249 (NH), 3090 (C–H_{arom}), 2978, 2934 (C–H_{aliph}), 1641 (C=$O_{triazinone}$), 1581 (C=N), 1217 (P=O), 1060 (P–O–C), 659 (C–Cl). ^1H NMR (DMSO), δ: 1.30 (t, 3H, $J = 7$ Hz, OCH_2***CH_3***), 2.22 (s, 3H, CH_3), 4.24 (q, 2H, $J = 7$ Hz, O***CH_2***CH_3), 5.41 (br, 1H, ***CH***–P), 7.31 (d, 2H, $J = 6.7$ Hz, aromatic protons), 7.98 (d, 2H, $J = 6.5$ Hz, aromatic protons), 8.24 (br, 1H, NH). ^{13}C NMR (DMSO), δ: 16.7 (OCH_2***CH_3***, $J = 6.3$ Hz), 17.0 ($CH_{3,triazine}$), 48.8 (***CH***–P, $J_{C,P} = 150$ Hz), 59.8 (O***CH_2***CH_3, $J = 6.3$ Hz), 129.0–138.0 (phenyl carbons), 149.4 (C=N), 170.0 (C=O), 172.3 (C–S). ^{31}P NMR (DMSO), δ: +38.9 ppm. MS (*m*/*e*, %): 374 (M + 1, 0.25%), 373 (M^+, 2.34), 360 (10.45), 327 (13.45), 270 (10.45), 239 (65.76), 216 (67.90), 124 (100), 111 (12.57), 52 (3.45). Calcd for: $C_{13}H_{14}ClN_4O_3PS$ (372.76): C, 41.88; H, 3.78; N, 15.03; Found: C, 41.60; H, 3.56; N, 14.91.

5.4. Synthesis of 7-(4-chlorophenyl)-8-(diphenylphosphoryl)-3-methyl-4-oxo-4,8-dihydropyrazolo[5,1-c][1,2,4]triazine-8-carbonitrile (13)

A mixture of **7** (0.005 mol, 1.40 g) and diphenylphosphoryl acetonitrile (0.005 mol, 1.20 g) in THF (30 mL) containing amount of sodium hydride (0.01 mol, 0.24 g) was heated under reflux for 12 h. The reaction mixture was evaporated under reduced pressure, giving crude solid which was crystallized from ethanol. Pale brown crystals in 46% yield; mp 208–209 °C. IR (KBr), ν (cm^{-1}): 3092 (C–H_{arom}), 2924 (C–H_{aliph}), 2226 (C≡N), 1667 (C=$O_{triazinone}$), 1594 (C=N), 1262 (P=O), 587 (C–Cl). ^1H NMR (DMSO), δ: 2.08 (s, 3H, CH_3), 7.50–7.66 (m, 14H, aromatic protons). ^{13}C NMR (DMSO), δ: 17.1 ($CH_{3,triazine}$), 45.8 (***C***–P=O, $J_{C,P} = 62.5$ Hz), 110.1 (C≡N), 127.9–137.3 (phenyl carbons), 145.4 (C=$N_{pyrazole}$), 150.0, 152.0 (2C=$N_{triazine}$), 170.0 (C=O). ^{31}P NMR (DMSO), δ: +32.8 ppm. Calcd for: $C_{25}H_{17}ClN_5O_2P$ (485.86): C, 61.80; H, 3.52; N, 14.41; Found: C, 61.62; H, 3.34; N, 14.23.

5.5. Synthesis of 3,7-dimethyl-2-ethoxy-2-oxido-1,2,3,4-tetrahydro-6H-[1,2,4]triazino[4,3-b][1,2,4,5]triazaphosphinin-6-one (16) and 9-amino-3,7-dimethyl-4-ethoxy-4-oxido-2,3,4,9-tetrahydro-8H-[1,2,4]triazino[3,2-c][1,2,4,5]triazaphosphinin-8-one (21)

A mixture of 3,4-diamino-6-methyl-1,2,4-triazin-5(4*H*)-one (**14**) (0.005 mol, 0.70 g) or 4-amino-3-hydrazino-6-methyl-1,2,4-triazin-5(4*H*)-one (**17**) (0.005 mol, 0.78 g) and acetaldehyde (0.005 mol, 0.22 g) in THF (30 mL) containing amount of sodium hydride (0.005 mol, 0.12 g) was warmed for 10 min, then added to a solution of diethyl phosphite (0.007 mol, 0.966 g) in THF (3 mL)

and heated under reflux for 12 h. The reaction mixture was evaporated under reduced pressure, giving crude solids which were crystallized from ethanol to give **16** and **21**, respectively.

16: Pale brown crystals in 49% yield; mp 108–111 °C. IR (KBr), ν (cm^{-1}): 3212, 3146 (NH, NH), 2958 (C–H_{aliph}), 1695 (C═$O_{triazinone}$), 1615, 1596 (C═N), 1287 (P═O), 1088 (P–O–C). ^{1}H NMR (DMSO), δ: 1.12 (d, 3H, J = 6.7 Hz, NCH***CH*****$_3$**), 1.17 (t, 3H, J = 7.5 Hz, OCH_2***CH*****$_3$**), 2.13 (s, 3H, CH_3), 3.77 (q, 2H, J = 7.2 Hz, O***CH*****$_2$**CH_3), 5.38 (br, 1H, ***CH***–P), 6.49 (br, 1H, NH), 8.24 (s, 1H, NH). ^{13}C NMR (DMSO), δ: 16.4 (OCH_2***CH*****$_3$**, J = 6.3 Hz), 16.7 (P–CH–***CH*****$_3$**), 17.1 ($CH_{3,triazine}$), 47.5 (***CH***–P, $J_{C,P}$ = 147.2 Hz), 62.5 (O***CH*****$_2$**CH_3, J = 7.5 Hz), 150.2 (C═N), 151.9 (N═***C***–N), 172.3 (C═O). ^{31}P NMR (DMSO), δ: +21.5 ppm. MS (*m*/*e*, %): 261 (M + 2, 0.35%), 259 (M^+, 3.82), 214 (16.44), 213 (100), 176 (15.75), 103 (30.07), 77 (14.47), 64 (12.68), 51 (11.26). Calcd for: $C_8H_{14}N_5O_3P$ (259.20): C, 37.07; H, 5.44; N, 27.01; Found: C, 36.85; H, 5.23; N, 26.81.

21: Yellow crystals in 59% yield; mp 131–133 °C. IR (KBr), ν (cm^{-1}): 3420 (br, NH_2), 3250 (NH), 2950 (C–H_{aliph}), 1665 (C═$O_{triazinone}$), 1624 (C═N), 1210 (P═O), 1091 (P–O–C). ^{1}H NMR (DMSO), δ: 1.12 (d, 3H, J = 6.9 Hz, NCH***CH*****$_3$**), 1.17 (t, 3H, J = 7.5 Hz, OCH_2***CH*****$_3$**), 2.13 (s, 3H, CH_3), 3.72 (q, 2H, J = 7 Hz, O***CH*****$_2$**CH_3), 5.38 (br, 1H, ***CH***–P), 6.49 (s, 2H, NH_2), 7.70 (s, 1H, NH). ^{13}C NMR (DMSO), δ: 16.2 (OCH_2***CH*****$_3$**, J = 6.3 Hz), 16.7 (P–C–***CH*****$_3$**), 17.1 ($CH_{3,triazine}$), 49.5 (***CH***–P, $J_{C,P}$ = 145.2 Hz), 60.5 (O***CH*****$_2$**CH_3, J = 6.9 Hz), 151.2 (C═N), 152.4 (N═***C***–N), 172.8 (C═O). ^{31}P NMR (DMSO), δ: +22.3 ppm. MS (*m*/*e*, %): 275 (M + 1, 1.34%), 274 (M^+, 2.45), 257 (3.54), 228 (10.23), 211 (100), 110 (65.03), 102 (34.11), 84 (22.11), 74 (20.14), 56 (15.09). Calcd for: $C_8H_{15}N_6O_3P$ (274.21): C, 35.04; H, 5.51; N, 30.64; Found: C, 34.87; H, 5.32; N, 30.52.

5.6. Synthesis of 3-[ethylideneamino]-6-methyl-2-oxido-2-phenyl-2,3-dihydro[1,2,4,3] triazaphospholo[5,1-c][1,2,4]triazin-7-one (**18**)

A mixture of **17** (0.005 mol, 0.78 g) and acetaldehyde (0.005 mol, 0.22 g) in THF (30 mL) containing triethylamine (0.01 mol, 1.01 g) was warmed for 10 min, then added to a solution of phenylphosphonic dichloride (0.005 mol, 0.97 g) in THF (3 mL) and heated under reflux for 4 h. The reaction mixture was evaporated under reduced pressure, giving crude solid which was crystallized from ethanol. Yellow crystals in 77% yield; mp 196–198 °C. IR (KBr), ν (cm^{-1}): 3180 (NH), 3077 (C–H_{arom}), 2961 (C–H_{aliph}), 1694 (C═$O_{triazinone}$), 1644, 1609 (C═N), 1284 (P═O). ^{1}H NMR (DMSO), δ: 2.13 (s, 3H, CH_3), 2.50 (d, 3H, ***CH*****$_3$**CHN), 5.77 (br, 1H, N═CH), 7.44–7.99 (m, 5H, aromatic protons), 8.29 (s, 1H, NH). ^{13}C NMR (DMSO), δ: 16.8 (***CH*****$_3$**–C–N), 17.0 ($CH_{3,triazine}$), 128.5–130.1 (phenyl carbons), 142.3 (N═***CH***–CH_3), 151.1 (C═N), 152.2 (N═***C***–N), 172.2 (C═O). ^{31}P NMR (DMSO), δ: +26.4 ppm. Calcd for: $C_{12}H_{13}N_6O_2P$ (304.24): C, 47.37; H, 4.30; N, 27.62; Found: C, 47.12; H, 4.17; N, 27.45.

5.7. Synthesis of 2,7-dimethyl-2-oxido-1,2,3,4-tetrahydro-8H-[1,2,4]triazino[4,3-e][1,2,4,5,3] tetrazaphosphinin-8-one (**22**)

A mixture of **17** (0.005 mol, 0.78 g) and bis(dimethylamino)-methylphosphonate (0.005 mol, 1.03 g) in THF (30 mL) containing few drops of concentrated hydrochloric acid, was heated under reflux for 5 h. The reaction mixture was evaporated under reduced pressure, giving crude solid which was crystallized from ethanol. Pale brown crystals in 81% yield; mp 175–177 °C. IR (KBr), ν (cm^{-1}): 3533 (br, NHNH), 3146 (NH), 2957, 2925 (C–H_{aliph}), 1690 (C═$O_{triazinone}$), 1618 (C═N), 1269 (P═O). ^{1}H NMR (DMSO), δ: 2.13 (s, 3H, CH_3), 2.50 (s, 3H, ***CH*****$_3$**–P), 5.34 (br, 1H, NH), 6.48 (br, 1H, NH), 7.68 (s, 1H, NH). ^{13}C NMR (DMSO), δ: 16.6 ($CH_{3,tetrazaphosphinine}$), 17.0 ($CH_{3,triazine}$), 149.9 (C═N), 150.4 (N═***C***–N), 173.2 (C═O). ^{31}P NMR (DMSO), δ: +27.1 ppm. MS (*m*/*e*, %): 219 (M + 3, 0.06%), 216 (M^+, 1.09), 208 (13.45), 176 (23.09), 165 (34.76), 162 (23.90), 149 (45.98), 105 (100), 100 (56.98), 91 (45.87), 78 (22.65), 56 (31.56). Calcd for: $C_5H_9N_6O_2P$ (216.13): C, 27.78; H, 4.19; N, 38.88; Found: C, 27.53; H, 3.92; N, 38.63.

5.8. Synthesis of 4,8-dimethyl-3,3,3-triphenyl-2,3-dihydro-3-λ^5-[1,2,4]triazino[4,3-f][1,2,5,6,3]tetrazaphosphepin-7(1H)-one (**23**)

A mixture of **17** (0.005 mol, 0.78 g) and acetyl triphenylphosphonium chloride (0.005 mol, 1.70 g) in DMF (30 mL) containing few drops of piperidine, was heated under reflux for 12 h, cooled and then poured into ice. The obtained solid was filtered off and crystallized from methanol. Brown crystals in 43% yield; mp 154–155 °C. IR (KBr), ν (cm^{-1}): 3295, 3217 (NH, NH), 3096 (C–H_{arom}), 2907 (C–H_{aliph}), 1662 (C═$O_{triazinone}$), 1601 (C═N). ^{1}H NMR (DMSO), δ: 2.08 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 7.43–7.96 (m, 15H, aromatic protons), 8.67 (s, 1H, NH), 10.0 (br s, 1H, NH). ^{13}C NMR (DMSO), δ: 16.0 ($CH_{3,tetrazaphosphepine}$), 17.0 ($CH_{3,triazine}$), 128.2–130.0 (phenyl carbons), 145.9 (N═***C***–P, $J_{C,P}$ = 62.5 Hz), 152.0 (C═N), 152.5 (N═***C***–N), 172.0 (C═O). ^{31}P NMR (DMSO), δ: −22.3. Calcd for: $C_{24}H_{23}N_6OP$ (442.45): C, 65.15; H, 5.23; N, 18.99; Found: C, 64.87; H, 5.05; N, 18.82.

5.9. Antibacterial activity

All the newly synthesized compounds were evaluated *in vitro* for their antibacterial activity. The antibacterial activity is carried out against four bacterial strains, *S. aureus* (MTCCB 737), *S. epidermidis* (MTCCB 1824), *E. coli* (MTCCB 1652) and *P. aeruginosa* (ATCC 27853) employing the nutrient agar disc diffusion method [25] at 100 μg/mL concentration. DMSO was used as blank exhibited no activity against any of the used organisms. The antibacterial activity was determined by measuring the inhibition zone (Table 1), after 16–20 h of incubation at 37 °C for bacterial strains. Tetracycline was used as reference drug against bacterial strains at 30 μg/mL concentration.

5.10. 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 agents. The tube dilution technique [26] was applied for the determination of MIC of the tested compounds against. Dilution series were set up with 3.12, 6.25, 12.5, 25 and 50 μg/mL of nutrient broth medium to each tube, 100 μL of standardized suspension of the test microbes (10^7 cell/mL) were added and incubated at 37 °C for 24 h (Table 2).

5.11. Cytotoxicity bioassay

Brine shrimp lethality bioassay [33,34] 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 1 L 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). Samples of the tested compounds were prepared by dissolving 10 mg of each compound in 2 mL of DMSO. From this stock, a series of solutions 80, 40, 20, 10

and 5 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 [35] for the determination of LC_{50} values for the five tested compounds (Table 3).

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