Synthesis, biological activity and molecular modeling study of novel 1,2,4-triazolo[4,3-b][1,2,4,5]tetrazines and 1,2,4-triazolo[4,3-b][1,2,4]triazines

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Different novel 1,2,4-triazolo[4,3-b][1,2,4,5]tetrazines and 1,2,4-triazolo[4,3-b][1,2,4]triazines have been obtained from heterocyclization of 3-substituted-4-amino-5-substituted-amino-1,2,4-triazoles (3a-d) and 3-substituted-4-amino-5-hydrazino-1,2,4-triazoles (9a,b) with α and β bifunctional compounds like chloromethyl biphenyl-phosphanoxide, pyruvic acid, phenacyl bromide, diethyl oxalate, triethyl orthoformate, triethyl phosphite, fluorinated benzaldehydes, carbon disulfide and ethyl chloroformate under different experimental settings. Fourier transformer infrared analysis (FTIR), Proton nuclear magnetic resonance (1H NMR) and 13C nuclear magnetic resonance (13C NMR), as well as that of the mass spectral data, were used as the appropriate characterization techniques for the chemical structures of all newly synthesized compounds. The newly prepared compounds were examined as an anti-inflammatory, antibacterial agents (against E. coli (Escherichia coli) and P. aeruginosa (Pseudomonas aeruginosa) as examples for Gram-negative bacteria and S. aureus (Staphylococcus aureus) as examples for Gram-positive bacteria), as well as antifungal (against C. albicans (Candida albicans)) agents. The newly prepared compound showed high antibacterial, antifungal, and anti-inflammatory activities in comparing with the commercial antibiotics Indomethacin, Nalidixic acid, Imipenem, and Nystatin. Docking of the most active compounds was performed depending on the results of antibacterial screening and the anti-inflammatory assay.

Studies on heterocyclic compounds containing bridgehead nitrogen atom particularly those holding (1,2,4,5)-tetrazine, (1,2,4)-triazole and (1,2,4)-triazine derivatives have received much interest recently as they can be used in a variety of applications, especially in the medicinal field. For example, many of 1,2,4-triazole rings are found into a wide range of pharmaceutical drugs including antimicrobial agents1,2, antifungal3,4, antibacterial5–9, antmycobacterial10, antiviral11,12, anticancer13, antitubercular14, antmycocytic activity15,16, antimitragni agents, anti-inflammatory and analgesic17–19, anticonvulsants20, antinoceptive21, anti-urease22, antioxidant23, CNS stimulants, and antidepressant24 properties.

1,2,4-triazole rings possess not only diverse pharmacological activities but also to have herbicidal, insecticidal, plant growth regulatory and antifungal activities25. Thus, for many years, the biochemistry of these molecules has been investigated26. Also, Heterobicyclic nitrogen systems containing 1,2,4-Triazines derivatives and 1,2,4-Triazines themselves have been found to display a diversity of biological applications such as Lamotrigine as anti-epileptic drug27, Tiraizapine as anti-tumor28, and fused 1,2,4-triazines as antimicrobial29,30, anti-viral31, antmycobacterial32, angioytic33 and antidepressant34 activities. They also have shown anti-HIV and anticancer activities35. Moreover, derivatives of the tetrazine ring have attracted extensive attention from numerous research groups because of their interesting and diverse biological activities as antitumor and antiviral36. In this respect, Abdel-Rahman et al.37–39 found that 1,2,4-triazines, 1,2,4-triazoles and/or 1,2,4-triazolo-1,2,4,5-tetrazines can be used as a molluscicidal and antimicrobial as well as they have anticancer drugs activity. Due to their novel energetic properties37–39, organic compounds with high-nitrogen content currently attract the attention of many researchers.

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In light of these remarks, researchers were prompted to design and synthesize new drugs containing heterocyclic compounds, especially, those containing triazoles, triazines and tetrazines rings as a result of the fact that Nitrogen–Nitrogen bond is difficult to be produced in living organisms in addition to its therapeutic activities. Thus, because of these remarks, the target of this work is to synthesize some new 1,2,4-triazole fused with 1,2,4-triazine and/or 1,2,4,5-tetrazine nucleus holding different types of functional groups to enhance their biological activity, in a hope to design a semi-drug. To demonstrate whether COX-II was a potential target for our newly prepared compounds, molecular modeling studies have been conducted on these compounds.

Experimental
General Method for the preparation of novel 1,2,4-triazolo[4,3-b][1,2,4,5]tetrazines and 1,2,4-triazolo[4,3-b][1,2,4]triazines. A mixture of compounds 1a,b, and 2a,b with 1:1 molar ratio in 100 mL Ethanol-DMF was refluxed for 5 h, then poured into ice and the formed solids were collected by filtration and crystallized to give compounds 3a to 3d, respectively. To prepare the compounds 5a–d, a mixture of (0.01 mol) of 3a–d and (0.01 mol) of chloromethyl-diphenylphosphonoxide in (100 mL) THF with (0.5 mL) TEA was refluxed for 5 h and cooled. The obtained solids were then were collected by filtration and crystallized to give 5a–d, respectively. When (0.01 mol) of 3a–d were mixed with (0.01 mol) of phenacyl bromide in 50 mL from 5% ethanolic KOH and heated under reflux for 3 h, then poured into ice and acidified with few drops of HCl. The yielded solids were filtered and crystallized to give compounds 6a–d respectively. Compounds 7a–d were prepared by mixing a mixture of (0.01 mol) 3a–d and (0.01 mol) sodium pyruvate, in 10 mL H₂O with 50 mL 5% aqueous NaOH. After that, the mixture was refluxed for 3 h, then poured into ice and acidified with few drops of HCl. The products filtered and recrystallized to give 7a–d compounds respectively. Equimolar ratio mixture of both compounds 3a–d and diethyl oxalate in 100 mL THF was refluxed for 5 h and cooled. The obtained products were filtered and crystallized from the proper solvent to give 8a–d respectively. A mixture of each of 1a,b (0.01 mol) and hydrazine hydrate (0.04 mol) in ethanol (100 mL) was refluxed for 6 h and cooled. Then a few drops of acetic acid were added. The produced solids were filtered off and recrystallized to give 9a,b respectively. When the equimolar ratio of each of 9a,b and triethylphosphite in tetrahydrofuran (100 mL) and triethylamine (0.5 mL) was refluxed 6 h and cooled. The obtained solids were filtered off and recrystallized to give compounds 10a,b respectively. A mixture of 9a,b (0.01 mol) and triethylthioformate (0.012 mol) in tetrahydrofuran (100 mL) was refluxed for 6 h and cooled. The produced solids were filtered off and recrystallized to give 11a,b respectively. The spectral data together with the physical constants of 11a,b are listed below. A mixture of 9a,b (0.01 mole) and 2-chloro-6-fluorobenzaldehyde (0.01 mol) in ethanol (100 mL)/ piperidine (0.5 mL) was refluxed 12 h and then cooled. The produced solids were filtered off and crystallized to give 12a,b respectively. A mixture of each of 9a,b (0.01 mol), Cs₂ (0.02 mol) in DMF (50 mL) was refluxed for 3 h and cooled, then poured onto the ice. The resulting solids so formed were filtered off and recrystallized to give 13a,b respectively. A mixture of each of 9a,b (0.01 mol) and ethyl chloroformate (0.012 mol) with tetrahydrofuran (100 mL) and triethylamine (0.5 mL, added dropwise) was refluxed for 3 h and then cooled. The solids obtained were filtered off and recrystallized to give 14a,b respectively.

The first compound (1) was prepared by direct hydrazinolysis of 4-pyridyl-CONHNH₂, K according to Reid et al. reported method.

Characterization of the newly synthesized samples. The spectral data together with the physical constants of all of the previously prepared compounds were identified as follows:

To confirm the occurrence of the reaction, Fourier transformer infrared (FTIR) spectrometer (a Perkin Lemer Spectrum RXI FT-IR systems No. 55529) was used. FT-IR spectra within the wavenumber ranged from 4000–6000 cm⁻¹ were recorded at room temperature in ATR discs.

¹H/¹³C-NMR Spectra were recorded in (DMSO-d₆) at 400 MHz with a Bruker NMR Advance DPX 400 MH Spectrometer. TMS was used as an internal reference. Chemical shifts were supposed to be due to the presence of the solvent.

The melting points of the synthesized compounds were determined by using SMP (Stuart Scientific melting point) (USA).

GCMS-Q 1000 Ex spectrometer was used to measure the mass spectra of the prepared materials. Shimadzu UV-visible 3101 PC Spectrophotometer was used to record the electronic absorption spectra of the synthesized materials in DMF.

Antimicrobial activity. A bacterial suspension having a density of about 1 to 2 × 10⁸ (CFU) colony-forming units/mL was prepared by aging a single colony on an agar plate for 24 hours. The antibacterial activity of some newly synthesized compounds was investigated on Mueller-Hinton agar using Agar diffusion techniques at concentrations of 100, 50 and 25 μg/mL, respectively. Muller Hinton agar (MHA) plates were inoculated with test inoculum (E. coli, Pseudomonas aeruginosa and Staphylococcus aureus) of standard inoculum (0.5 McFarland). Nalidixic acid and Imipenem were used as a reference drug for bacteria. Briefly, MHA agar plates were inoculated with bacterial strains under sterile conditions, and disc (diameter = 8 mm) was loaded with 50 μL of the tested sample. The plate was incubated at 37 °C for 24 hours. Nalidixic acid and Imipenem were used as a reference drug for bacteria. After the incubation period, the antimicrobial agent usually diffuses into the agar and inhibits the germination and growth of the test microorganism, and then according to the Clinical Laboratory Standards (CLSI), the diameter of the growth-inhibiting zone was measured. The MICs of the prepared compounds were determined by the agar dilution plate technique following the standard procedure of the Clinical and Laboratory Standards. The MICs were calculated from the X-axis intercept of the linear graph between log (Inhibitory concentration) and the growth inhibitory zone area.
The fungal strain was grown in 5 mL Sabourad Glucose Broth (glucose: peptone; 40: 10) for 3–4 days to reach 10^5 CFU/mL cells. Fungal cultures (0.1 mL) were spread evenly on Sabourad dextrose agar plates. The plates were inoculated with fungal strains under sterile conditions, and disc (diameter = 8 mm) was loaded with 50 μL of the test sample. The plate was then incubated at 30 °C for 3–4 days and according to the Clinical Laboratory Standards (CLSI), the diameter of the growth-inhibiting zone was measured. Nystatin was used as an antifungal standard drug.

Anti-inflammatory activity of the newly synthesized compounds. Carrageenan-induced paw edema method was used to evaluate the anti-inflammatory effect of the selected prepared compounds. Indomethacin drug as a suspension in 24 tweens 80 was used as the reference drug, using Winter et al. method. The inhibition percentage of inflammation was calculated using the following equation:

\[
\text{Inhibition} \% = \frac{\text{weight of paw edema of control} - \text{weight of paw edema of related}}{\text{weight of paw edema of control}} \times 100
\]

Microanalyses (CHNS elemental) and anti-inflammatory activity of the prepared compounds were carried out in the pharmaceutical Microbiology Department, National Center for Radiation Research and Technology (NCRRT), Nasr City, Egypt.

**Molecular docking.** Based on the results of antibacterial screening and the anti-inflammatory assay, docking of the most active compound 5d and the positive control, *Nalidixic acid*, in case of antibacterial screening was performed with the binding site of DNA Gyrase (topoisomerase II) enzyme (PDB ID: 2XCT). Also, docking of compounds 10a,b, 12a,b and the positive control *Indomethacin* in case of anti-inflammatory effect was performed with the binding site of COX-2 (PDB ID: 1CX2). Docking was done to shed light on their potential binding modes and investigate their similarity to the standard ligand binding modes.

**Molecular docking procedure.** X-ray crystal structure of DNA Gyrase (topoisomerase II) complexed with a Ciprofloxacin was determined at 3.35 Å resolution and X-ray crystal structure of COX-2 complexed with a selective inhibitor, sc-558 at 3 Å resolution. All molecular modeling calculations and docking studies were carried out using Molecular Operating Environment 2019.0101 software (MOE of Chemical Computing Group Inc., on a Core i7 2.2 GHz workstation) running on a Windows 10 PC.

**Preparation of the targets (DNA Gyrase (topoisomerase II) enzyme and COX-2).** The X-ray crystallographic structures of DNA Gyrase (topoisomerase II) enzyme (PDB ID: 2XCT) and cyclo-oxygenase 2 (PDB ID: 1CX2) were downloaded from the protein data bank (http://www.rcsb.org)\(^4\). The enzyme was prepared for docking study by removal of chain B, C and D of its dimmers, water molecules, and ligands that are not involved in the binding. The enzyme preparation was then carried out using quick preparation protocol in MOE with default options.

**Docking validation.** To confirm whether the applied docking protocol is valid or not, Re-docking of the co-crystallized ligand into the enzyme was done. Based on the binding mode and rmsd (root mean square deviation), the coordinates of the native ligand in the co-crystallized PDB file were compared with the coordinates of the greatest scoring docking pose of the native ligand. The docking validation results revealed a near-perfect alignment with the original ligand as attained from the resolved X-ray PDB file. In the case of antibacterial screening, docking validation was confirmed from the small root mean square deviation (rmsd = 0.3155) between the docked pose and the co-crystallized ligand (S (energy score) of −11.2658 kcal/mol). Docking validation was also demonstrated by the ability of the docking poses to restore the main interactions that occur between the co-crystallized ligand and the active site’s hot spots, Manganese (Mn) atom and DG 5 of DNA Gyrase enzyme. In case of anti-inflammatory activity, the re-docked ligand showed a small root mean square deviation value (rmsd = 0.0215) between the docked compound and the co-crystallized ligand (S of −15.569 kcal/mol), they also showed a high ability to repeat the main interactions that occur between the co-crystallized ligand and the active site’s hot spots, Arg513 and His90 of COX-II.

**Active compounds preparation for docking.** Active compounds preparation for docking was done as follows: firstly, Marvin Sketch was used to build up the 2D structures of the docked ligands and copied onto MOE. This step is followed by 3D protonation of the active compounds structure. Then the systemic search was used for the running of the conformational analysis and then the smallest energetic conformer was selected. An identical docking protocol was applied with the ligand.

**Running of docking.** Docking studies were performed using DNA Gyrase (topoisomerase II) enzyme co-crystallized with the native ligand of protein data bank file ID: 2XCT (PDB ID: 2XCT) and also COX-II enzyme co-crystallized with the native ligand of protein data bank file ID: 1CX2 (PDB ID: 1CX2). Posing compounds 5d and *Nalidixic acid*, in case of antibacterial screening, and compounds 10a,b, 12a,b and the positive control *Indomethacin*, in case of anti-inflammatory effect, was scored by London dG scoring function, that was used for docking, and Triangle Matcher placement protocol. The docked compounds of the greatest scoring pose were documented. Interactions between ligand and receptor in the formed complexes were tested in both 3D and 2D styles.
Results and Discussion
The spectral data and physical constants of the newly synthesized compounds.

For compound (3a) (N3-(5-chloropyrimidin-2-yl)-5-phenyl-1H,1,2,4-triazole-3,4-diamine):

Yield 68% crystal from dioxan; mp 213–215°C. Analysis calculated for C30H25ClN7O (507): C, 50.90; H, 3.44; N, 34.08; Cl, 3.50; C12.32. The UV spectrum gave \( \lambda_{max} \) (Log e): 374 nm. IR characteristic peaks appear at (υ cm\(^{-1}\)): 3449, 3318 (NH\(_2\)), 1319 (NH), 1601, 1581 (C=\( \equiv \))N. \(^1\)H NMR characteristic peaks appear at δ ppm: 5.84(s, 2H), NH\(_2\)), 4.0(s, 1H), NH), 8.05(s, 2H, (CH)-pyrimidine), 7.61–8.28 (m, 5H, CH-benzene). \(^13\)C NMR characteristic peaks appear at δ ppm: 144.1 (C2 of triazole), 129.1 (C1 of benzene attached with triazole), 129.2 (C3 of benzene attached with triazole), 131.1 (C4 of benzene attached with triazole), 151.1 (C5 of pyrimidine, C-Cl), 126.5,129.2 (C2 and C3 of benzene), 131.1 (C4 of benzene), 121.3 (C2 of benzene), 149.8 (C2 and C6 of pyrimidine). MS (Int.%) : 338 (8.7, M\(^+\)).

For compound (5a) (8-(5-chloropyrimidin-2-yl)-3,6,6-triphenyl-5,6,7,8-tetrahydro-[1,2,4]triazolo-[3,4-f][1–3,5]triazaphosphinin-6-ol):

Yield 61% crystal from dioxan; mp 222–225°C. Analysis calculated for C\(_{24}\)H\(_{20}\)ClN\(_8\)O\(_2\) (502): C, 57.32; H, 4.23; Cl, 7.06; N, 19.53; P, 6.17. The analyses found for the compound are: C, 57.80; H, 3.42; Cl, 19.56; P, 6.18. IR characteristic peaks appear at (υ cm\(^{-1}\)): 3408 (aromatic (CH)), 2925 (aliphatic (CH)), 1630 (P=O), 1522 (C=\( \equiv \))N. \(^1\)H NMR characteristic peaks appear at δ ppm: 2.8(s, 1H, OH), 2.0(s, 1H, NH), 3.1(s, 2H, CH\(_2\)), 7.41–8.05 (m, 15H, CH-benzene), 8.08(s, 2H, CH-pyrimidine). \(^13\)C NMR characteristic peaks appear at δ ppm: 119.0 (C5 of pyrimidine, C-Cl), 134.5 (C6 of benzene, C-Cl), 127.2 (C2 of triazole), 167.9 (C2 of pyrimidine), 156.8 (C4 and C6 of pyrimidine). MS (Int.%): 287 (5.1, M\(^+\)), 271(12.5, M\(^+\) - NH\(_2\)), 174 (22.5, M\(^+\) - 4-chloropyrimidyl), 159 (34.8, M\(^+\) - 4-chloropyrimidyl-NH\(_2\)), 128 (15.1, 4-chloropyrimidyl-NH), 113 (12.2, 4-chloropyrimidyl).
For compound (5c) (8-(5-Bromopyrimidin-2-yl)-3,6,6-triphenyl-5,6,7,8-tetrahydro[1,2,4]triazolo[3,4-f]
[1-3,5]triazaphosphin-6-ol):
Yield 39%, crystals from dioxan; mp 267–269°C. Analysis calculated for C25H21BrN7OP (554): C, 54.96;
H, 3.87; Br, 14.62; N, 17.95; O, 2.93; P, 5.67. The analyses found for the compound are: C, 54.94; H, 3.86;
Br, 14.65; N, 18.00; O, 2.93; P, 5.70. IR characteristic peaks appear at (ν cm⁻¹): 1307 (aromatic (CH)),
1520 (C-Br of pyrimidine), 1310 (C6 of benzene), 152.1 (C5 of benzene attached with triazole), 129.2 (C6 and C8 of benzene attached with triazole), 131.1 (C3 of benzene attached with triazole), 131.2 (C-P of benzene), 84.3 (N-C-P of triazaphosphine), 157.2 (C5 of triazole), 151.1 (C6 of triazole), 168.4 (C6 of pyrimidine, C-N), 159.1 (C4 and C6 of pyrimidine).

For compound (6c) (8-(5-bromopyrimidin-2-yl)-6-phenyl-3-(pyridin-2-yl)-7,8-dihydro-[1,2,4]
triazolo[4,5-b][1,2,4]triazine):
Yield 49%, crystals from dioxan/diluted by methanol, mp 189–191°C. Analysis calculated for C18H12BrN9
(433): C, 49.79; H, 2.79; Br, 18.40; N, 29.03. The analyses found for the compound are: C, 49.7; H, 2.8;
Br, 18.4. IR characteristic peaks appear at (ν cm⁻¹): 3078, 2959 cm⁻¹ (aromatic & aliphatic (CH)), 1520 (C=N).
1H NMR characteristic peaks appear at δ ppm: 3.3 (s, 2H, (CH)-pyrimidine), 7.4–7.8 (m, 5H, CH-benzene), 8.4 (s, 2H, (CH)-pyrimidine).

For compound (6d) (8-(5-bromopyrimidin-2-yl)-6-phenyl-3-(pyridin-2-yl)-7,8-dihydro-[1,2,4]
triazolo[4,5-b][1,2,4]triazine):
Yield 49%, crystals from dioxan/diluted by methanol, mp 189–191°C. Analysis calculated for C18H12BrN9
(433): C, 49.79; H, 2.79; Br, 18.40; N, 29.03. The analyses found for the compound are: C, 49.7; H, 2.8;
Br, 18.4. IR characteristic peaks appear at (ν cm⁻¹): 3078, 2959 cm⁻¹ (aromatic & aliphatic (CH)), 1520 (C=N).
1H NMR characteristic peaks appear at δ ppm: 3.3 (s, 2H, (CH)-pyrimidine), 7.4–7.8 (m, 5H, CH-benzene), 8.4 (s, 2H, (CH)-pyrimidine).
For compound (8c) (8-(5-bromopyrimidin-2-yl)-3-phenyl-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-7(8H)-one):
Yield 55% crystal from ethanol; mp 300–303 °C. Analysis calculated for C_{14}H_{8}BrN_{7}O_{2}: C, 43.54; H, 2.06; Br, 20.9; IR characteristic peaks appear at (ν cm⁻¹): 3079, 2977 cm⁻¹ (aromatic & aliphatic (CH)), 1532 (C=C). ¹H NMR characteristic peaks appear at (δ ppm): 7.82 (s, (1H), NH), 8.42 (s, (2H), (CH)-pyrimidine), 7.94 (d, (2H), (CH)-pyrimidine). MS (m/z, %): 383 (M⁺, 100.0%), 226 (M⁺ + 5-bromopyrimidine, 32.4%), 228 ((M⁺ + 5) - 5-bromopyrimidine, 3.2%), 158 (5-bromopyrimidine, 2.1%).

For compound (7b) (8-(5-chloropyrimidin-2-yl)-6-methyl-3-phenyl-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-7(8H)-one):
Yield 42% crystal from ethanol; mp 244–246 °C. Analysis calculated for C_{14}H_{8}BrN_{7}O_{3}: C, 46.89; H, 2.62; Br, 20.8; IR characteristic peaks appear at (ν cm⁻¹): 3077, 2987 cm⁻¹ (aromatic & aliphatic (CH)), 1520 (C=C). ¹H NMR characteristic peaks appear at (δ ppm): 7.03–8.28 (m, 5H, CH-benzene). MS (m/z, %): 385 (M⁺ + 2, 32.4%), 387 (M⁺ + 3, 13.4%), 228 (M⁺ + 5 - 5-bromopyrimidine, 12.3%).

For compound (7c) (8-(5-bromopyrimidin-2-yl)-6-methyl-3-(pyridin-4-yl)-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-7(8H)-one):
Yield 36% crystal from ethanol; mp 274–276 °C. Analysis calculated for C_{14}H_{8}BrNO_{3}: C, 43.66; H, 2.36; Br, 20.74; N, 29.09. The analyses found for the compound are: C, 43.7; H, 2.4; N, 29.1; Br, 20.7. IR characteristic peaks appear at (ν cm⁻¹): 3079, 2977 cm⁻¹ (aromatic & aliphatic (CH)), 1532 (C=C). ¹H NMR characteristic peaks appear at (δ ppm): 7.81 (s, (1H), NH), 8.42 (s, (2H), (CH)-pyrimidine), 7.94 (d, (2H), (CH)-pyrimidine). MS (m/z, %): 383 (M⁺, 100.0%), 385 (M⁺ + 2, 33.4%), 226 (M⁺ + 5 - 5-bromopyrimidine, 11.7%), 228 ((M⁺ + 5) - 5-bromopyrimidine, 4.1%), 158 (5-bromopyrimidine, 15.3%), 116 (5-bromopyrimidine, 5.1%).

For compound (7d) (8-(5-chloropyrimidin-2-yl)-3-phenyl-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-6,7(5H,8H)-dione):
Yield 36% crystal from ethanol; mp 215–215 °C. Analysis calculated for C_{14}H_{8}BrN_{7}O_{2}: C, 49.21; H, 2.36; Br, 20.74; N, 29.09. The analyses found for the compound are: C, 49.2; H, 2.4; N, 28.7, Cl, 10.4. IR characteristic peaks appear at (ν cm⁻¹): 1520 (C=N). ¹H NMR characteristic peaks appear at (δ ppm): 7.99 (d, (2H), (CH)-pyrimidine), 8.75 (d, (2H), (CH)-pyrimidine). MS (m/z, %): 340 (M⁺, 100.0%), 342 (M⁺ + 2, 32.4%), 228 ((M⁺ + 2) - 5-bromopyrimidine, 3.2%), 158 (5-bromopyrimidine, 21.3%), 160 (5-bromopyrimidine, 7.1%).

For compound (8a) (8-(5-chloropyrimidin-2-yl)-3-(pyridin-4-yl)-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-6,7(5H,8H)-dione):
Yield 42% crystalized from ethanol; mp 152–155 °C. Analysis calculated for C_{14}H_{8}ClN_{7}O_{3}: C, 49.21; H, 2.36; Cl, 10.38; N, 28.69. The analyses found for the compound are: C, 49.2; H, 2.4; N, 28.7, Cl, 10.4. IR characteristic peaks appear at (ν cm⁻¹): 1522 (C=N). ¹H NMR characteristic peaks appear at (δ ppm): 7.6 (s, (1H), NH), 8.05 (s, (2H), (CH)-pyrimidine), 7.03–8.28 (m, 5H, CH-benzene). MS (m/z, %): 385 (M⁺ + 2, 32.4%), 387 (M⁺ + 3, 13.4%), 226 (M⁺ + 5 - 5-bromopyrimidine, 11.7%), 228 ((M⁺ + 5) - 5-bromopyrimidine, 3.2%), 158 (5-bromopyrimidine, 21.3%), 160 (5-bromopyrimidine, 5.1%).

For compound (8b) (8-(5-bromopyrimidin-2-yl)-3-phenyl-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-6,7(5H,8H)-dione):
Yield 38% crystallized from ethanol; mp 215–217 °C. Analysis calculated for C_{14}H_{8}BrN_{7}O_{2}: C, 45.56; H, 2.06; Cl, 10.35; N, 32.70. The analyses found for the compound are: C, 45.6; H, 2.1; N, 32.7, Cl, 10.3. IR characteristic peaks appear at (ν cm⁻¹): 3197 (NH), 1729, 1666 (C=O), 1520 (C=N). ¹H NMR characteristic peaks appear at (δ ppm): 7.9 (s, (1H), NH), 8.12 (s, (2H), (CH)-pyrimidine), 7.96 (d, (2H), (CH)-pyrimidine), 8.05 (d, (2H), (CH)-pyrimidine).

For compound (8c) (8-(5-bromopyrimidin-2-yl)-3-phenyl-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-6,7(5H,8H)-dione):
Yield 52% crystallized from ethanoic/acid mixture; mp 197–199 °C. Analysis calculated for C_{14}H_{8}BrN_{7}O_{2}: C, 43.54; H, 2.09; Br, 20.69; N, 25.39. The analyses found for the compound are: C, 43.5; H, 2.1; N, 25.4, Br, 20.7. IR characteristic peaks appear at (ν cm⁻¹): 3188 (NH), 3079 (aromatic (CH)), 1734, 1658 (2C=O), 1522 (C=N). ¹H NMR characteristic peaks appear at (δ ppm): 7.81 (s, (1H), NH), 8.42 (s, (2H), (CH)-pyrimidine), 8.05 (d, (2H), (CH)-pyrimidine).

For compound (8d) (8-(5-bromopyrimidin-2-yl)-3-(pyridin-4-yl)-[1,2,4]triazolo[4,3-b][1,2,4]
triazin-6,7(5H,8H)-dione):
Yield 57% crystallized from diluted ethanol; mp 223–225 °C. Analysis calculated for C_{14}H_{8}BrN_{7}O_{2}: C, 40.33; H, 1.82; Br, 20.64; N, 28.94. The analyses found for the compound are: C, 40.3; H, 1.8; N, 28.9, Br, 20.6. IR characteristic peaks appear at (ν cm⁻¹): 3180 (NH), 3088 (aromatic (CH)), 1735, 1668 (C=O), 1520 (C=N). ¹H NMR characteristic peaks appear at (δ ppm): 7.82 (s, (1H), NH), 8.43 (s, (2H), (CH)-pyrimidine), 7.87 (d, (2H), (CH)-pyrimidine), 8.15 (d, (2H), (CH)-pyrimidine).
For compound (9a) (3-hydrazinyl-5-phenyl-4H-1,2,4-triazol-4-amine):
Yield 67% crystal from ethanol; mp 155–157°C. Analysis calculated for C_{19}H_{22}N_{11} (436): C, 50.52; H, 5.30; N, 44.18. The analyses found for the compound are: C, 50.5; H, 5.3; N, 44.2. UV (DMF, λ_{max} nm (Log ε)): 316 (2.4) nm. IR characteristic peaks appear at (ν cm⁻¹): 3344 (NH₂), 3174 (NH), 3076 (aromatic (CH)), 1602 (deformation NH₂), 1522 (C=N). ¹H NMR characteristic peaks appear at δ ppm: 4.11 (s, 2H), NH₂ of hydrazine), 5.77 (s, 2H), NH₂ of triazole), 7.41–8.08 (m, 5H, CH-benzene), 9.23 (s, 1H, NH of hydrazine).

For compound (9b)(3-hydrazinyl-5-(pyridin-4-yl)-4H-1,2,4-triazol-4-amine):
Yield 58% crystal from ethanol; mp 187–189°C. Analysis calculated for C_{19}H_{21}N_{11} (411): C, 43.97; H, 4.74; N, 51.28. The analyses found for the compound are: C, 44.0; H, 4.7; N, 51.3. UV (DMF, λ_{max} nm (Log ε)): 312 (2.1) nm. IR characteristic peaks appear at (ν cm⁻¹): 3356, (NH₂), 3181 (NH), 1600 (deformation NH₂), 1518 (C=N). ¹H NMR characteristic peaks appear at δ ppm: 4.23 (s, 1H, NH₂ of hydrazine), 5.53 (s, 1H, NH₂ of triazole), 7.83 (d, (2H), (CH)-pyridine), 8.87 (d, (2H), (CH)-pyridine), 9.76 (s, 1H, NH).

For compound (10a) (7-phenyl-3,4-dihydro-[1,2,4]triazolo[4,3-e][1–5] tetrazaphosphinine:
Yield 58% crystal from THF; mp 267–269°C. Analysis calculated for C_{20}H_{21}N_{11}P (218): C, 44.04; H, 3.23; N, 38.52; P 14.20. The analyses found for the compound are: C, 44.0; H, 3.3; N, 38.50; P 14.2. IR characteristic peaks appear at (ν cm⁻¹): 3120–2962 (broad, CH=N-H), 1630 (3NH), 1525 (C=N). ¹H NMR characteristic peaks appear at δ ppm: 4.25 (s, 1H, NH-N), 6.32 (s, 1H, NH-P), 7.41–8.28 (m, 5H, CH-benzene). MS (m/z, %): 218 (M⁺, 4.34%), 194 (M⁺ - NH-NH-P, 100%), 163 (M⁺ - NH-NH-P-P, 44%), 148 (M⁺ - NH-NH-P-P, 2.21%).

For compound (10b) (7-phenyl-4-yl)-3,4-dihydro-[1,2,4]triazolo[4,3-e][1–5] tetrazaphosphinine:
Yield 44% crystal from THF; mp 226–228°C. Analysis calculated for C_{20}H_{21}N_{11}P (219): C, 38.37; H, 2.76; N, 44.74; P 14.13. The analyses found for the compound are: C, 38.4; H, 2.8; N, 44.8; P 14.1. IR characteristic peaks appear at (ν cm⁻¹): 3120–2965 (broad, C=H-NH), 1634 (P-NH), 1522 (C=N). ¹H NMR characteristic peaks appear at δ ppm: 4.31 (s, 1H, NH-NH), 6.52 (s, 1H, NH-P), 7.94 (d, (2H), (CH)-pyridine), 8.79 (d, (2H), (CH)-pyridine). MS (m/z, %): 219 (M⁺, 6.22%), 195 (M⁺ - NH - NH-P, 100%), 164 (M⁺ - NH-NH-P, 37%), 149 (M⁺ - NH-NH-P-P, 5.11%).

For compound (11a) (3-phenyl-1,7-dihydro-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazine):
Yield 37% crystallized from dioxane; mp 252–254°C. Analysis calculated for C_{19}H_{20}N_{11} (200): C, 53.99; H, 4.03; N, 41.98. The analyses found for the compound are: C, 54.0; H, 4.0; N, 42.0. IR characteristic peaks appear at (ν cm⁻¹): 3184,3132 (NH, NH of tetrazine and triazole), 1521 (C=N). ¹H NMR characteristic peaks appear at δ ppm: 3.41 (s, 1H, NH-tetrazine), 5.82 (s, br., 1H, NH-triazole), 7.02 (s, 1H, CH-tetrazine), 7.51–7.82 (m, 5H, CH-benzene).

For compound (11b) (3-phenyl-4-yl)-1,7-dihydro-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazine:
Yield 41% crystallized from dioxan; mp 271–273°C. Analysis calculated for C_{19}H_{20}N_{11} (201): C, 47.76; H, 3.51; N, 48.73. The analyses found for the compound are: C, 47.7; H, 3.5; N, 48.7. IR characteristic peaks appear at (ν cm⁻¹): 3188,3129 (NH, NH of tetrazine and triazole), 1519 (C=N). ¹H NMR characteristic peaks appear at δ ppm: 3.62 (s, br., 1H, NH-tetrazine), 6.22 (s, br., 1H, NH-triazole), 7.63 (s, 1H, CH-tetrazine), 7.90 (d, (2H), (CH)-pyridine), 8.63 (d, (2H), (CH)-pyridine).

For compound (12a) (6-(2-chloro-6-fluorophenyl)-3-phenyl-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazine):
Yield 53% crystal from dioxan; mp 234–236°C. Analysis calculated for C_{19}H_{19}ClF_{2}N_{11} (330): C, 54.47; H, 3.66; Cl, 10.72; F, 5.74; N, 25.41. The analyses found for the compound are: C, 54.5; H, 3.7; N, 25.4; F, 5.7; Cl, 10.7. UV (DMF, λ_{max} nm (Log ε)): 265 (1.13) and 263 (1.03) nm. IR characteristic peaks appear at (ν cm⁻¹): 3354,3169 and 3132 cm⁻¹ (3NH), 3078 (aromatic (CH)), 1522 cm⁻¹ (C=N). ¹H NMR characteristic peaks appear at δ ppm: 2.31 (s, 1H, NH-tetrazine at 2-position), 4.32 (s, 1H, NH-tetrazine at 4-position), 5.04 (s, 1H, CH-tetrazine), 6.32 (s, 1H, NH-tetrazine at 5-position), 7.07–8.28 (m, 8H, CH-aromatic). ¹³C NMR characteristic peaks appear at δ ppm: 157.2 (C₁ of triazole), 151.1 (C₁ of triazole), 130.6 (C₁ of non-substituted benzene), 127.5 (C₂ of non-substituted benzene), 129.2 (C₃ of benzene), 131.1 (C₄ of non-substituted benzene), 137.3 (C₅ of tetrazine), 133.8 (C₁Cl), 160.8 (C₁F), 129.8 (C₂ of substituted benzene), 124.2, 129.7, 113.4 (3C of substituted benzene).

For compound (12b) (6-(2-chloro-6-fluorophenyl)-3-(pyridin-4-yl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazine):
Yield 45% crystal from THF; mp 276–278°C. Analysis calculated for C_{19}H_{19}ClF_{2}N_{11} (232): C, 46.54; H, 3.47; N, 36.18; S, 13.81. The analyses found for the compound are: C, 46.5; H, 3.5; N, 36.2; S, 13.8. UV (DMF, λ_{max} nm (Log ε)): 314 (2.1) nm. IR characteristic peaks appear at (ν cm⁻¹): 3332, 3178 (NH-NH), 1528 (C=S). ¹H NMR characteristic peaks appear at δ ppm: 3.8 (s, 2H, -NH-tetrazine at position 2), 5.3 (s, 2H, 1H, NH-tetrazine at position 4), 6.1 (s, br., 1H, NH-tetrazine at position 5), 7.52–8.07 (m, 5H, CH-benzene). ¹³C NMR
characteristic peaks appear at δ ppm: 157.2 (C₃ of triazole), 151.1 (C₅ of triazole), 182.1 (C₃ of tetrazine), 130.6 (C₁ of benzene), 127.5 (C₂, 6 benzene), 129 (C₃, 5 of benzene), 131.1 (C₄ of benzene).

• For compound (13b) (3-(pyridin-4-yl)-7,8-dihydro-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazine-6(5H)-thione):
  Yield 51% crystal from THF; mp 255–258 °C. Analysis calculated for C₈H₇N₇S (233): C, 41.19; H, 3.02; N, 42.03; S, 13.75. The analyses found for the compound are: C, 41.2; H, 3.0; N, 42.0; S, 13.8. UV [λ max (Log ε)]: 312 (1.8) nm. IR characteristic peaks appear at (ν cm⁻¹): 3325, 3182 (NH-NH), 1522 (C=S). 1H NMR characteristic peaks appear at δ ppm: 3.2 (s, br., 1H, -NH- tetrazine at position 2), 5.1 (s, br., 1H, NH-tetrazine at position 4), 5.9 (s, br., 1H, NH-tetrazine at position 5), 7.99 (d, 2H of pridine at C₃, C₅), 8.77 (d, 2H of pyridine at C₂, C₆). 13C NMR characteristic peaks appear at δ ppm: 157.2 (C₃ of triazole), 151.1 (C₅ of triazole), 182.1 (C₃ of tetrazine), 134.0 (C₄ of pyridine), 149.8 (C₂₆ of pyridine), 121.3 (C₂₂ of pyridine).

• For compound (14a) (3-phenyl-7,8-dihydro-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6(5H)-one):
  Yield 43% crystal from ethanol; mp 191–193 °C. Analysis calculated for C₉H₈N₆O (216): C, 50.00; H, 3.73; N, 38.87. The analyses found for the compound are: C, 50.1; H, 3.7; N, 38; 9. UV [λ max (Log ε)]: 324 (1.51) nm. IR characteristic peaks appear at (ν cm⁻¹): 3167 (NH-NH), 1643 (amidic CO), 1563 (C=N); 1H NMR characteristic peaks appear at δ ppm: 2.9 (s, br., 1H, NH-tetrazine at position 2), 4.8 (s, br., 1H, NH-tetrazine at position 4), 5.6 (s, br., 1H, NH-tetrazine at position 5), 7.87–8.08 (m, 5H of benzene). 13C NMR characteristic peaks appear at δ ppm: 157.2 (C₃ of triazole), 151.1 (C₅ of triazole), 152.4 (C₃ of tetrazine), 130.6 (C₁ of benzene), 127.5 (C₂, 6 benzene), 129.2 (C₃, 5 benzene), 131.1 (C₄ benzene). MS (m/z, %): 216 (M⁺, 4.7%), 188 (M⁺ - CO, 100%), 173 (M⁺ - CO-NH, 4.3%).

• For compound (14b) (3-(pyridin-4-yl)-7,8-dihydro-[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6(5H)-one):

Figure 1. Synthesis of triazolotriazine derivatives 5–8.
Yield 38% crystal from ethanol; mp 170–173 °C. Analysis calculated for C₈H₇N₇O (217): C, 44.24; H, 3.25; N, 45.14. The analyses found for the compound are: C, 44.2; H, 3.3; N, 45.1. UV [λ max (Log ε)]: 320 (1.21) nm. IR characteristic peaks appear at (ν cm⁻¹): 3172 (NH-NH), 1651 (amidic CO), 1534 (C=N). ¹H NMR characteristic peaks appear at δ ppm: 3.1 (s, br., 1H, -NH- tetrazine at position 2), 5.1 (s, br., 1H, NH-tetrazine at position 4), 5.9 (s, br., 1H, NH-tetrazine at position 5), 7.98 (d, 2H of pyridine at C₂,6), 8.67 (d, 2H, of pyridine at C₃,5). ¹³C NMR characteristic peaks appear at δ ppm: 157.2 (C₃ of triazole), 151.1 (C₅ of triazole), 152.4 (C₃ of tetrazine), 134.0 (C₄ of pyridine), 149.8 (Cₓₓ of pyridine), 121.3 (Cₓₓ of pyridine). MS (m/z, %): 217 (M⁺, 6.2%), 189 (M⁺ - CO, 100%), 174 (M⁺ - CO-NH, 3.7%).

Samples characterization. From the above mentioned spectral data and physical constants of the newly synthesized compounds, we concluded that: the mercapto group in the 4-amino-5-phenyl-4H-1,2,4-triazole-3-thiol (1a) is simply displaced by the amino group (nucleophilic group) in heterocyclic primary amines such as 5-chloropyrimidin-2-amine (2a) in Ethanol-DMF mixture (1:1) under reflux to yield 4-amino-5-substituted amino-1,2,4-triazole derivative (3a). Structure of 3a was elucidated mainly from the disappearance of the mercapto group peak at 2600–2500 cm⁻¹ in the IR spectrum, the new NH proton with NH₂ proton of aminotriazole of compound 3a showed through the ¹H NMR spectrum at δ 14.22 ppm 5.87 ppm respectively. Also, the UV absorption of 3a shows λ max at 372 nm while the λ max of 1a appears at 317 nm, which proves the structure of compound 3a (Fig. 1). Ring closure reactions⁹ of compound 3a with diphenyl(chloromethyl)-phosphanoxide (4) under reflux in tetrahydrofuran (THF) produces 3,8-diaryl-4,5,6,7-tetrahydro-6-hydroxy-1,2,4-triazole[4,3-b] [1–3,5]-phosphotriazine (5a) (Fig. 1). The presence of C-H, P-OH, P-N and NH, P-N, P-OH, and C-H functional groups peaks at 1522, 1650, 2925 and 3080 cm⁻¹ in its IR spectrum were used to deduce the structure of 5a. This structure was confirmed also by ¹H NMR spectrum which showed resonated signals at δ 2.8 (s, 1H, OH), 3.1 (s, (2H), CH₂), 2.0 (s, 1H, N-H), 7.41–8.05 (m, 15H, CH-benzene) and 8.08 (s, 2H, (CH)-pyrimidine).

Derivatives of 1,2,4-Triazolo[4,3-b]-1,2,4-triazine compounds (6a–8a) have been obtained from the hetero-cyclization of compound 3a with phenacyl bromide dissolved in ethanolic potassium hydroxide, pyruvic acid dissolved sodium hydroxide and diethyloxalate dissolved in DMF/THF under reflux, respectively (Fig. 1). The chemical structures of compounds 6a–8a were elucidated from both the spectral measurements and the elemental analyses. Infrared spectrum analysis of compound 6a showed representative bands at ν 3079 (cm⁻¹) and 2966 (cm⁻¹) for aromatic & aliphatic (CH) and band at 1522 for (C=N). While, representing bands of compound 7a were recorded at ν = 2978 (cm⁻¹), 1480 (cm⁻¹) for (stretching and bending vibration
### Table 1. The antibacterial activity for some of the newly prepared Compounds.

| S. aureus | Enterococcus | E. coli | Bacteria | Inhibitory Concentrations |
|----------|--------------|---------|----------|----------------------------|
| MIC 25   | 25 | 25 | 25 | 25 |
| 50 | — | — | — | — |
| 100 | — | — | — | — |
| Growth inhibition Zone (mm) | — | — | — | — |
| Compounds | — | — | — | — |
| 75.7 — 21 | 49.6 — 8 | 28 | 38.6 — 17 | 28 |
| 4.5 — 9 | 23 | 68 | — — 26 | 44.3 — 15 | 31 |
| 49.6 — 5 | 20 | 49.7 — 8 | 33 | 32 — 20 | 29 |
| 48.9 — 8 | 18 | 46.9 — 11 | 30 | 31.7 — 22 | 32 |
| 68.2 — 19 | 47.9 — 10 | 22 | 38.3 — 15 | 24 |
| 67.2 — 24 | 79.6 — 20 | 37.3 — 17 | 27 | 8b |
| 46.9 — 10 | 22 | 43.3 — 11 | 19 | 26.6 — 19 | 24 | 8c |
| 48.4 — 9 | 24 | 79.5 — 17 | 44.6 — 14 | 29 | 8d |
| 68.7 — 21 | 49 — 6 | 25 | 42.6 — 15 | 28 | 9a |
| 46.6 — 11 | 25 | 61.3 — 22 | 44.5 — 13 | 26 | 9b |
| 75.1 — 23 | 75.4 — 21 | 45.5 — 13 | 28 | 10a |
| 49.5 — 7 | 22 | 45 — 10 | 20 | 45.7 — 12 | 25 | 10b |
| 66.5 — 22 | 43.8 — 9 | 14 | 25.7 — 14 | 29 | 11a |
| 48.2 — 8 | 19 | 40.4 — 11 | 17 | 43.8 — 16 | 32 | 11b |
| 75.3 — 14 | 44.4 — 11 | 20 | 44.6 — 14 | 29 | 12a |
| 39.5 — 10 | 15 | 53.5 — 25 | 38.7 — 16 | 26 | 12b |
| 61.7 — 19 | 49.3 — 8 | 23 | 30.3 — 16 | 22 | 13a |
| 37.2 — 11 | 16 | 49 — 9 | 26 | 45.2 — 13 | 27 | 13b |
| 73.6 — 13 | 47.7 — 10 | 25 | 44.5 — 12 | 24 | 14a |
| 31.6 — 10 | 13 | 49.6 — 8 | 28 | 43.7 — 14 | 27 | 14b |
| 23.3 6 | 26 | 31 | 45.3 — 10 | 20 | — — — Nalidixic acid |
| 14.2 17 | 20 | 26 | 15.9 14 | 19 | 23 | 13.9 18 | 22 | 28 | Imipenem |

### Table 2. The antifungal activity for some of the newly prepared Compounds.

| C. albicans | Fungi |
|-------------|-------|
| 25 | 50 | 100 | Inhibitory Concentrations |
| Growth inhibition Zone (mm) | — | — | — | — |
| Compounds | — | — | — | — |
| — | — | 14 | 5a |
| — | — | 17 | 5b |
| — | — | 16 | 5c |
| — | — | 14 | 5d |
| — | — | 13 | 8a |
| — | — | 14 | 8b |
| — | — | 16 | 8c |
| — | — | 14 | 8d |
| — | — | 12 | 9a |
| — | — | 11 | 9b |
| — | — | 13 | 10a |
| — | — | 12 | 10b |
| — | — | 12 | 11a |
| — | — | 14 | 11b |
| — | — | 12 | 12a |
| — | — | 10 | 12b |
| — | — | 11 | 13a |
| — | — | 13 | 13b |
| — | — | 12 | 14a |
| — | — | 11 | 14b |
| — | — | 40 | Nystatin |

• Synthesized compounds (Inhibitor) Concentrations: 100, 50 and 25 μg/mL. Highly active: DIZ ≥ 12. Moderately active: DIZ = 9–12. Slightly active: DIZ = 6–9; Not sensitive: DIZ < 6 mm.
of CH₃) and a band at 1725 (cm⁻¹) for (C=O). In addition to that compound 8a bands were recorded at ν 3233 (cm⁻¹) for NH, 1734 (cm⁻¹) and 1658 (cm⁻¹) for (2C=O), C=N and substituted pyrimidine and aromatic rings bands appear at 1522 (cm⁻¹) and 850–730 (cm⁻¹). Also, 4 amino-5-(4 pyridyl)-4H-1,2,4-triazole-3-thiol compound (1b) reacted with 5-chloro-pyrimidine-2-amine (2b) in Ethanol/DMF mixture (1:1) under reflux to give 4-amino-5-substituted amino-1,2,4-triazole derivative 3b. Compound 3b in turn, undergo ring closure with each of diphenyl (chloromethyl) - phosphanoxide (4), phenacyl bromide in ethanolic KOH, pyruvic acid in NaOH, and/or diethyloxalate in THF/DMF to provide the compounds 5b to 8b, respectively. The chemical structures of compounds 5b-8b were confirmed by considering both spectral measurements and elemental analyses. In a similar way, under the same above-mentioned experimental conditions, the reaction of compound 1a,b with 5-bromopyrimidin-2-amine (2c) takes place to give 3c,d compounds respectively. The triazole-3,4-diamines 3c,d were cyclized with each of diphenyl (chloro-methyl) phosphanoxide (4), phenacyl bromide, pyruvic acid, and diethyloxalate to give the corresponding compounds 5c,d–8c,d, respectively. The chemical structures of compounds 5c,d–8c,d were elucidated by their spectral measurements and elemental analyses data.

The reported results of the high potency of 1,2,4-triazolo[1,2,4,5]tetrazines system as antimicrobial and anti-inflammatory agents were our motivation to synthesize several derivatives of these rings. Thus, it was found that refluxing compound 1a,b with hydrazine hydrate dissolved in ethanol gives the corresponding 5 hydrazine-4-amino-1,2,4-triazoles (9a,b) which was used as new synthons for the present study aiming to build new 1,2,4-triazolotetrazines. A hydrazino group is a stronger nucleophilic group and more basic if it is.
compared to the amino group in both compounds 9a,b, which enhances the cyclization firstly followed by the amino center (Fig. 2). The triazolo[4,3-e][1–5]tetrazaphosphinines (10a,b) were obtained from refluxing 9a,b with triethylphosphite in tetrahydrofuran (THF), respectively. Treating compound 9a,b with triethylorthoformate under the previously mentioned conditions, produced the corresponding 3-(pyridin-4-yl)- and/or 3-phenyl-1,7-dihydro-[1,2,4,5]-tetrazine 11a and/or 11b, respectively. Cycloaddition reaction, in boiling ethanol with a few drops of piperidine, of compounds 9a,b with 2-chloro-6-fluorobenzaldehyde furnished the corresponding triazolotetrazine derivatives 12a,b, respectively (Fig. 2).

Structures of compounds 10a,b, 11a,b and 12a,b were elucidated by examining the data of IR spectra. The IR absorption bands at $\nu$ (cm$^{-1}$): 3123–2962, 3120–2965 of (broad NH-NH), 1650, 1643 of (P-NH), 1528, 1522 of (C=N) for 10a, b, respectively. The IR absorption bands at $\nu$ (cm$^{-1}$): 3184–3132, 3188–3129 of (NH, NH of tetrazine and triazole), 1521, 1519 (C=N) for 11a,b, respectively. The IR absorption bands at $\nu$ (cm$^{-1}$): 3354–3132, 3343–3129 (3NH), 3078, 3072 (aromatic (CH)), 1522, 1518 (C=N) for 12a,b, respectively. The mass spectra of each of 10a and 10b gave molecular ion peaks at 218 and 219 which corresponded to the molecular weights of C$_8$H$_7$N$_6$P and C$_7$H$_6$N$_7$P of the assigned structures 10a,b, respectively.

UV spectral data of both compounds 3a,b and 12a,b explains that the hetero-cyclization would inhibit the electronic transition and this caused the hypochromic effect "shift to the shorter wavelength". Thus, $\lambda_{max}$ of 3a,b was 372 and 374 nm while that of 12a,b was 265 and 263 nm, respectively. $^{13}$C NMR (DMSO) of compound 12a showed the presence of thirteen different signals for thirteen different carbon atoms, which, for
NH-tetrazine at position 4), 6.1 (s, 1H, NH-tetrazine at position 5) and 7.52–8.07 (m, 5H of benzene). 13C NMR followed by elimination of one molecule of ethanol in each case52. The chemical carried via esterification of 9a,b structures of 13a,b and 14a,b ν 3167, 3172 and 1643, 1651 cm−1 gives us good evidence for the structure of 13a with ethyl chloroformate in THF/TEA, respectively (Fig. 2). Formation of 12b occurred in two steps, the first, by addition to S=C=S and the second step is the elimination of H2S, while the formation of compounds 14a,b was carried via esterification of 9a,b followed by elimination of one molecule of ethanol in each case52. The chemical structures of 13a,b, and 14a,b were characterized by their elemental and spectral analyses. Thus, the infrared spectra of 13a,b showed absorption bands at 3332, 3325 cm−1 of (C=O) while that of 14a,b showed the absorption bands at ν 3332, 3325 cm−1 of NH and 1528, 1522 cm−1 of (C=N). The 1H NMR spectra of 14a,b showed resonant signals at 2.86–3.06 (m, 3H of THF), 3.85–4.06 (m, 3H of THF), 6.11–6.15 (m, 2H), 6.75–7.07 (m, 4H), 7.18–7.37 (m, 4H), 8.05–8.28 (m, 1H), respectively, while that of 13a,b showed the absorption bands at ν 3332, 3325 cm−1 of NH and 1528, 1522 cm−1 of (C=S). The 1H NMR spectra of 13a,b recorded signals of 3.8 (s, 1H, -NH- tetrazine at position 2), 3.9 (s, 1H, NH-tetrazine at position 4), 6.1 (s, 1H, NH-tetrazine at position 5) and 7.52–8.07 (m, 5H of benzene). 13C NMR gives us good evidence for the structure of 13a where it showed resonated signals at 157 (C3 -triazole), 151 (C5 -triazole), 182 (C7 -tetrazine) and 130, 131,129, 127 ppm of benzene carbons. Mass spectra of both compounds 14a,14b showed molecular ion peaks at 216 (4.7%) for 14a and at 217 (6.2%) for 14b, which confirmed their structures. In addition, 13C NMR data of 14a,14b showed six different carbon signals, which were in good agreement with the proposed structures (Fig. 2).

12b, showed twelve signals for twelve different carbons. Finally, the compounds 1,2,4-trizolo[4,3-b][1,2,4,5] tetrazine-5(4H)-thiones/ones (13a,b and 14a,b) were synthesized from refluxing of 9a,b with CS2 in DMF and/or with ethyl chloroformate in THF/TEA, respectively (Fig. 2). Formation of 13a,b occurs in two steps, the first, by addition to S=C=S and the second step is the elimination of H2S, while the formation of compounds 14a,b was carried via esterification of 9a,b followed by elimination of one molecule of ethanol in each case52. The chemical structures of 13a,b, and 14a,b were characterized by their elemental and spectral analyses. Thus, the infrared spectra of 13a,b showed absorption bands at 3332, 3325 cm−1 of (C=O) while that of 14a,b showed the absorption bands at ν 3332, 3325 cm−1 of NH and 1528, 1522 cm−1 of (C=N). The 1H NMR spectra of 14a,b showed resonant signals at 2.86–3.06 (m, 3H of THF), 3.85–4.06 (m, 3H of THF), 6.11–6.15 (m, 2H), 6.75–7.07 (m, 4H), 7.18–7.37 (m, 4H), 8.05–8.28 (m, 1H), respectively, while that of 13a,b showed the absorption bands at ν 3332, 3325 cm−1 of NH and 1528, 1522 cm−1 of (C=S). The 1H NMR spectra of 13a,b recorded signals of 3.8 (s, 1H, -NH- tetrazine at position 2), 3.9 (s, 1H, NH-tetrazine at position 4), 6.1 (s, 1H, NH-tetrazine at position 5) and 7.52–8.07 (m, 5H of benzene). 13C NMR gives us good evidence for the structure of 13a where it showed resonated signals at 157 (C3 -triazole), 151 (C5 -triazole), 182 (C7 -tetrazine) and 130, 131,129, 127 ppm of benzene carbons. Mass spectra of both compounds 14a,14b showed molecular ion peaks at 216 (4.7%) for 14a and at 217 (6.2%) for 14b, which confirmed their structures. In addition, 13C NMR data of 14a,14b showed six different carbon signals, which were in good agreement with the proposed structures (Fig. 2).

Biological activity. The different biological activities of the synthesized compounds have been evaluated by studying their antibacterial activity against Pseudomonas aeruginosa and Escherichia coli as examples for Gram-negative bacteria and Staphylococcus aureus as an example for Gram-positive bacteria, in addition, the antifungal activity against Candida albicans using the technique reported by Barry et al53,54. Dimethylformamide was used as a solvent. Nystatin was used as a reference drug for fungi while Nalidixic acid and Imipenem were used as reference drugs for bacteria. The diameter of the growth inhibition zone (DIZ) was presented in Tables 1, 2 and Figs. 3–6. The Minimum Inhibitory Concentrations (MIC) in antibacterial activity were presented in Table 1. The results in Table 1 show that the antibacterial activity of the tested compounds could be classified to higher to moderate activity against the used bacteria E.coli, S. aureus and P. aeruginosa in comparison with Nalidixic acid at concentrations 100, 50 and 25µg/mL. All the tested Compounds showed higher activity against E. coli bacteria at 100 µg/mL concentrations and higher to moderate activity at 50 µg/mL concentrations. For S. aureus bacteria,
all the tested Compounds showed higher activity at 100 µg/mL concentrations. Compounds 5a, 5c, 8a, 8b, 9a, 10a, 11a, 12a, 13a and 14a were not sensitive toward S. aureus while compounds 5d, 10b and 11b were slightly active at 50 µg/mL concentrations. On the other hand, the remaining compounds showed moderate activity at 50 µg/mL concentrations. For P. aeruginosa bacteria, all the tested compounds showed higher activity at 100 µg/mL concentrations. Compounds 5b, 8b, 8d, 9b, 10a and 12b were not sensitive toward P. aeruginosa bacteria.
while compounds 5a, 5c, 9a, 13a and 14b were slightly active at 50 µg/mL concentrations. On the other hand, the remaining compounds showed moderate activity at 50 µg/mL concentrations. The MICs for all the tested compounds against *E. coli, S. aureus, and P. aeruginosa* were presented in Table 1. The data in Table 2 showed moderate to high antifungal activities of the tested compounds against *C. albicans* in comparison with Nystatin.

**Figure 8.** Molecular docked model of compounds 10a,b, 12a,b and Indomethacin with COX–II enzyme (the target is presented as thin sticks; the ligands are drawn as ball–and–stick). Images (a,c,e,g,i) represent the 2D docking styles for COX–II enzyme with compounds 10a,b, 12a,b and Indomethacin, respectively. Images (b,d,f,h,j) represent the 3D docking styles for COX–II enzyme with compounds 10a,b, 12a,b and Indomethacin, respectively.
at 100 µg/mL concentration. The MIC for all the tested compounds against *C. albicans* fungi was 100 µg/mL. The higher activity of some of the tested compounds was mainly due to containing chlorine, fluorine, bromine and phosphorus elements within the chemical structure of 1,2,4-triazine and 1,2,4,5-tetrazine. On the other hand, the obtained results in Table 3 indicated that: Compounds 10a,b, 12a,b and 13b carrying phenyl and pyridine groups having both the chlorine and fluorine elements had good anti-inflammatory activity, in comparison with the standard anti-inflammatory drug used (Indomethacin). Compounds 10a,b, and 12a,b contained mainly triazole and tetrazine rings with the presence of both fluorine and phosphorus elements, incorporated with pyridil moiety. The activity of the new materials depends on the existence of those new moieties which have a high biological activity. The higher biological activity of the synthesized compounds was in a good agreement with the previously stated results in the field of fluorine and phosphorus-bearing nitrogen heterocyclic systems. Molecular modeling studies (Structure-based drug design) Table 4 and Fig. 7 illustrate the results of the bonding interactions for the docking of compound 5d and *Nalidixic acid* with amino acids of DNA Gyrase enzyme (PDB ID: 2XCT) active site. From these results, we found that the active compound 5d showed extra binding modes to DA13 and Arg 458 in addition to interaction with the essential binding sites Mn metal and DG5.

Table 5 and Fig. 8 illustrate the results and the bonding interactions of the docked compounds and Indomethacin, respectively, with active sites of COX-2 (PDB ID: 1CX2).

From these results, it appears that, generally, the tested compounds and Indomethacin showed a comparable binding pattern. Compound 10a showed well interaction with Arg513 residue. Compound 10b showed well interaction with Ser353 and Tyr355 which is going alongside with the screening results. Also, compound 12a showed three binding interactions to Leu352, His90 and Val523 amino acid. Finally, compound 12b binds to Ala527 and Tyr355 residues.

**Conclusions**

Some novel heterocyclic compounds 5a,b–14a,b containing fluorine, chlorine, bromine and phosphorus elements were synthesized utilizing 3-substituted-4-amino-5-substituted amino-1,2,4-triazoles 3a–d and 3-substituted-4-amino-5-hydrazino-1,2,4-triazole 9a,b compounds as building units in the synthesizing process. These newly prepared compounds were fully characterized through the spectral and elemental analyses which were completely fit with the assigned structures. A number of the synthesized compounds were screened against gram-positive bacteria, gram-negative bacteria, and fungi, such as *Streptococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. Most of the newly prepared compounds showed a high antibacterial, antifungal, and anti-inflammatory in comparing with the standard commercial antibiotics Imipenem and Nalidixic acid, Nystatin and Indomethacin, respectively. Compounds 10a,b, and 12a,b containing both chlorine and fluorine elements showed high inflammation activity.

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**Author contributions**

Ahmed A.M. El-Reedy wrote the introduction and experimental parts and do the experimental work and also shared in the interpretation of data. N.K. Soliman prepared figures, wrote the results and discussion part and also shared in the interpretation of data.
Competing interests
The authors declare no competing interests.

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