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Synthesis and Exploring Novel Annulated 1,3-diphenylpyrazole Derivatives as Antimicrobial and Anticancer Agents

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Abstract:

A novel series of annulated diphenylpyrazoles were designed and synthesized through various strategies. The bifunctional enaminonitrile 1 used as the key intermediate to prepare the pyrrolopyrazole 2, pyrazolopyridine 3, pyrazolothiazine 8, and pyrazolopyrimidine 9 derivatives through its cyclocondensation with ethyl chloroacetate, diethyl malonate, and carbon disulfide under different conditions. The novel valuable derivatives 2 and 5 are subjected to amylolysis, while compound 9 is used to synthesize tricyclic compounds. The reaction of 1 with sodium azide afforded the tetrazole derivative 13. The behavior of 13 was studied with carbon disulfide, benzaldehyde and phenacyl bromide. The condensation behavior of compound 1 with furfural, cyclohexanone, chloroacetyl chloride, benzyl chloride, benzoyl chloride, ammonium hydroxide/H_2O_2, sulfuric acid (50%), sodium hydroxide (50%), triethyl orthoformate, and ammonium thiocyanate have also been taken into consideration. These entire novel scaffolds have been proofed using Elemental analysis, spectral data including IR, ^{1}H-NMR in addition to ^{13}C-NMR, and mass spectra. These new scaffolds were screened for *in vitro* antimicrobial and cytotoxic activities. Most analogs revealed excellent to good results. Finally, TEM investigations were screened and revealed that pyrazolopyrimidinethione derivative **9** showed completely lysed.

***Keywords*:** Antimicrobial, antitumor, pyrazole, pyrazolopyridine, pyrazolopyridinone, pyrazolopyrimidine,TEM.
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1. Introduction:

Research in the heterocyclic chemistry area is of great interest and various studies have determined their effect on living organisms (drugs or medicine). During the last decades, there was significant attention on five-membered heterocyclic compounds that comprise nitrogen and oxygen atoms due to their unique properties and various applications, especially pyrazole which considered the building block of heterocyclic compounds. Pyrazole and its analogs have gained pharmacological properties such as antioxidant, [1] antiviral, [2] antibacterial, [3, 4] antimicrobial, [5] anticancer, [6] analgesic, [7] anti-inflammatory, [8] insecticidal, [9] anthelmintic, [10] antimitotic, [11] herbicidal [12] and antagonist activities [13].

Several synthetic pyrazole compounds are essential as medicinal products and dyes. Several pyrazole-based drugs such as antipyrine used as an analgesic and febrifugal; tartrazine widely used as yellow colorant for foods; phenyl-butazone as an anti-inflammatory drug for arthritis [14].

On the other hand, Bacteria and fungi are liable for several illnesses, most of which have public health concerns around the world.

From these findings and as continuation of our recent studies [15-18], the syntheses of a new series of compounds containing the pyrazole moiety conveying other heterocyclic are now reported. The various compounds prepared are outlined in Schemes 1-5.

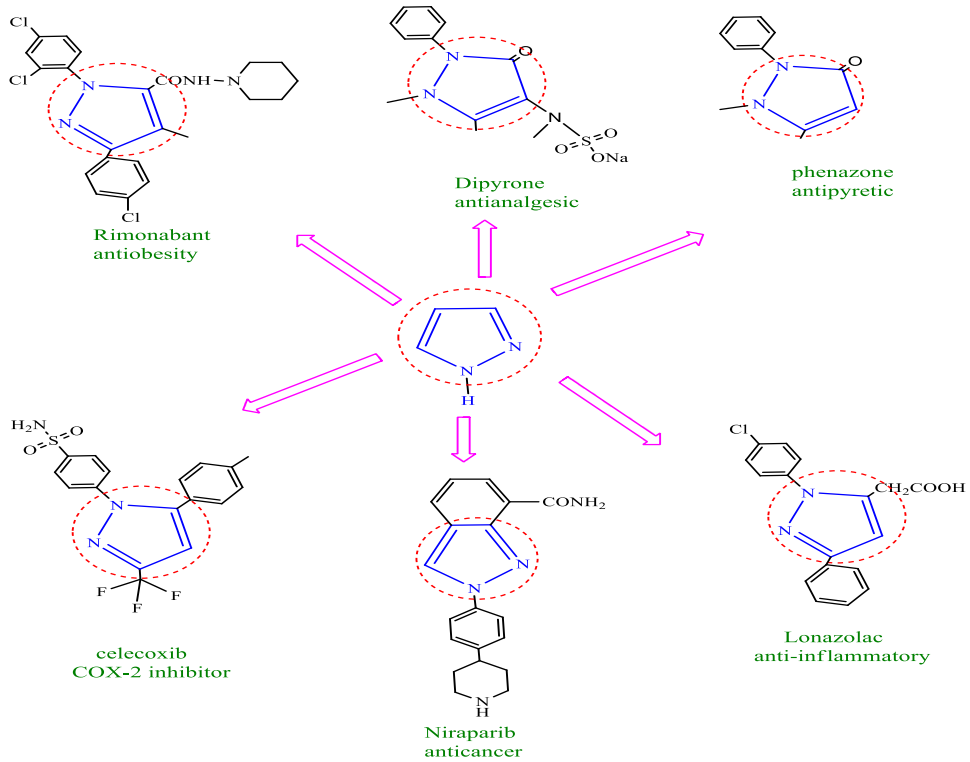


Fig. 1: The pyrazole moiety abidance in various medication

2. Material and methods

2.1.

2.2. Chemistry

All melting points were estimated on a digital Stuart "SM.P.3" instrument. Infrared spectra were recorded on "PerkinElmer 293 spectrophotometer" using KBr disks. ^{1}H-NMR and ^{13}C-NMR spectra were determined on Varian Mercury "300 MHz" spectrometer using "DMSO-d6" as solvent and TMS as an internal standard. Chemical shift are expressed as "δ_{ppm}" and coupling constants (J) in Hz. Mass spectra were recorded on a Shimadzu Gas chromatography "GC-2010" instrument mass spectrometer (70 eV) with electron ionization technique. Elemental analyses were performed on a PerkinElmer CHN-2400 analyzer and the microanalyses were within ±0.4% comparative to the theoretical values. Thin layer chromatograph (TLC) was carried out for the monitoring of the progress of all reactions and homogeneity of the synthesized compounds. TLC was performed using aluminum sheet silica gel F_{254} (Merck).The biological evaluation of the products at The Regional Center for Biotechnology and Mycology, Al-Azhar University, Nasr city, Cairo, Egypt. Merck chemicals and solvents were used in all the reactions.

2.2.1. Formation of ethyl 4-amino-1,3-diphenyl-1,6-dihydropyrrolo[2,3-c]pyrazole-5-carboxylate (2)

To a solution of pyrazole derivative **1** (2.6 g, 0.01 mol) in DMF (20 mL), ethyl chloroacetate (1.2 mL, 0.01 mol) was added. The mixture was refluxed for 8h. After cooling, the mixture was poured onto water (100 mL), the solid precipitated was filtered off, washed with water, dried and recrystallized from absolute ethanol to give compound (**2**) as brown powder.

Yield (%) = 3.23 g (91%), m.p. 180-182 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3442, 3312 (NH_2), 3217 (NH), 1736 (C=O), 1643 (C=N); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 1.22 (t, *J*= 6.6 Hz, 3H, $\underline{CH_3}CH_2$), 4.21 (q, *J*= 6.8 Hz, 2H, $CH_3\underline{CH_2}$), 6.74-7.92 (m, 10H, Ar-H), 9.25 (s, 2H, NH_2, D_2O exchangeable), 10.19 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 15.9 (CH_3), 58.6 (OCH_2), 101.2, 111.7, 118.2, 125.4, 125.8, 127.7, 128.1, 128.7, 129.7, 135.4, 145.4, 146.2, 147.8, 155.9 (ester, C=O); MS *m/z (%): 346 (M+, 48.9)*; Anal. Calcd. for $C_{20}H_{18}N_4O_2$ (346): C, 69.35; H, 5.24; N, 16.17, Found: C, 69.01; H, 5.45; N, 16.32.

2.2.2. Formation of 4-amino-1,3-diphenyl-1,6-dihydropyrrolo[2,3-c]pyrazole-5-carbohydrazide (3)

A mixture of compound **2** (3.46 g, 0.01 mol), hydrazine hydrate (0.5 mL, 0.01 mol) in ethanol (50 mL) was refluxed for 6h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from benzene to afford compound (**3**) as off-white powder.

Yield (%) = 2.95 g (89%), m.p. 242-244 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3442, 3400, 3390, 3322 (NH_2), 3266, 3217 (NH), 1690 (C=O), 1631(C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 3.46 (s, 2H, CONH$\underline{NH_2}$, D_2O exchangeable), 4.50 (s, 2H, NH_2, D_2O exchangeable), 6.75-7.92 (m, 10H, Ar-H), 8.78 (s, 1H, CO$\underline{NH}NH_2$, D_2O exchangeable), 10.34 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 112.0, 118.7, 125.5, 125.7, 127.3, 127.9, 128.4, 128.7, 129.1, 129.4, 135.8, 136.4, 145.3, 146.2, 148.6, 159.2 (C=O); MS *m/z (%): 332 (M^+, 21.4)*; Anal. Calcd. for $C_{18}H_{16}N_6O$ (332): C, 65.05; H, 4.85; N, 25.29, Found: C, 65.24; H, 4.77; N, 25.13.

2.2.3. Formation of 4-amino-1,3-diphenyl-N-(p-tolyl)-1,6-dihydropyrrolo[2,3-c]pyrazole-5-carboxamide (4)

A mixture of compound **2** (3.46 g, 0.01 mol), p-toluidine (1.07 g, 0.01 mol) in ethanol (50 mL) was refluxed for 6h. After cooling, the obtained solid was filtered off, washed with ethanol, dried and recrystallized from petroleum ether (80-100^{O}C) to furnish compound (**4**) as black crystals.

Yield (%) = 3.58 g (88%), m.p. 170-172^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3438, 3381 (NH_2), 3264, 3242 (NH), 1666 (C=O), 1592 (C=N); ^{1}H-NMR (DMSO-d6): δ_{ppm}=2.33 (s, 3H, CH_3), 6.3 (S, 1H, CONH, D_2O exchangeable), 6.73-7.92 (m, 14H, Ar-H), 9.33 (s, 1H, NH, D_2O exchangeable), 10.87 (s, 2H, NH_2, D_2O exchangeable); MS *m/z (%): 407 (M^+, 34.0)*; Anal. Calcd. for $C_{25}H_{21}N_5O$ (407): C, 73.69; H, 5.19; N, 17.19, Found: C, 73.42; H, 5.30; N, 17.21.

2.2.4. Formation of ethyl 4-amino-6-oxo-1,3-diphenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylate (5)

To a solution of pyrazole derivative **1** (2.6 g, 0.01 mol) in sodium ethoxide freshly prepared (20 mL absolute ethanol+1 g of sodium metal), diethyl malonate (1.6 mL, 0.01 mol) was added. The reaction mixture was refluxed for 6h. The reddish crystals formed during reflux was filtered, washed with ethanol, dried and recrystallized from absolute ethanol to produce compound (**5**).

Yield (%) = 2.84 g (76%), m.p. 198-200^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3479, 3417 (NH_2), 3236 (NH), 1660, 1635 (C=O), 1600 (C=N); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 1.24 (t, *J*= 6.4 Hz, 3H, CH_3), 4.23 (q, *J*= 6.8 Hz, 2H, CH_2), 6.38 (s, 2H, NH_2, D_2O exchangeable), 6.71-7.92 (m, 10H, Ar-H), 10.68 (S, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 16.2 (CH_3), 57.4 (OCH_2), 100.8, 112.2, 118.6, 125.3, 125.6, 127.6, 128.3, 128.9, 129.4, 130.0, 135.4, 145.4, 146.2, 147.8, 158.6 (pyridine, C=O), 160.7 (hydrazide, C=O); MS *m/z (%): 374 (M^+, 41.4)*; Anal. Calcd. for $C_{21}H_{18}N_4O_3$ (374): C, 67.37; H, 4.85; N, 14.96, Found: C, 67.55; H, 4.71; N, 15.01.

2.2.5. Formation of 4-amino-6-oxo-1,3-diphenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carbohydrazide (6)

A mixture of pyridine derivative (**5**) (3.74 g, 0.01 mol), hydrazine hydrate (0.5 mL, 0.01 mol) in ethanol (50 mL) was refluxed for 6h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from ethanol to give compound (**6**) as yellow crystals.

Yield (%) = 3.24 g (90%), m.p. 166-168 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3443, 3400, 3390, 3342 (NH_2), 3245, 3224 (NH), 1635 (C=O), 1603(C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 3.48 (s, 2H, CONH<u>NH_2</u>, D_2O exchangeable), 4.62 (s, 2H, NH_2, D_2O exchangeable), 6.75-7.92 (m, 10H, Ar-H), 8.49 (s, 1H, CO<u>NH</u>NH_2, D_2O exchangeable), 10.34 (s, 1H, pyridine-H_7); ^{13}C NMR (DMSO-d6): δ_{ppm}= 101.3, 111.8, 118.9, 125.5, 127.8, 128.3, 128.6, 129.0, 135.5, 136.3, 145.4, 146.8, 148.2, 154.4 (C=O), 162.2 (hydrazide-CO); MS *m/z (%): 360 (M^+, 49.7)*; Anal. Calcd. for $C_{19}H_{16}N_6O_2$ (360): C, 63.33; H, 4.48; N, 23.32, Found: C, 63.01; H, 4.62; N, 23.24.

2.2.6. Formation of 4-amino-6-oxo-1,3-diphenyl-N-(p-tolyl)-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxamide (7)

A mixture of pyridine derivative (**5**) (3.74 g, 0.01 mol), p-toluidine (1.07 g, 0.01 mol) in ethanol (50 mL) was refluxed for 6h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from butanol to afford compound (**7**) as brown crystals.

Yield (%) = 3.44 g (79%), m.p. 234-236 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3444, 3404, (NH_2), 3258, 3210 (NH), 1645 (C=O), 1603, 1591 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 2.32 (s, 3H, CH_3), 3.51 (s, 2H, NH_2, D_2O exchangeable), 6.75-7.92 (m, 14H, Ar-H), 8.60 (s, 1H, NH, D_2O exchangeable), 10.36 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 21.9 (CH_3), 106.3, 112.0, 118.7, 125.6, 127.8, 128.6, 128.7, 129.1, 135.8, 136.3, 145.3, 158.7 (pyridine-C_6), 162.3 (-CONH-); MS *m/z (%): 435 (M^+, 39.9)*; Anal. Calcd. for $C_{26}H_{21}N_5O_2$ (435): C, 71.71; H, 4.86; N, 16.08, Found: C, 71.59; H, 4.92; N, 15.97.

2.2.7. Formation of 4-imino-1,3-diphenyl-4,7-dihydropyrazolo[3,4-d][1,3]thiazine-6(1H)-thione (8)

A solution of pyrazole derivative **1** (2.6 g, 0.01 mol) in CS_2 (20 mL) was refluxed for 2h on water bath. After cooling, the mixture was poured onto water (100 mL), the obtained solid was filtered off, washed with water, dried and recrystallized from petroleum ether (80-100 ^{O}C) to furnish compound (**8**) as beige crystals.

Yield (%) = 2.99 g (89%), m.p. 258-260 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3223, 3211 (NH), 1630, 1592 (C=N), 1259 (C=S); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 6.77-8.05 (m, 10H, Ar-H), 9.98 (s, 1H, CH=NH), 10.32 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}=112.0, 118.8, 125.6, 127.8, 128.5, 128.6, 129.0, 129.1, 129.4, 135.5, 135.8, 145.3, 151.7 (C=NH), 182.3 (C=S); MS *m/z* (%): *336 (M^+, 27.9)*; Anal. Calcd. for $C_{17}H_{12}N_4S_2$ (336): C, 60.69; H, 3.60; N, 16.65; S, 19.06, Found: C, 60.77; H, 3.68; N, 16.42; S, 19.13.

2.2.8. Formation of 1,3-diphenyl-1,7-dihydro-4H-pyrazolo[3,4-d]pyrimidine-4,6(5H)-dithione (9)

To a solution of pyrazole derivative **1** (2.6 g, 0.01 mol) in pyridine (5 mL), CS_2 (5 mL) was added. The reaction mixture was refluxed for 8h. After cooling, the mixture was poured onto dil. HCl (100 mL), the separated solid was filtered off, washed with water, dried and recrystallized from absolute methanol to produce compound (**9**) as reddish-brown crystals.

Yield (%) = 2.76 g (82%), m.p. 120-122 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3300, 3280 (NH), 1592 (C=N), 1288, 1299 (C=S); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 6.72 (s, 1H, NH, D_2O exchangeable), 6.75-7.87 (m, 10H, Ar-H), 10.31 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 111.0, 133.5, 117.4, 124.7, 126.2, 128.3, 128.7, 129.1, 129.8, 130.2, 135.4, 136.2, 142.4, 176.2 (C=S), 182.3 (C=S); MS *m/z (%):336 (M^+, 16.5)*; Anal. Calcd. for $C_{17}H_{12}N_4S_2$ (336): C, 60.69; H, 3.60; N, 16.65; S, 19.06, Found: C, 60.70; H, 3.42; N, 16.77; S, 19.11.

2.2.9. General method for preparation of compounds (10-12)

A mixture of pyrimidine derivative (**9**) (3.36 g, 0.01 mol) in sodium ethoxide freshly prepared (20 mL absolute

ethanol+1 g of sodium metal), each of, 2-(ethoxymethylene)malononitrile (1.22 g, 0.01 mol), 2-benzylidenemalononitrile (1.54 g, 0.01 mol) and/or benzalacetophenone (2.08 g, 0.01 mol) was refluxed for 15h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from methanol.

2.2.9.1. Formation of 6-amino-8-ethoxy-1,3-diphenyl-4-thioxo-1,4-dihydro-6H-pyrazolo[3',4' :4,5]pyrimido[2,1-b][1,3]thiazine-7-carbonitrile (10)

Straw yellow powder, yield (%)= 3.62 g (79%), m.p. 222-224 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3330, 3310 (NH_2), 2257 (CN), 1597 (C=N), 1271 (C=S); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 1.03 (t, *J*= 6.6 Hz, 3H, $CH_2\underline{CH_3}$), 2.74 (s, 1H, CH), 3.77 (q, *J*= 6.8 Hz, 2H, $O\underline{CH_2}CH_3$), 6.76-7.88 (m, 10H, Ar-H), 9.55 (s, 2H, NH_2, D_2O exchangeable); ^{13}C-NMR (DMSO-d6): δ_{ppm}= 16.2 (CH_3), 59.7, 63.4 (CH_2), 82.5, 113.6, 114.3, 119.7, 120.2, 121.0, 125.3, 126.4, 127.0, 127.4, 128.1, 128.5, 129.3, 129.9, 135.5, 136.7, 143.9, 144.4, 145.0, 149.2, 183.6 (C=S); MS *m/z (%): 458 (M^+, 45.2)*; Anal. Calcd. for $C_{23}H_{18}N_6OS_2$ (458): C, 60.24; H, 3.96; N, 18.33; S, 13.98, Found: C, 60.39; H, 4.01; N, 18.17; S, 13.87.

2.2.9.2. Formation of 6-amino-1,3,8-triphenyl-4-thioxo-1,4-dihydro-8H-pyrazolo[3',4':4,5] pyrimido[2,1-b][1,3]thiazine-7-carbonitrile (11)

Brown powder, yield (%)=3.72 g (76%), m.p. 276-278 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3328, 3313(NH), 2195 (CN), 1596 (C=N), 1248 (C=S); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 5.09 (s, 1H, CH), 6.79-7.73 (m, 15H, Ar-H), 10.18 (s, 2H, NH_2, D_2O exchangeable); ^{13}C-NMR (DMSO-d6): δ_{ppm}= 31.6 (thiazine-C8), 64.3 (thiazine-C7), 112.4, 118.7 (CN), 120.4, 125.4, 126.2, 128.2, 128.7, 129.1, 129.7, 130.1, 133.3, 135.2, 136.8, 137.6, 139.2, 143.5, 150.6, 172.6 (C=S; MS *m/z (%): 490 (M^+,34.9)*; Anal. Calcd. for $C_{27}H_{18}N_6S_2$ (490): C, 66.10; H, 3.70; N, 17.13; S, 13.07, Found: C, 66.02; H, 3.82; N, 17.25; S, 12.91.

2.2.9.3. Formation of 1,3,6,8-tetraphenyl-8H-pyrazolo[3',4':4,5]pyrimido[2,1-b][1,3]thiazine-4(1H)-thione (12)

Black powder, yield (%)= 4.10 g (78%), m.p. 152-154 ^{O}C; IR (KBr) ν_{max}/cm^{-1}:1630, 1595 (C=N), 1579 (C=C), 1253 (C=S); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 5.04 (d, *J*= 5.1 Hz, =CH-$\underline{CH}$), 6.68 (d, *J*= 4.8 Hz, =$\underline{CH}$-CH), 6.80-7.89 (m, 20H, Ar-H); ^{13}C-NMR (DMSO-d6): δ_{ppm}= 34.1, 89.1, 111.2, 113.5, 114.2, 119.6, 120.4, 121.1, 125.2, 126.8, 127.1, 127.4, 128.1, 128.4, 129.3, 129.5, 133.5, 135.5, 136.7, 140.2, 143.5, 145.9, 147.3, 150.2, 176.3 (C=S); MS *m/z* (%): 526 (M^+,70.4); Anal. Calcd. for $C_{32}H_{22}N_4S_2$ (526): C, 72.98; H, 4.21; N, 10.64; S, 12.17, Found: C, 73.05; H, 4.14; N, 10.59; S, 12.22.

2.2.10. Formation of 1,3-diphenyl-4-(1H-tetrazol-5-yl)-1H-pyrazol-5-amine (13)

A mixture of pyrazole derivative **1** (2.6 g, 0.01 mol), sodium azide (0.78 mL, 0.01 mol) and ammonium chloride (0.64 g, 0.01 mol) in DMF (20 mL). The reaction mixture was refluxed for 12h. After cooling, the reaction mixture was poured onto water (100 mL), the separated solid was filtered off, washed with water, dried and recrystallized from ethanol to afford compound (**13**) as brown powder.

Yield (%) = 2.79 g (92%), m.p. 130-132 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3310, 3300 (NH_2), 3150 (NH), 1600, 1591 (C=N), 1564 (N=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 6.73-7.92 (m, 10H, Ar-H), 9.36 (s, 1H, NH, D_2O exchangeable), 9.69 (s, 2H, NH_2, D_2O exchangeable); MS *m/z (%): 303 (M^+, 4.3)*; Anal. Calcd. for $C_{16}H_{13}N_7$ (303): C, 63.36; H, 4.32; N, 32.32, Found: C, 63.49; H, 4.28; N, 32.23.

2.2.11. Formation of 7,9-diphenyl-6,7-dihydro-5H-pyrazolo[4,3-e]tetrazolo[1,5-c]pyrimidine-5-thione (14)

To a solution of compound **13** (3.03 g, 0.01 mol) in pyridine (5 mL), CS_2 (5 mL) was added. The reaction mixture was refluxed for 10h. After cooling, the reaction mixture was poured onto dil. HCl (200 mL, 10%), the separated solid was filtered off, washed with water, dried and recrystallized from methanol to furnish compound (**14**) as black powder.

Yield (%) = 3.04 g (88%), m.p. 184-186 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3200 (NH), 1600, 1591 (C=N), 1564 (N=N), 1288 (C=S); ^{1}H-NMR (DMSO-d6): δ_{ppm}= 6.74-8.01 (m, 10H, Ar-H), 10.36 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 112.0, 113.0, 118.7, 119.2, 125.6, 126.0, 127.6, 127.8, 128.1, 128.9, 129.1, 129.4, 135.8, 136.4, 145.2, 150.2 (C=N), 172.2 (C=S); MS *m/z (%): 345 (M^+, 21.3)*; Anal. Calcd. for $C_{17}H_{11}N_7S$ (345): C, 59.12; H, 3.21; N, 28.39; S, 9.28, Found: C, 58.99; H, 3.24; N, 28.52; S, 9.25.

2.2.12. Formation of 5,7,9-triphenyl-6,7-dihydro-5H-pyrazolo[4,3-e]tetrazolo[1,5-c]pyrimidine (15)

A mixture of compound **13** (3.03 g, 0.01 mol), benzaldehyde (1.06 mL, 0.01 mol) with few drops of HCl in methanol (25 mL) was refluxed for 16h. After cooling, the mixture was poured onto dil. Na_2CO_3 solution (100 mL, 25%), the separated solid was filtered off, washed with water, dried and recrystallized from methanol to give compound (**15**) green powder.

Yield (%) = 3.32 g (85%), m.p. 288-290 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3222 (NH), 3026 (CH-SP^3), 1611, 1598 (C=N), 1565 (N=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 5.45 (s, 1H, CH), 6.71–7.97 (m, 15 H, Ar-H), 10.62 (s, 1H, NH, D_2O exchangeable); MS *m/z (%): 391 (M^+, 7.4)*; Anal. Calcd. for $C_{23}H_{17}N_7$ (391): C, 70.57; H, 4.38; N, 25.05, Found: C, 70.48; H, 4.19; N, 25.33.

2.2.13. Formation of 6,8,10-triphenyl-5,8-dihydropyrazolo[4,3-f]tetrazolo[1,5-d][1,4]diazepine (16)

To a solution of compound **13** (3.03 g, 0.01 mol) in sodium ethoxide freshly prepared (20 mL absolute ethanol+1 g of sodium metal), phenacyl bromide (1.85 mL, 0.01 mol) was added. The reaction mixture was refluxed for 10h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from butanol to afford compound (**16**) as beige powder.

Yield (%) =3.47 g (86%), m.p. 166-168 ^{O}C; IR (KBr) ν_{max}/cm^{-1}:1600, 1559 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 4.46 (s, 2H, CH_2), 6.77-7.92 (m, 15H, Ar-H); ^{13}C NMR (DMSO-d6): δ_{ppm}= 58.6 (CH_2), 105.1, 119.7, 120.1, 121.6, 125.3, 126.7, 127.0, 127.9, 128.2, 129.8, 130.2, 135.5, 136.7, 142.7, 145.5, 150.2 (tetrazole, C=N), 151.9 (azepine, C=N); MS *m/z (%): 403 (M^+, 26.9)*; Anal. Calcd. for

$C_{24}H_{17}N_7$ (403): C, 71.45; H, 4.25; N, 24.30, Found: C, 71.62; H, 4.12; N, 24.26.

2.2.14. Formation of 5-((furan-2-ylmethylene)amino)-1,3-diphenyl-1H-pyrazole-4-carbonitrile (17)

A mixture of pyrazole derivative **1** (2.6 g, 0.01 mol), furfuraldehyde (0.96 mL, 0.01 mol) with few drops of glacial AcOH in ethanol (25 mL) was refluxed for 6h. After cooling, the reaction mixture was poured onto ice, the separated solid was filtered off, washed with water, dried and recrystallized from acetic acid to produce compound (**17**) as pale-yellow powder.

Yield (%) = 3.12 g (92%), m.p. 150-152 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 2221 (CN), 1591 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 6.71-8.22 (m, 14H, Ar-H); MS *m/z* (%): *338 (M^+, 34.0)*; Anal. Calcd. for $C_{21}H_{14}N_4O$ (338): C, 74.54; H, 4.17; N, 16.56, Found: C, 74.43; H, 4.22; N, 16.61.

2.2.15. Formation of 5-(3-chloro-2-(furan-2-yl)-4-oxoazetidin-1-yl)-1,3-diphenyl-1H-pyrazole-4-carbonitrile (18)

To a solution of compound (**17**) (3.38 g, 0.01 mol) in DMF (20 mL), chloroacetyl chloride (1.13 mL, 0.01 mol) was added. The reaction mixture was refluxed for 5h. After cooling, the mixture was poured onto ice, the separated solid was filtered off, washed with water, dried and recrystallized from methanol to give compound (**18**) as black crystal.

Yield (%) = 3.60 g (87%), m.p. 138-140 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 2200 (CN), 1682 (C=O), 1598 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 4.27 (d, 1H, *J*= 4.7 Hz, CH-furfural), 5.04 (d, 1H, *J*= 4.8Hz, CH-Cl), 6.71-8.17 (m, 13H, Ar-H); ^{13}C NMR (DMSO-d6): δ_{ppm}= 43.5 (azetidine-C_3), 43.8 (azetidine-C_2), 72.2, 111.9, 119.2, 123.7, 125.5, 127.0, 127.3, 128.6, 128.7, 129.0, 129.1, 129.5, 129.9, 130.1, 133.5, 134.7, 141.8, 153.4, 167.0 (C=O); MS *m/z (%): 414 (M^+, 18.0)*; Anal. Calcd. for $C_{23}H_{15}ClN_4O_2$ (414): C, 66.59; H, 3.64; Cl, 8.55; N, 13.51, Found: C, 66.62; H, 3.58; Cl, 8.48; N, 13.66.

2.2.16. Formation of 5-(cyclohexylideneamino)-1,3-diphenyl-1H-pyrazole-4-carbonitrile (19)

A mixture of pyrazole derivative **1** (2.6 g, 0.01 mol), cyclohexanone (0.78 mL, 0.01 mol) was fused for 2h. After cooling, the mixture was poured onto ice, the separated solid was filtered off, washed with water, dried and recrystallized from ethanol to afford compound (**19**) as brown powder.

Yield (%) = 3.03 g (89%), m.p. 230-232 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 2220 (CN), 1597, 1590 (C=N), 1564 (C=C); ^{1}H NMR (DMSO-d6): δ_{ppm}= 1.51-2.13 (m, 10H, CH_{Al}), 6.76-7.92 (m, 10H, Ar-H); MS *m/z (%): 340 (M^+, 7.9)*; Anal. Calcd. for $C_{22}H_{20}N_4$ (340): C, 77.62; H, 5.92; N, 16.46, Found: C, 77.78; H, 5.83; N, 16.33.

2.2.17. Formation of 2-(1',3'-diphenyl-1',7'-dihydrospiro[cyclohexane-1,6'-pyrazolo[3,4-d]pyrimidin]-4'(5'H)-ylidene)malononitrile (20)

A mixture of compound (**19**) (4.06 g, 0.01 mol), malononitrile (0.66 mL, 0.01 mol) in sodium ethoxide freshly prepared (20 mL absolute ethanol+1 g of sodium metal) was refluxed for 6h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from butanol to furnish compound (**20**) as black powder.

Yield (%) = 3.41 g (84%), m.p. 146-148 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3201, 3156 (NH), 2257, 2221 (CN), 1592 (C=N), 1564 (C=C); ^{1}H NMR (DMSO-d6): δ_{ppm}= 1.50-2.16 (m, 10H, CH_{Al}), 6.72-7.90 (m, 10H, Ar-H), 9.56 (s, 1H, NH, D_2O exchangeable), 10.40 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 23.2, 26.1, 29.3, 40.2, 60.0, 70.4, 88.2 (Spiro-C), 100.5, 111.9, 113.3, 113.6, 114.3, 118.7, 125.5, 126.5, 127.8, 128.2, 128.6, 128.9, 129.4, 135.8, 136.4, 145.3, 146.2, 148.9, 150.3, 162.3 ($C(CN)_2$); MS *m/z (%): 406 (M^+, 12.0)*; Anal. Calcd. for $C_{25}H_{22}N_6$ (406): C, 73.87; H, 5.46; N, 20.67, Found: C, 73.74; H, 5.53; N, 20.73.

2.2.18. Formation of 2-chloro-N-(4-cyano-1,3-diphenyl-1H-pyrazol-5-yl)acetamide (21)

A mixture of pyrazole derivative **1** (2.6 g, 0.01 mol), chloroacetyl chloride (1.13 mL, 0.01 mol) in butanol (20 mL) was refluxed for 6h. After cooling, the mixture was poured onto ice, the separated solid was filtered off, washed with water, dried and recrystallized from ethanol to produce compound (**21**) as green powder.

Yield (%) = 2.62 g (78%), m.p. 228-230 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3284 (NH), 2211 (CN), 1699 (C=O), 1596 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 4.27 (s, 2H, CO$\underline{CH_2}$Cl), 7.01-7.87 (m, 10H, Ar-H), 10.02 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 40.3 (CH_2), 78.3, 112.2, 116.9, 118.5, 125.7, 127.3, 128.4, 129.1, 129.4, 135.8, 136.4, 145.3, 146.2, 148.6, 151.1, 161.4 (C=O); MS *m/z (%): 336 (M^+, 6.2)*; Anal. Calcd. for $C_{18}H_{13}ClN_4O$ (336): C, 64.20; H, 3.89; Cl, 10.53; N, 16.64, Found: C, 64.14; H, 3.96; Cl, 10.48; N, 16.59.

2.2.19. Formation of N,2-bis(4-cyano-1,3-diphenyl-1H-pyrazol-5-yl)acetamide (22)

Method 1: A mixture of compound **21** (3.36 g, 0.01 mol)**,** pyrazole derivative **1** (2.6 g, 0.01 mol) in sodium ethoxide freshly prepared (20 mL absolute ethanol+1 g of sodium metal) was refluxed for 12h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from ethanol to give compound (**22**) as brown powder. Yield (%) = 4.89 g (87%)

Method 2: A mixture of pyrazole derivative **1** (5.2 g, 0.02 mol), chloroacetyl chloride (1.13 mL, 0.01 mol) in sodium ethoxide freshly prepared (20 mL absolute ethanol+1 g of sodium metal) was refluxed for 12h. After cooling, the separated solid was filtered off, washed with ethanol, dried and recrystallized from ethanol to afford compound (**22**) as brown powder.

Yield (%) = 5.04 g (90%), m.p. 188-190 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3294 (NH), 2222, 2207 (CN), 1695 (C=O), 1610, 1595 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 4.23 (s, 2H, CO$\underline{CH_2}$N), 6.76-7.92 (m, 20H, Ar-H), 8.28 (s, 1H, $\underline{NH}CH_2$, D_2O exchangeable), 9.88 (s, 1H, CO$\underline{NH}$, D_2O exchangeable); MS *m/z (%): 560 (M+, 3.6)*; Anal. Calcd. for $C_{34}H_{24}N_8O$ (560): C, 72.84; H, 4.32; N, 19.99, Found: C, 72.99; H, 4.21; N, 20.08.

2.2.20. Formation of 5-(benzylamino)-1,3-diphenyl-1H-pyrazole-4-carbonitrile (23)

A mixture of compound **1** (2.6 g, 0.01 mol), benzyl chloride (1.26 mL, 0.01 mol) with few drops of TEA in

dioxane (20 mL) was refluxed for 8h. After cooling, the reaction mixture was poured onto ice, the separated solid was filtered off, washed with water, dried and recrystallized from methanol to produce compound (**23**) as green powder.

Yield (%) =2.91 g (83%), m.p. 126-128 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3220 (NH), 2222 (CN), 1600 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 5.31 (s, 2H, $\underline{CH_2}$Ph), 6.75-7.89 (m, 15H, Ar-H), 9.78 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 39.9 (CH_2), 79.7, 112.4, 114.2, 118.1, 125.1, 126.6, 127.9, 128.3, 129.5, 129.7, 135.7, 144.9, 146.7, 148.6, 152.3; MS *m/z (%): 350 (M+, 21.8)*; Anal. Calcd. for $C_{23}H_{18}N_4$ (350): C, 78.83; H, 5.18; N, 15.99, Found: C, 78.60; H, 5.35; N, 16.05.

2.2.21. Formation of N-(4-cyano-1,3-diphenyl-1H-pyrazol-5-yl)benzamide (24)

A mixture of compound **1** (2.6 g, 0.01 mol), benzoyl chloride (1.40 mL, 0.01 mol) with few drops of TEA in dioxane (20 mL) was refluxed for 8h. After cooling, the reaction mixture was poured onto ice, the separated solid was filtered off, washed with water, dried and recrystallized from ethanol to give compound (**24**) as black powder.

Yield (%) = 2.80 g (77%), m.p. 156-158 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3289 (NH), 2224 (CN), 1690 (C=O), 1610 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 6.74-7.98 (m, 15H, Ar-H), 11.37 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}=76.2, 113.3, 112.4, 118.2, 125.5, 126.4, 127.6, 128.0, 129.2, 129.7, 135.7, 145.2, 146.7, 149.6, 150.2; MS *m/z (%): 364 (M+, 32.1)*; Anal. Calcd. for $C_{23}H_{16}N_4O$ (364): C, 75.81; H, 4.43; N, 15.38, Found: C, 75.66; H, 4.35; N, 15.49.

2.2.22. Formation of 5-amino-1,3-diphenyl-1H-pyrazole-4-carboxamide (25)

To a solution of pyrazole derivative **1** (2.6 g, 0.01 mol) in ammonium solution (50 mL), hydrogen peroxide (20 mL) was added. The reaction mixture stirred at room temperature for 3h. The reaction mixture was poured onto ice, the separated solid was filtered off, washed with water, dried and recrystallized from ethanol to afford compound (**25**) as off white powder.

Yield (%) = 2.25 g (81%), m.p. 186-188 ^{O}C; IR (KBr) ν_{max}/cm^{-1}:3446, 3400, 3312, 3290 (NH_2), 1706 (C=O), 1600, 159 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 6.76-7.92 (m, 10H, Ar-H), 9.09 (s, 2H, NH_2, D_2O exchangeable), 10.64 (s, 2H, NH_2, D_2O exchangeable); MS *m/z (%): 278 (M^+, 31.6)*; Anal. Calcd. for $C_{16}H_{14}N_4O$ (278): C, 69.05; H, 5.07; N, 20.13, Found: C, 68.89; H, 5.14; N, 20.19.

2.2.23. Formation of 1,3-diphenyl-1H-pyrazol-5-amine (26)

Method 1: A solution of pyrazole derivative **1** (2.6 g, 0.01 mol) in sodium hydroxide (100 mL, 50%) freshly prepared was stirred for 5h at 0^{O}C. The reaction mixture was poured onto cold dil. HCl (100 mL, 50%), the separated solid was filtered off, washed with water, dried and recrystallized from methanol to give compound (**26**) as green powder. Yield (%) = 1.96 g (83%)

Method 2: A solution of pyrazole derivative **1** (2.6 g, 0.01 mol) in sulphuric acid (100 mL, 50%) freshly prepared was stirred for 5h at 0^{O}C. The reaction mixture was poured onto cold dil. NH_4OH (150 mL, 33%), the separated solid was filtered off, washed with water, dried and recrystallized from methanol to give compound (**26**) as green powder.

Yield (%) = 1.88 g (80%), m.p. 128-130 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3439, 3310 (NH_2), 1594 (C=N); ^{1}H NMR (DMSO-d6): δ_{ppm}= 5.39 (s, 1H, CH), 6.88-7.84 (m, 10H, Ar-H), 10.28 (s, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ_{ppm}= 100.2, 112.3, 117.9, 125.4, 125.7, 127.2, 127.4, 127.6, 128.1, 128.6, 129.3, 129.9, 135.8, 136.5, 145.3, 146.7, 148.8, 149.2; MS *m/z (%): 235 (M^+, 48.3)*; Anal. Calcd. for $C_{15}H_{13}N_3$ (235): C, 76.57; H, 5.57; N, 17.86, Found: C, 76.74; H, 5.41; N, 17.85.

2.2.24. Formation of ethyl N-(4-cyano-1,3-diphenyl-1H-pyrazol-5-yl)formimidate (27)

A mixture of pyrazole derivative **1** (2.6 g, 0.01 mol), TEOF (1.48 mL, 0.01 mol) was fused for 2h. After cooling, the reaction mixture was poured onto water, the separated solid was filtered off, washed with water, dried and recrystallized from toluene to afford compound (**27**) as brown powder.

Yield (%) = 2.46 g (78%), m.p. 252-254 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 2200 (CN), 1592 (C=N), 1564 (C=C); ^{1}H NMR (DMSO-d6): δ_{ppm}= 1.17 (t, *J*= 6.7Hz, 3H, CH_3), 4.12 (q, *J*= 6.8Hz, 2H, CH_2O), 6.76-7.92 (m, 10H, Ar-H), 8.34 (s, 1H, N=CH); MS *m/z (%): 316 (M^+, 77.7)*; Anal. Calcd. for $C_{19}H_{16}N_4O$ (316): C, 72.13; H, 5.10; N, 17.71, Found: C, 72.25; H, 5.02; N, 17.93.

2.2.25. Formation of 1-(4-cyano-1,3-diphenyl-1H-pyrazol-5-yl)thiourea (28)

A mixture of pyrazole derivative **1** (2.6 g, 0.01 mol), ammonium thiocyanate (0.76 g, 0.01 mol) was fused for 1h. After cooling, the reaction mixture was poured onto water (100 mL), the separated solid precipitated was filtered off, washed with water, dried and recrystallized from ethanol to give compound (28) as black powder.

Yield (%) = 2.62 g (82%), m.p. 142-144 ^{O}C; IR (KBr) ν_{max}/cm^{-1}: 3400, 3310 (NH_2), 3183 (NH), 2220 (CN), 1594 (C=N), 1248 (C=S); ^{1}H NMR (DMSO-d_6): δppm= 6.76-7.92 (m, 10H, Ar-H), 9.21 (s, 2H, NHCS$\underline{NH_2}$, D_2O exchangeable), 10.14 (s, 1H, $\underline{NH}$CSNH$_2$, D_2O exchangeable); MS *m/z (%): 319 (M^+, 17.2)*; Anal. Calcd. for $C_{17}H_{13}N_5S$ (319): C, 63.93; H, 4.10; N, 21.93; S, 10.04, Found: C, 64.05; H, 4.26; N, 21.77; S, 9.92.

2.3. Biological assessment

2.3.1. Antimicrobial evaluation

The Susceptibility Tests were performed according to NCCLS recommendations (National Committee for clinical laboratory Standards, 1993). Screening tests regarding the inhibition zone were carried out by the well diffusion method. [19, 20] The inoculum suspension was prepared from colonies grown overnight on an agar plate and inoculated into Mueller-Hinton broth (fungi using malt broth). A sterile swab was immersed in the suspension and used to inoculate Mueller-Hinton agar plates (fungi using malt agar plates). The compounds were dissolved in dimethyl sulfoxide (DMSO) with different concentrations (50 mg/ml). The inhibition zone was measured around each well after 24h at 37^{o}C (for fungi after 48h), controls using DMSO were adequately done.

2.3.2. Electron microscopy

For TEM preparation, the samples were fixed in 3% glutaraldehyde, rinsed in phosphate buffer, and post-fixed in potassium permanganate solution for 5 min. at room temperature. The samples were dehydrated in an ethanol series ranging from 10% to 90% for 15 min in each alcohol dilution and finally with absolute ethanol for 30 min. Samples were infiltrated with epoxy resin and acetone through a graded series till finally in pure resin. Ultrathin sections were collected on copper grids. Sections were then double-stained in uranyl acetate followed by lead citrate. Stained sections were observed with a JEOL - JEM 1010 transmission electron microscope at 70 kV at The Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University [21, 22].

2.3.3. Anticancer evaluation

Some of the newly synthesized compounds were evaluated in vitro against three human cancer cell lines, which are Hepatocellular carcinoma (HePG-2), colorectal carcinoma (HCT-116), mammary gland breast cancer (MCF-7) and human lung fibroblast normal cells (WI-38). The cell lines were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. The reagents RPMI-1640 medium, MTT, and DMSO (sigma co., St. Louis, USA), Fetal Bovine serum (GIBCO, UK). 5-Fluorouracil was used as a standard anticancer drug for comparison. The different cell lines mentioned above were used to determine the inhibitory effects of compounds on cell growth using the MTT assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100µg/ml streptomycin at 37^{O}C in a 5% CO_2 incubator. The cells were seeded in a 96-well plate at a density of 1.0x104 cells/well. At 37 ^{O}C for 48 h under 5% CO_2. After incubation, the cells were treated with different concentrations of compounds and incubated for 24h. After 24h of drug treatment, 20 µl of MTT solution at 5mg/ml was added and incubated for 4h. Dimethyl sulfoxide (DMSO) in a volume of 100 µl is added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at an absorbance of 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of the untreated sample) X 100. [15-18]

3. Results and Discussion:

3.1. Chemistry

The bifunctional starting material 5-amino-1,3-diphenyl-1H-pyrazole-4-carbonitrile **1** was prepared by a literature known procedure [23] using benzaldehyde, malononitrile, and phenyl hydrazine in a one-pot reaction.

The bifunctional compound 1 was used for the preparation of pyrrolopyrazole 2 and pyrazolopyridine 5 derivatives.

Thus, the reaction of 1 with ethyl chloroacetate in refluxing DMF afforded the pyrrolopyrazole derivative 2 through the nucleophilic attack of the amino group on the carbon of CH_2 group followed by elimination of HCl molecule, attack of the carbanion ion formed on the cyano group and rearrangement afforded compound 2 [15]. Its IR spectrum was devoid of νCN and revealed a strong band at 1736 cm^{-1} specific for the C=O of the ester group, while its ^{1}H-NMR spectrum showed bands at δ: 1.22, 4.21, 9.25, and 10.19 ppm corresponding to $CH_2\underline{CH_3}$, $\underline{CH_2}CH_3$, NH_2, and NH protons, respectively. Its ^{13}C-NMR spectrum showed signals at δ: 15.9, 58.6, and 155.9 ppm characteristic for CH_3, CH_2, and CO, respectively. Its mass spectrum showed a molecular ion peak at *m/z = 346 [M^{+}]* corresponding to molecular formula $C_{20}H_{18}N_4O_2$.

Compound 2 undergo amylolysis through reaction with hydrazine hydrate and p-toluidine to give the corresponding carbohydrazide 3 and carboxamide 4 derivatives, respectively. The absence of the carbonyl of the ester group in their IR spectra supported the proposed structures [15].

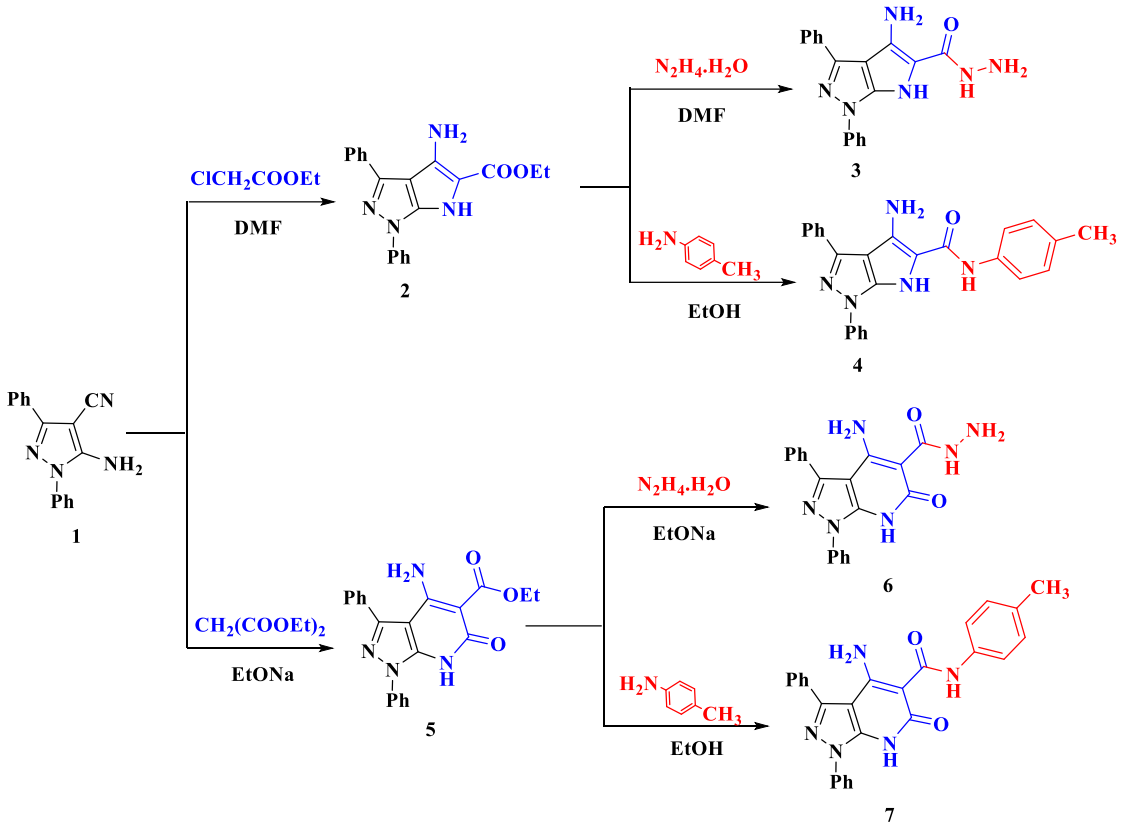
However, the reaction of compound 1 with diethyl malonate in the presence of sodium ethoxide furnished the corresponding pyrazolopyridine derivative 5. Compound 5 can be obtained through the nucleophilic attack of the amino group on the carbonyl group of ester followed by elimination of ethanol molecule, attack of the carbanion ion formed on the cyano group, and rearrangement afforded compound 5 [17]. The disappearance of the CN group from its IR spectrum, besides the appearance of the two absorption bands at 1660, 1635 for the two carbonyl groups establish evidence for the structure of the product 5. Its ^{1}H-NMR spectrum showed bands at δ: 1.24, 4.23, 6.38, and 10.68 ppm corresponding to $CH_2\underline{CH_3}$, $\underline{CH_2}CH_3$, NH_2, and NH protons, respectively. Its ^{13}C-NMR spectrum showed signals at δ: 158.6 and 160.7 ppm characteristic for two CO groups. Its mass spectrum showed a molecular ion peak at *m/z = 374 [M^{+}]* corresponding to molecular formula $C_{21}H_{18}N_4O_3$.

Similarly, compound 5 undergo amylolysis through reaction with hydrazine hydrate and p-toluidine to afford the corresponding carbohydrazide 6 and carboxamide 7 derivatives, respectively. (Scheme 1)

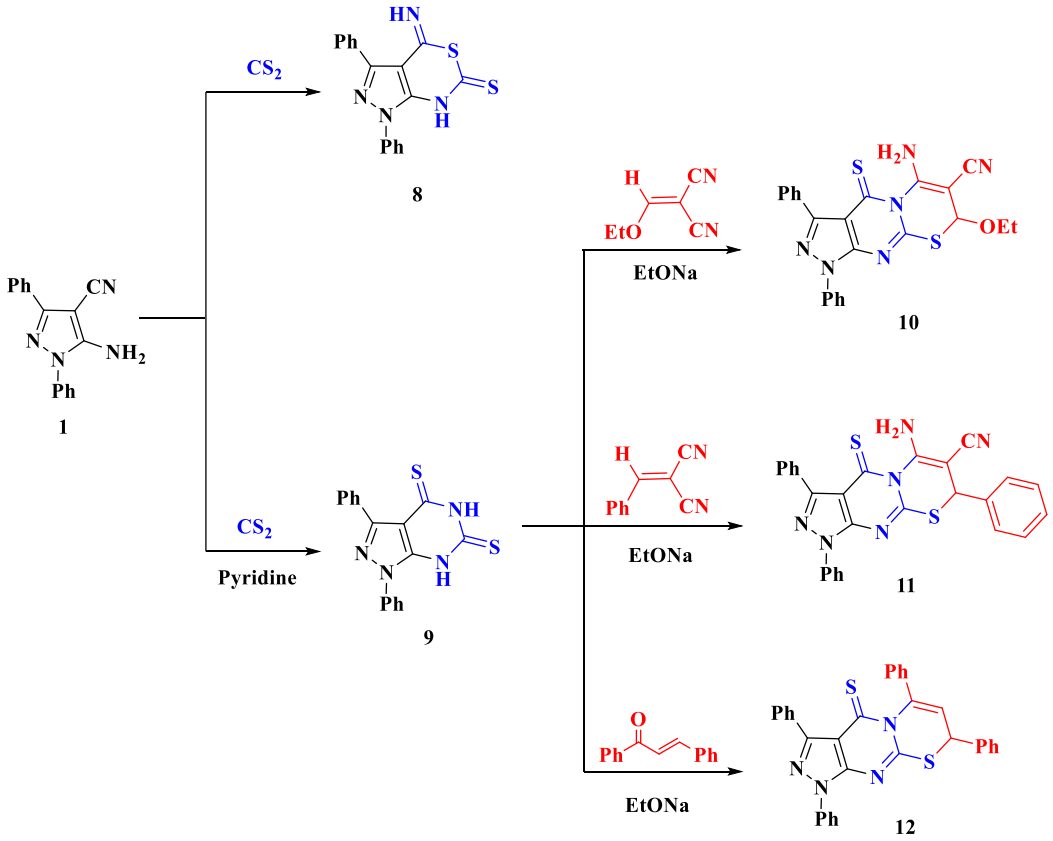
The cyclized product obtained from the reaction of enaminonitrile 1 with carbon disulfide was found to depend upon the conditions of the reaction. Thus, refluxing enaminonitrile 1 with carbon disulfide for 2h, the corresponding pyrazolothiazine derivative 8 was obtained, while refluxing enaminonitrile 1 with carbon disulfide in pyridine for 8h, the pyrazolopyrimidine derivative 9 was produced.

Compound 9 was obtained through the first formed compound 8, which rearranged according to Dimroth rearrangement when boiled in pyridine to give the pyrazolopyrimidine derivative 9. (Fig. S53)

Compound 9 can be used as a key intermediate for the synthesis of the tricyclic compounds 10-12, via its reactions with 2-(ethoxymethylene) malononitrile, 2-benzylidene malononitrile, and benzalacetophenone, respectively. (Fig. S54). The structure of the obtained derivatives 8-12 was confirmed from their spectroscopic data (IR, ^{1}H-NMR, ^{13}C-NMR, MS) and elemental analysis. (Scheme 2)



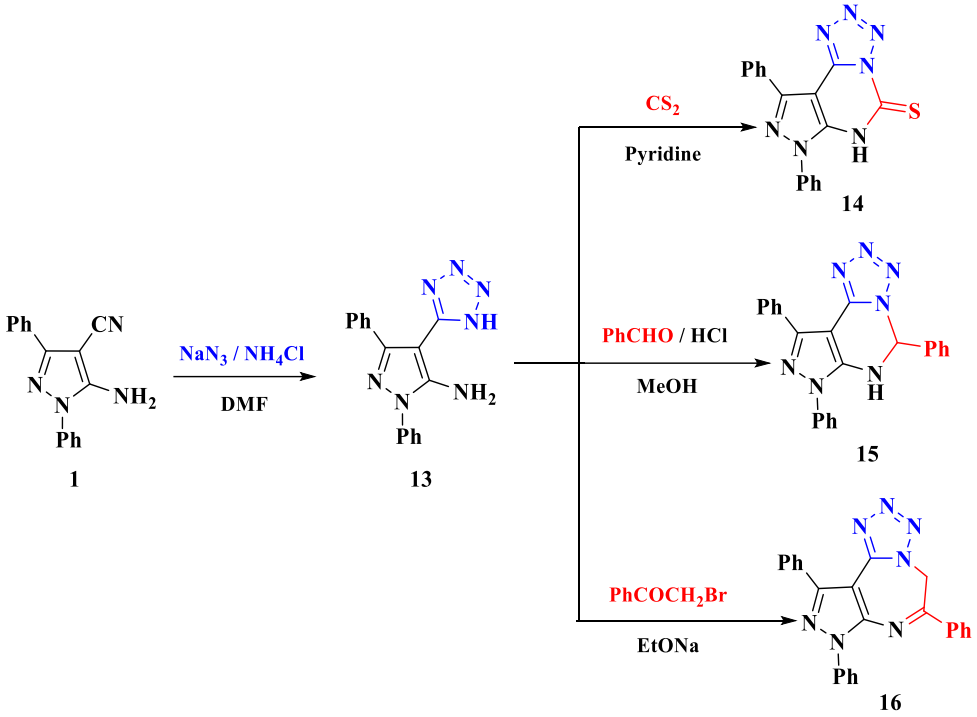
Scheme 1. Synthesis of pyrrolopyrazole and pyrazolopyridine derivatives 2-7.



Scheme 2. Synthesis of pyrazolothiazine, pyrazolopyrimidine, pyrazolopyrimidinothiazole and pyrazolopyrimidinothiazine derivatives 8-12.

A large number of tetrazole derivatives are reported to exhibit anticancer [24], antibacterial [25], antifungal [26], and anti-inflammatory [27] activities.

This prompted the authors to synthesize a new tetrazolopyrazole derivative 13, through the reaction of the enaminonitrile 1 with sodium azide in the presence of a catalytic amount of ammonium chloride in refluxing DMF. Its IR spectrum showed the Ar-H characteristic band of tetrazole ring at 1564 cm^{-1}, its ^{1}H-NMR spectrum revealed signals at δ 9.36 and 9.69 ppm corresponding to NH and NH_2 protons, respectively. Its mass spectrum revealed a molecular ion peak at *m/z=303 [M⁺]* which corresponding to molecular formula $C_{16}H_{13}N_7$. On the other hand, tetrazole derivative 13 was considered as a key intermediate in the preparation of some novel derivatives 14-16, through its reactions with CS_2, benzaldehyde, and phenacyl bromide. The structure of the new derivatives was confirmed by using different spectroscopy data of IR, ^{1}H-NMR, ^{13}C-NMR, mass spectra, and elemental analysis. (Fig. S55 & 56) (Scheme 3)



Scheme 3. Synthesis of tetrazole derivatives 13-16.

Condensation of enaminonitrile 1 with furfur aldehyde in refluxing ethanol in the presence of catalytic amount of glacial acetic acid gave the corresponding Schiff base 17, which was formed through the nucleophilic attack of the amino group on the carbonyl of the aldehyde followed by elimination of one molecule of water.

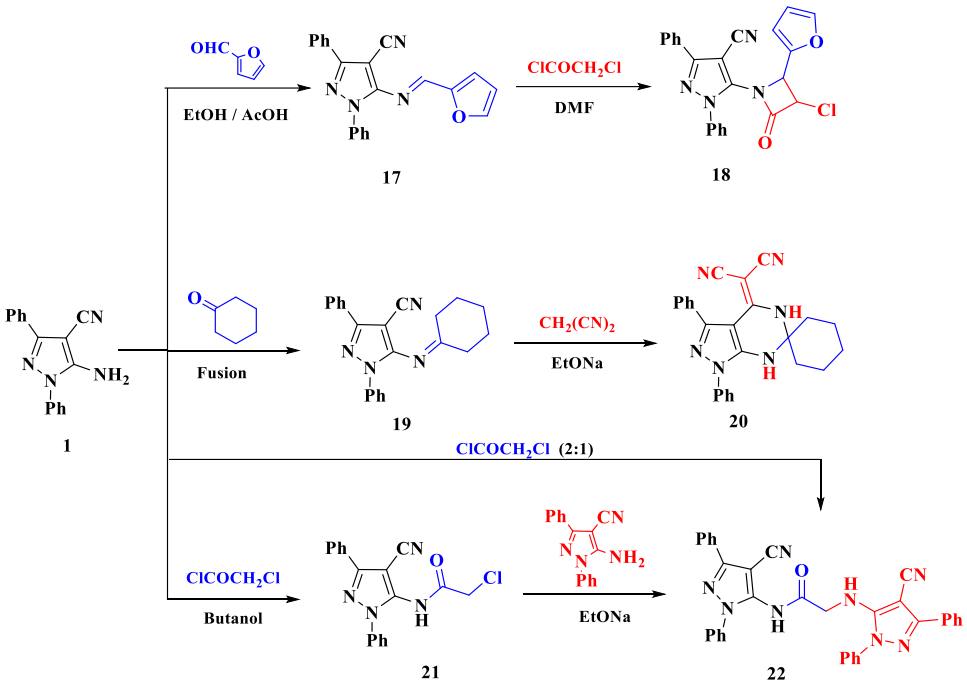
Due to the biological significance of azetidinones as anticancer [28], antibacterial [29], antifungal [30], and anti-inflammatory [31], we have synthesized a new hybrid compound containing both entities through the reaction of the obtained Schiff base derivative 17 with chloroacetyl chloride to give the azetidinone derivative 18 this is in accordance with the previous publication [32]. The structure of azetidinone derivative 18 was established from its IR spectrum which revealed a band at ν 1682 cm^{-1} specific for the C=O group. Its ^{1}H-NMR spectrum exhibit signals at δ: 4.72 and 5.04 ppm corresponding to CH-Cl and CH-Ar protons, respectively. Its ^{13}C-NMR spectrum revealed signals at 43.5, 43.8, and 167.0 ppm characteristic for CH-Cl, CH-furan, and C=O group, respectively. Its mass spectrum revealed a molecular ion peak at *m/z=414 [M⁺]* which corresponding to molecular formula $C_{23}H_{15}N_4O_2Cl$.

Similarly, the fusion of compound 1 with cyclohexanone gave the corresponding Schiff base 19, which on reaction with malononitrile in the presence of sodium ethoxide gave the corresponding spiro derivative 20. The IR of the spiro derivative 20 revealed bands at ν 2257, 2221, 3156, and 3201 cm^{-1} which corresponding to 2CN and 2NH groups. Its mass spectrum showed a molecular ion peak at *m/z=496 [M⁺]* which corresponding to molecular formula $C_{25}H_{22}N_6$.

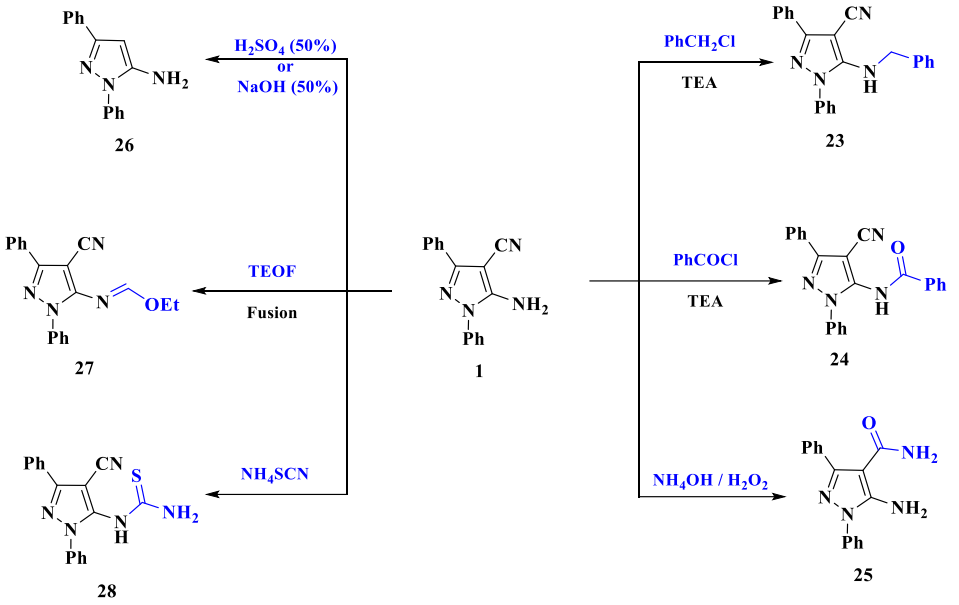
Refluxing enaminonitrile 1 with chloroacetyl chloride in butanol afforded the corresponding 2-chloro-N-acetamide derivative 21. Its IR spectrum revealed a band at ν 1699 cm^{-1} specific for the C=O group. Its ^{1}H-NMR spectrum exhibit signal at δ: 4.27 ppm corresponding to CH_2 proton. Its ^{13}C-NMR spectrum revealed signals at 40.3, and 161.4 ppm characteristic for CH_2, and C=O groups, respectively. While its mass spectrum showed a molecular ion peak at *m/z=336 [M⁺]* which corresponding to molecular formula $C_{18}H_{13}N_4OCl$.

The structure of compound 21 was further supported by its reaction with compound 1 to give the corresponding bispyrazole derivative 22.

Alternatively, compound 22 can be prepared by reaction of enaminonitrile 1 with chloroacetyl chloride in a 2:1 ratio. The prepared compound 22 by the two methods was a matching pair in m.p, mixed m.p, as well as TLC. (Scheme 4)



Scheme 4 . Synthesis of azetidinone, spiro pyrazolopyrimidine and bispyrazole derivatives 17-22.



Scheme 5. Synthesis of pyrazole derivatives 23-28.

Refluxing enaminonitrile **1** with benzyl chloride and/or benzoyl chloride in dioxane in the presence of few drops of TEA gave the corresponding N-pyrazolobenzylamine 23 and N-pyrazolobenzamide 24 derivatives, respectively.

Hydrolysis of enaminonitrile 1 using NH_4OH/H_2O_2 at room temperature gave the pyrazole carboxamide derivative 25, however, when hydrolysis was carried out with H_2SO_4 (50%) or NaOH (50%) at 0^OC, the known amino pyrazole derivative 26 was obtained through hydrolysis followed by decarboxylation [33].

The fusion of enaminonitrile 1 with triethyl orthoformate gave the known formimidate derivative 27 [34].

On the other hand, the fusion of enaminonitrile 1 with ammonium thiocyanate afforded the corresponding thiourea derivative 28. The structure of compounds 23-28 was confirmed by using different spectroscopy data of IR, ^{1}H-NMR, ^{13}C-NMR, mass spectra, and elemental analysis. (Scheme 5)

3.2. Biological studies:

3.2.1. Antimicrobial activity:

The prepared derivatives were evaluated in vitro for their expected antimicrobial activities versus methicillin-resistant *Staphylococcus aureus (MRSA)*, clinical isolate and *Bacillus subtilis* as Gram-positive bacteria and

Escherichia coli and *Proteus vulgaris* as Gram-negative bacteria, *Aspergillus fumigates* and *Candida albicans* as fungi. The antibacterial and antifungal activities was determinate by using the agar diffusion method. Ketoconazole and Gentamycin were used as slandered references for antibacterial and antifungal activities. The tested compounds results were recorded as inhibition zones average diameter (IZ) of bacterial and/or fungal which growth in mm around the discs. The zone diameters of inhibition referred to preliminary test concentration (5 mg/mL) which is shown in Table 1 and Fig. 2-7 (see supplementary file). Unfortunately, all the prepared compounds were inactive against S. aureus. This due to S. aureus can become resistant to methicillin and other β-lactam antibiotics through the expression of a foreign PBP, PBP2a, that is resistant to the action of methicillin, but which can perform the functions of the host PBPs [35, 36]. It is known that most of the compounds used have an effect on staph, but the tested isolate used in this research is from MERSA, and consequently, the properties of the compounds are high, and we hoped that our compounds used in this research would have an effect on it, but unfortunately, this did not happen. Transmission Electron Microscopy TEM is one of the important techniques which produce images of biological specimens with high resolution. It is used to investigate the mode of action of the prepared pyrazole derivatives of the highest antibacterial and antifungal activities on the structure of bacteria and fungi The TEM images high resolution obtained from the used package of electrons "which have very short wavelengths" as the illuminating radiation source. The action mode referred to preliminary compounds which imaging by Transmission Electron Microscopy against bacteria and fungi are shown in Fig. 8.

Table 1. Antimicrobial activities of compounds 2-28 against Gram +ve bacterial, Gram -ve bacterial, and fungi expressed as inhibition diameter zones in millimeters.

Sample Code	Tested microorganisms					
	Gram positive bacteria		Gram negative bacteria		Fungi	
	S. aureus	*B. subtilis*	*E. coli*	*P. vulgaris*	*A. fumigatus*	*C. albicans*
Ketoconazole	NT	NT	NT	NT	17	20
Gentamycin	24	26	30	25	NT	NT
2	NA	12	13	14	13	15
3	NA	NA	11	17	11	15
4	NA	16	13	23	8	25
5	NA	15	NA	NA	NA	24
6	NA	NA	14	20	10	18
7	NA	NA	13	17	12	19
8	NA	13	12	21	9	21
9	NA	27	NA	15	12	25
10	NA	14	NA	NA	NA	18
11	NA	16	NA	NA	NA	NA
12	NA	18	18	20	11	10
13	NA	22	10	25	12	26
14	NA	20	NA	11	11	23
15	NA	18	20	12	10	18
16	NA	NA	8	NA	9	18
17	NA	14	12	20	15	27
18	NA	21	15	NA	22	20
19	NA	12	10	NA	11	20
20	NA	NA	NA	NA	10	16
21	NA	19	12	20	15	28
22	NA	13	15	21	13	22
23	NA	NA	9	NA	10	21
24	NA	13	14	15	8	20
25	NA	10	13	9	11	14
26	NA	11	15	18	13	18
27	NA	14	15	22	10	18
28	NA	12	13	20	9	15

NT: Not tested. NA: No activity

3.2.2. Transmission Electron Microscopy investigation:

In TEM investigations, there were many different cellular changes of stained ultrathin sections (70 nm) of all tested organisms. Ultra-thin sections of treated B. subtilis with pyrazolopyrimidinethione derivative 9 showed completely lysed of cytoplasmic materials as shown in Fig. 8B, compared with control as shown in Fig. 8A. Control cells of P. vulgaris had ideal cell ultrastructure shape as shown in Fig. 8C, since, completely cellular alternations; absence of cytoplasmic materials was noticed after treatment with tetrazole derivative 13 which illustrated in as shown in Fig. 8D. Untreated cells of E. coli represented a unique and formed microstructure as shown in Fig. 8E, since, the cells affected with pyrazolotetrazolopyrimidine derivative 15 showed shrinkage and disappearance of cytoplasmic contents as shown in Fig. 8F. However, the ultra-thin sections of affected A. fumigatus with azetidinone derivative 18 represented deformations of both cell wall and cell membrane with the disappearance of the cytoplasmic materials as shown in Fig. 8H, compared with control ultrastructure as shown in Fig. 8G; no damage of cell wall (CW) and cell membrane (CM) with formed microorganelles; mitochondria (M) and nucleus (N). In case of ultra-thin sections of treated C. albicans with revealed clear findings of the ultrastructure of the yeast cells affected with pyrazole-N-acetamide 21; Irregularity of both cell wall and cell membrane with leakage of cytoplasmic materials as shown in Fig. 8J, compared with control cell; showed identified rounded cell with intact cell wall (CW), cell membrane (CM), Mitochondria (M) and vacuole (V) as shown in Fig. 8I.

3.2.3. Structure-activity relationship (SAR):

The present study revealed that the synthesized diazole derivatives (4, 5, 9, 13, 17, and 21) were potent against C. albicans, since their inhibition zones were much higher than those produced by the reference drug Ketoconazole. Additionally, derivatives (8, 14, 22, and 23) were higher than the reference drug but not as the previous derivatives. However, derivatives (18, and 24) showed the same inhibition zones as ketoconazole.

Compound 21 showed the highest antimicrobial activity, due to the presence of open chain 2-chloro-N-acetamide group $NHCOCH_2Cl$ which has a small size and three lone pairs centers NH, C=O and Cl. Inserting different big size side chain to the pyrazole derivative 1 as the presence of N-(furan-2-ylmethylene) in compound 17, oxoazetidine / furan rings in compound 18, pyrazole ring in compound 22 and benzene ring in compound 24, decreases the antimicrobial reactivity by comparison with compound 21. However, the presence of tetrazole ring in compound 13 increases the reactivity of the compound due to the presence of the NH group and other three lone pair centers, the tetrazole ring lower toxicity than the cyano group and not affect the organism's enzyme. Within, compound 23 lower than compound 17 due to the reactivity of benzene lower than furan ring. Also, the pyrazolopyrrole 4 activity return to the presence of the amide group and NH of the pyrrole ring. On the other hand, pyrazolopyrimidindithione 9 activity return to the presence of 2NH groups of the pyrimidine ring. Finally, the higher reactivity of the prepared compounds was due to the lone pair of NH group of amino, amide, pyrrole, and/or pyrimidine groups.

3.2.4. In Vitro Anticancer Screening:

Some of the newly prepared compounds were selected to evaluate as in vitro cytotoxic activity against *human hepatocellular carcinoma* "HePG-2", *Colorectal carcinoma* "HCT-116", *breast cell line* "MCF-7", and *human lung fibroblast normal cells* "WI-38" with a standard anticancer drug 5-Fluorouracil for comparison according to the reported method [37, 38]. The used cell lines have been obtained from "ATCC" via the biological products and vaccines holding company "VACSERA", Cairo, Egypt. Standard colorimetric assay method for determining cell growth using MTT assay. This method is used to determine potential medicinal agents cytotoxicity and other toxic substances. In brief, yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by mitochondrial dehydrogenases of living cells. A suitable solvent is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength. When the amount of purple formazan produced by cells treated with an agent is compared with that produced by unreacted control cells, the effectiveness of the agent in causing the death of cells can deduce through the production of a dose-response curve. The synthesized compounds IC_{50} were compared to the standard drug as shown in Table 2. The obtained results showed that the tested four compounds namely 5, 6, 9, 13, 16, 25, and 28 exhibited variable inhibitory activity degrees towards the three tested cell lines of cancer. Concerning activity against HepG-2 cell line, very strong cytotoxic activity was showed by compound 9 which showed the percentage viability IC_{50} at 8.48 mg/mL, whereas, strong cytotoxic activity was displayed by compounds 13 and 21 which showed the percentage viability IC_{50} at 8.27 and 8.34 mg/mL and strong inhibitory activity was also demonstrated by compounds 5, 16 and 28. The HCT-116 cell line showed very strong cytotoxic activity toward compounds 13 and 25 which have IC_{50} at 9.57 and 9.89 mg/mL, respectively, whereas, compounds 5, 6, 9, 16, and 28 displayed strong cytotoxic activities. On the other hand, compounds 13 and 25 showed very strong cytotoxic activity IC_{50} at 6.81 and 7.78 mg/mL toward MCF-7 cell line, compounds 5, 6, 28 showed the strong cytotoxic activity for the percentage viability IC_{50} at 12.44, 16.36 and 16.59 mg/mL against MCF-7 cell line. However, all the evaluated compounds showed weak cytotoxic activity for the percentage viability IC_{50} from 52.41 to 240.38 mg/mL against WI-38 cell line.

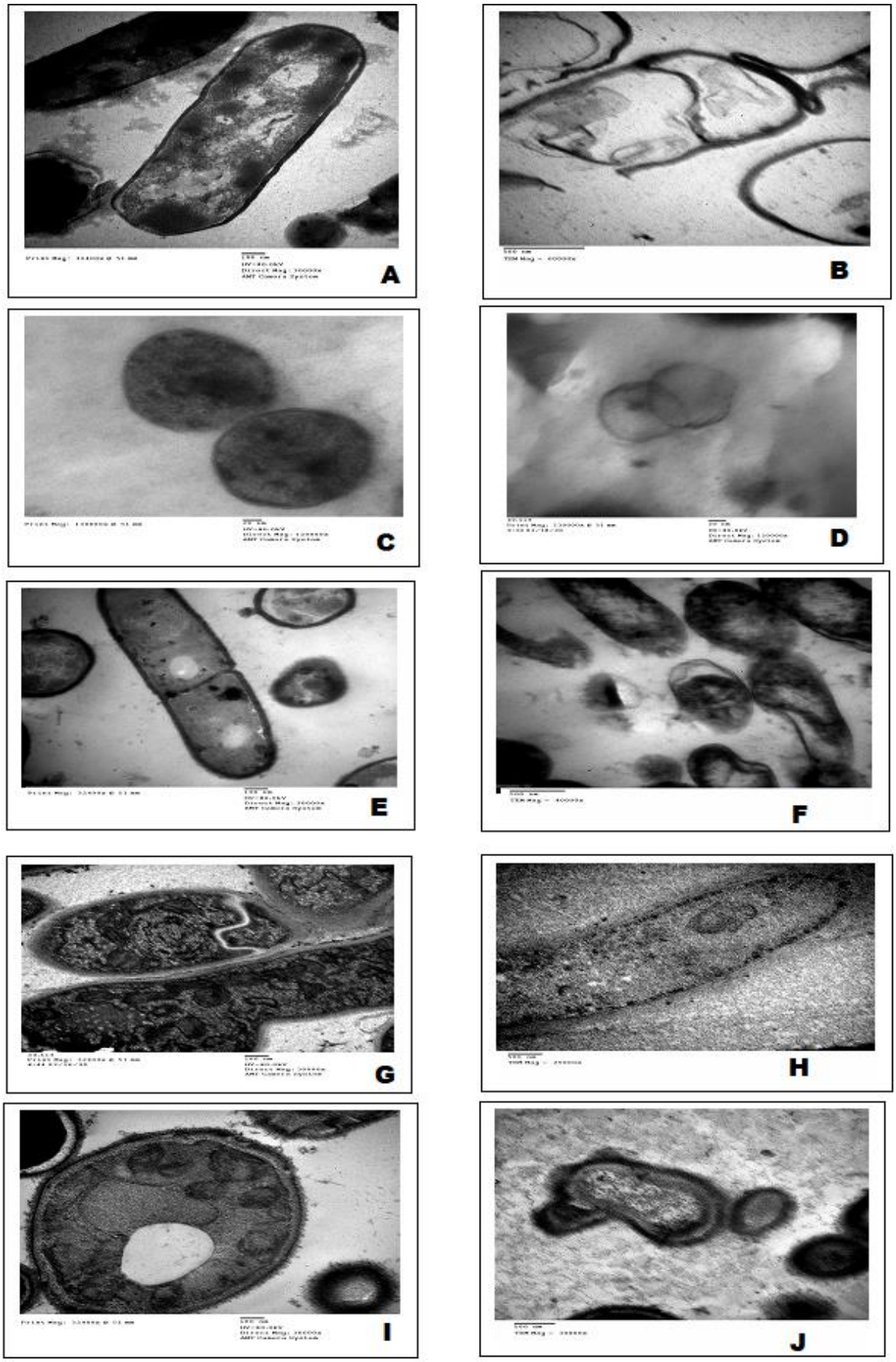


Fig. 8. TEM micrographs of B. subtilis A: control and B: affected with pyrazolopyrimidinethione derivative 9; C: control of P. vulgaris and D: affected with tetrazole derivative 13; E: control of E. coli and F: affected with pyrazolotetrazolopyrimidine derivative 15; G: control of A. fumigatus and H: affected with compounds azetidinone derivative 18; I: control of C. albicans and J: affected with pyrazole-N-acetamide 21.

Table 2. Cytotoxic activities of some compounds against human tumor cancer cells.

Compound number	In vitro Cytotoxicity IC50 (µM)*			
	HePG-2	HCT-116	MCF-7	WI-38
5-Fu	7.86±0.5	5.35±0.3	4.17 ± 0.2	4.39 ± 0.4
DMSO	0	0	0	0
2	28.05±1.9	33.29±1.7	19.46±1.9	154±0.9
3	19.48±1.5	28.74±2.1	19.38±1.6	92.5±1.7
5	14.25±0.9	18.16±1.5	12.44±1.3	176±1.0
6	9.89±0.7	16.75±1.3	16.36±0.9	240.38±0.7
8	13.71±1.0	22.29±1.7	29.60±2.2	56.4±2.2
9	8.48±0.9	14.52±2.5	9.78±1.2	78±2.4
11	38.25±2.4	42.38±2.7	25.12±2.0	94.5±1.4
13	8.27±2.7	9.57±1.4	6.81±1.9	55.3±2.4
14	24.06±1.9	11.63±0.9	11.29±1.1	67.1±1.3
15	53.98±3.1	62.40±3.3	42.64±2.2	214.3±0.8
16	15.26±0.9	27.25±2.3	26.98±2.3	187.4±1.5
18	48.44±3.0	57.17±3.2	29.23±2.5	180.7±2.3
20	42.98±1.5	61.24±2.4	45.39±0.9	127.6±2.4
22	53.6±2.3	43.94±1.6	30.68±2.7	87.5±0.9
23	32.12±2.2	36.01±2.4	32.06±2.8	153.7±1.2
25	8.43±0.9	9.89±1.7	7.78±0.7	94.25±0.9
26	45.50±2.8	50.08±2.9	39.25±3.0	59.26±2.4
28	12.27±0.9	19.79±2.4	16.59±1.4	52.41±2.3

* IC_{50} (µM): 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak) and above 100 (non-cytotoxic)

** 5-Fu: 5-Fluorouracil

** DMSO (solvent, negative control)

4. Conclusion:

In summary, efficient and useful synthetic protocol was used to construct novel pyrazole derivatives incorporated into different heterocycles in good to excellent yield. The antimicrobial evaluation of some novel derivatives showed that the most active compounds are 4, 5, 9, 13, 17 and 21, which inhibited the growth of Gram - negative and Gram - positive bacteria. Compound 21 showed the highest antimicrobial activity , this due to the presence of open chain 2-chloro-N-acetamide group $NHCOCH_2Cl$ which has a small size and three lone pairs Centers NH,C=O and Cl. On the other hand, some of the novel derivatives screened for their antitumor activity and the potency of selected compounds is 13>21>2 with reference to standard anticancer drug 5-fluorouracil.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethic approval and consent to participate

Not applicable.

Human and Animal rights

No animals/humans were used for studies that are the base of this research.

Consent for publication

Not applicable

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