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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF SOME NEW PHOSPHORUS COMPOUNDS CONTAINING PYRANOPYRAZOLE MOIETY

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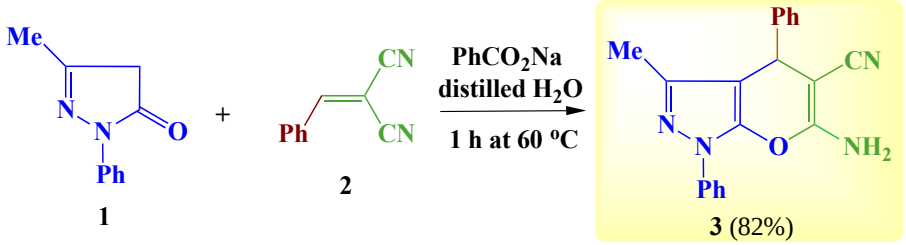
Abstract – A simple method for construction of functionalized pyranopyrazoles containing phosphoramide and phosphonate groups *via* one-pot reaction was achieved. The methodology depended on the reaction of 6-amino-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile (**3**) with triethyl phosphite and hexaethylphosphoramide and so with diethyl phosphite in the presence of chloroacetyl chloride or carbon disulfide. The products were screened for their antimicrobial, antioxidant and antiproliferative properties. Compound **7** displayed promising antimicrobial and antioxidative properties. Also, compound **8** showed potent cytotoxic effects against MCF-7, HepG-2 and HCT-116 cancer cells with IC_{50} values in range 5.3 ± 0.9 to 7.5 ± 0.6 μg/mL. Acridine orange and ethidium bromide were used for the detection of viable, apoptotic, and necrotic cells. The early apoptotic cell death was observed by the compounds in all types of the treated cells. Compounds **5, 7** and **8** induced high percentage of necrosis towards all treated cells. The late apoptosis was recorded as a high rate after treatment with compound **3** in HepG-2 cells.

INTRODUCTION

Organophosphorus compounds are essential substrates for the investigation of biochemical processes,[1] and tetracoordinate pentavalent phosphorus compounds are generally used as bioactive components.[2] Among them, compounds containing a phosphonate unit -P(O)(OR)$_2$ (in which R may be alkyl, or aryl), attracted interest in the production of isosteric or bioisosteric analogues of various natural products.[3-5] In addition, heterocyclic scaffolds bearing phosphonate moieties continue to remain at the focus of attention due to their wide synthetic applicability and therapeutic importance including antiviral,[6] antimicrobial,[7] antioxidant,[8] anticancer,[9] antimalarial,[10] antidiabetic[11] and anti‑HIV.[12] One of the most convenient methods for the construction of heterocyclic phosphonates is multicomponent reactions,[13] which usually use simple starting materials, using of a theoretical "ideal synthesis" such as fast accomplishment, save time, low used energy and being as environmentally-friendly.[14] On the other hand, pyrano[2,3-*c*]pyrazole moiety is a major structural motif in several natural products and synthetic compounds of widely recognized pharmacological properties.[15] In this context, pyranopyrazole systems have well been recognized for their potent biological activities including antimicrobial,[16] antioxidant,[17] antiviral[18] and antitumor.[19] Recently, we studied the reaction of the 6-amino-3-methyl-1,4-diphenyl-1,4-dihydropyrano-[2,3-*c*]pyrazole-5-carbonitrile (**3**) with some phosphorus halides.[20] Considering the above mentioned, we herein study the chemical reactivity of substrate **3** towards some other phosphorus amide and phosphorus esters in the presence of electrophilic reagents in a one-pot and three-components. These reactions led to the formation of some new pyrano[2,3-*c*]pyrazolyl phosphoramides and phosphonates that combine the advantages of pyranopyrazole systems and the phosphonate moieties. The isolated products were screened for the antimicrobial, antioxidant and antiproliferative activities.

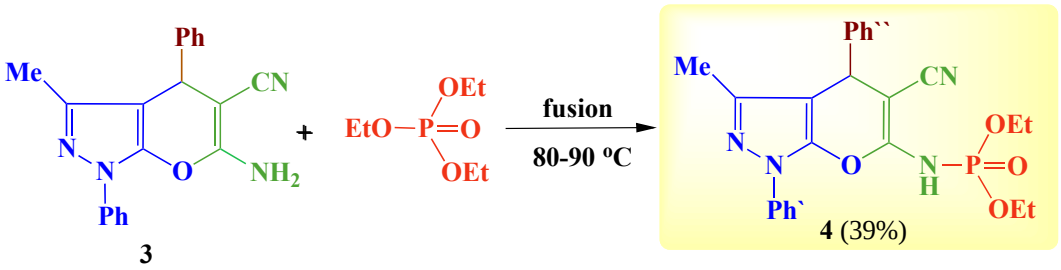
RESULTS AND DISCUSSION

The known starting material, 6-amino-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile (**3**) was synthesized in good yield as reported in the literature.[21] It was synthesized by the reaction of 3-methyl-1-phenyl-5-pyrazolone (**1**) with 2-benzylidenemalononitrile (**2**) in distilled water containing a catalytic amount of sodium benzoate (Scheme 1).



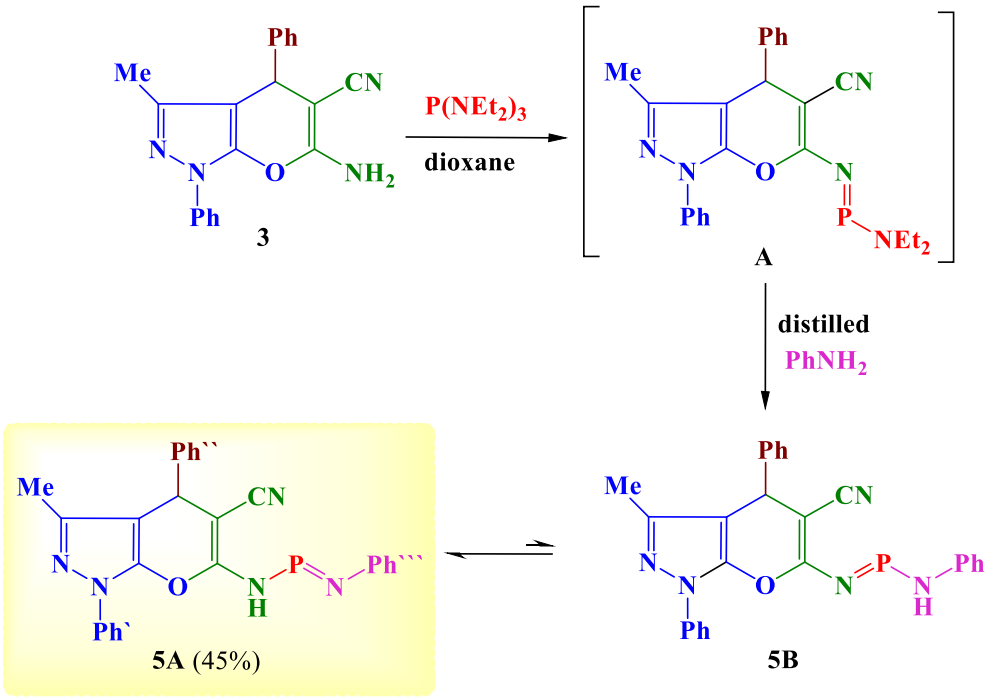
Scheme 1

Phosphorus esters have gained significant interest as versatile reagents in construction of various novel phosphorus heterocyclic compounds.[22,23] Thus, fusion of the starting material **3** with excess amount of triethyl phosphate at 80−90 °C for 10 hours afforded diethyl {[5-cyano-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazol-6-yl]amino}phosphoramidate (**4**) in 39% yield through removal of ethanol molecule (Scheme 2). The IR spectrum of product **4** revealed the existence of absorption bands relevant to NH, C≡N, P=O and P−O−C at 3384, 2209, 1179 and 1027 cm^{-1}, respectively. In addition, its ^{1}H-NMR spectrum displayed two multiplet signals for EtO groups at δ 1.15−1.24 (2 Me) and δ 3.88−4.18 (2 OCH_2) ppm. Moreover, its ^{13}C-NMR spectrum confirmed the proposed structure by recording of the specific carbon atoms Me, OCH_2 and C≡N at δ 14.9, 61.1 and 110.8 ppm, respectively. The ^{31}P-NMR spectrum for compound **4** recorded a singlet at δ 21.6 ppm. Besides, its M^+ peak was recorded at *m/z* 464 (18%) in its mass spectrum.



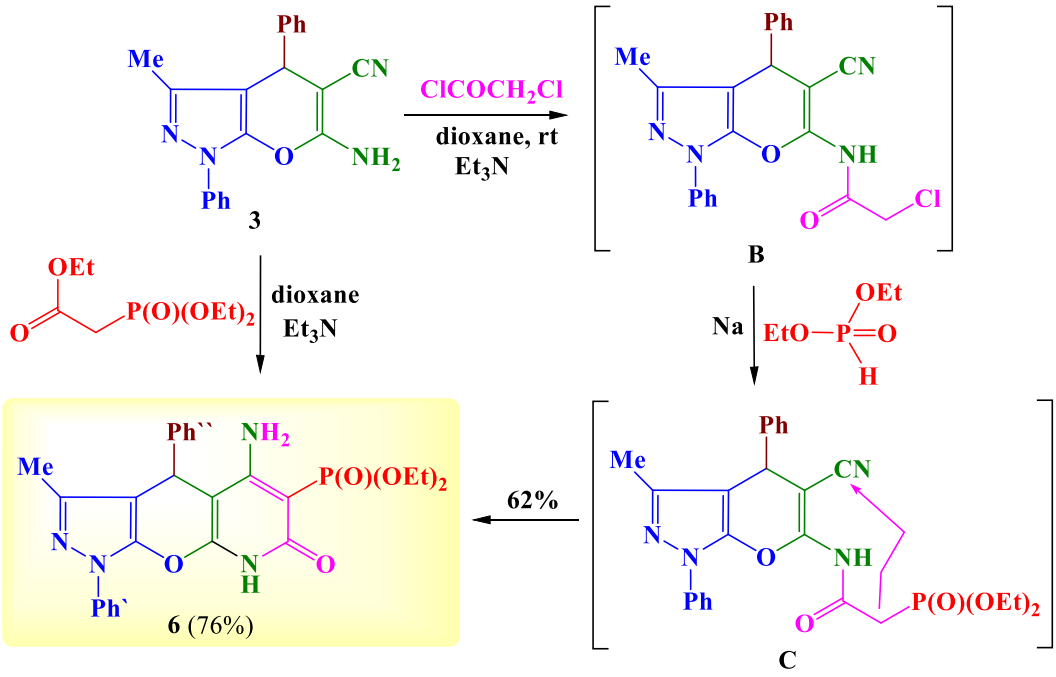
Scheme 2

Reaction of substrate **3** with hexaethylphosphoramide in dry dioxane may form the expected product **A** which was difficult to isolate in pure form. Thus, addition of equimolar amount of freshly distilled aniline to the nonisolable material **A** led to the formation of *N*-(5-cyano-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazol-6-yl)-*N*`-phenylphosphenimidous amide (**5**) in pure form in 45% yield (Scheme 3). The formation of product **5** can be interpreted in terms of condensation of amino group of compound **3** with $P(NEt_2)_3$ to give the intermediate **A** which then reacted with aniline molecule to afford the desired product **5** in preferred form **5A** and not **5B** according to its ^{1}H-NMR spectrum (Scheme 3).[24] The IR spectrum displayed characteristic absorption bands attributable to NH, C≡N and P=N functions at 3423, 2193 and 1454 cm^{-1}, respectively. The M^+ peak was recorded at *m/z* 449 (20%) in its mass spectrum. Besides, the ^{1}H- and ^{13}C-NMR spectra confirmed the suggested structure by recording of the additional phenyl group of P=N−Ph moiety. The presence of NH proton at downfield δ 8.42 ppm supported the form **5A** that explained difficulty of cyclization of compound **5**.



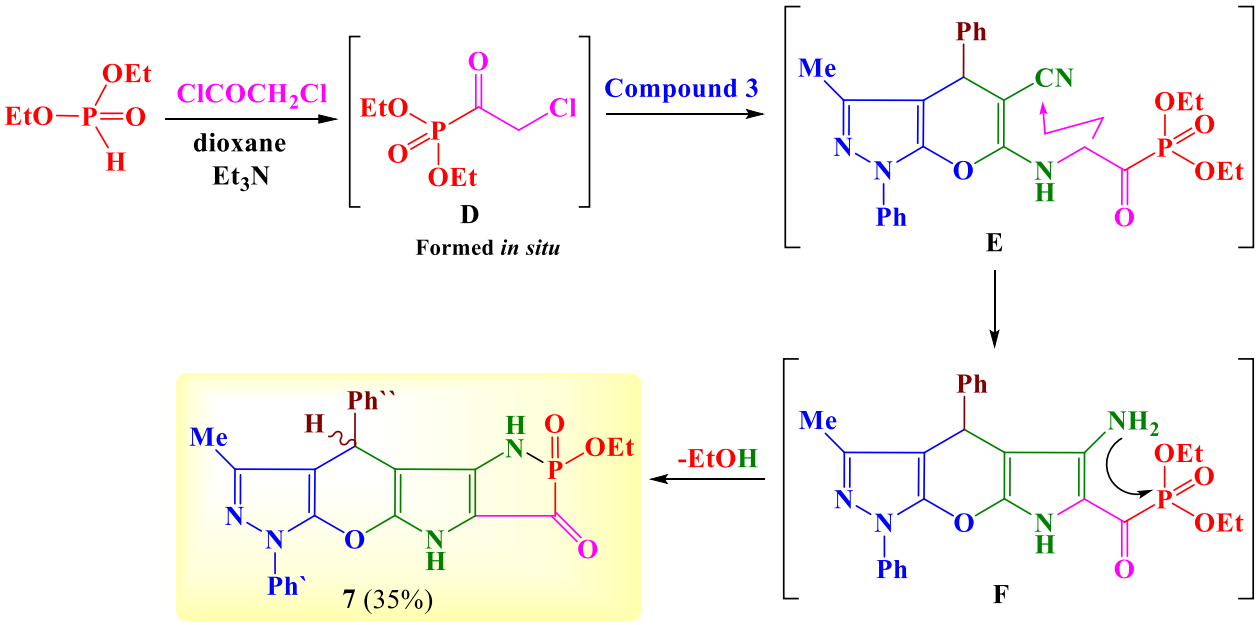
Scheme 3

In our recent work, we studied the chemical reactivity of substrate **3** with diethyl phosphite in the absence or presence of some electrophilic reagents such as benzaldehyde, triethyl orthoformate, and dimethyl-formamide dimethyl acetal.[25] Here, we extend our study to know the chemical behavior of substrate **3** towards diethyl phosphite in the presence of chloroacetyl chloride and carbon disulfide. Thus, stirring of substrate **3** with chloroacetyl chloride in dry dioxane containing a catalytic amount of triethylamine yielded the corresponding chloroacetamide intermediate **B**. A solution of diethyl phosphite and sodium metal as a catalyst in dioxane, was added to the previous mixture and heated under reflux to give the nonisolable intermediate **C** *via* removal of HCl molecule. Cyclization of the latter intermediate through nucleophilic addition of the active CH_2 group at the C≡N group produced diethyl {5-amino-3-methyl-7-oxo-1,4-diphenyl-1,4,7,8-tetrahydropyrazolo[4',3':5,6]pyrano[2,3-*b*]pyridin-6-yl}phosphonate (**6**) as a sole product in good yield (Scheme 4). The product **6** was also obtained in a higher yield *via* treatment of substrate **3** with triethyl phosphonoacetate in dry dioxane containing a catalytic amount of triethylamine (Scheme 4). The IR spectrum of the triheterocyclic compound **6** exhibited characteristic absorption bands attributable to NH_2, NH and C=O at 3430, 3312, 3262 and 1690 cm^{-1}, respectively, while the phosphonate moiety recorded a singlet at δ 20.0 ppm in its ^{31}P-NMR spectrum. In ^{1}H-NMR spectrum of product **6**, the chemical shift of NH_2 and NH protons resonated as three singlets at δ 8.41, 8.60 and 10.02 ppm, respectively. Additionally, its ^{13}C-NMR spectrum displayed the carbon atoms of diethoxy group at δ 13.9 (Me) and 62.4 (OCH_2) ppm, while C=O appeared at δ 165.4 ppm. Its mass spectrum agreed with the predicted structure and revealed the M^+ peak at *m/z* 506 (25%).



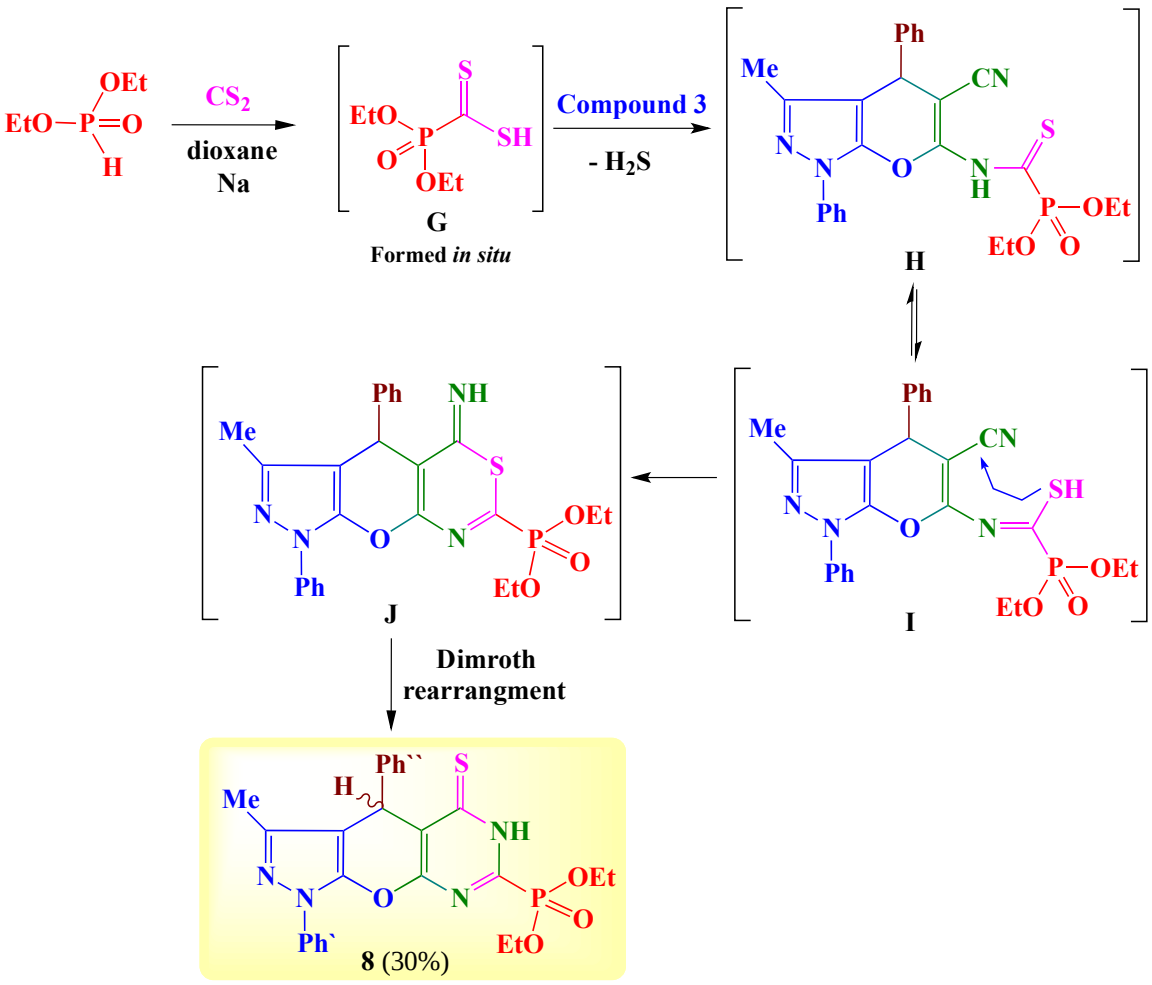
Scheme 4

Interestingly, treatment of compound **3** with diethyl (2-chloroacetyl)phosphonate (**D**) (formed *in situ* from refluxing diethyl phosphite with chloroacetyl chloride in dry dioxane containing a catalytic amount of triethylamine)[26] resulted the corresponding new pyrazolo[4",3":5',6']pyrano[3',2':4,5]pyrrolo[2,3-*d*][1,2]-azaphosphole system **7** in 35% yield (Scheme 5).



Scheme 5

The mass spectrum enhanced the proposed structure and displayed its M^+ peak at *m/z* 460 (20%). Also, the IR spectrum of compound **7** showed broad bands relative to two NH groups at 3446, 3163 cm^{-1} and new absorption band for C=O function at 1690 cm^{-1}. Furthermore, its ^{1}H-NMR and ^{13}C-NMR spectra agreed with the suggested structure (see experimental section). The formation of the isolated product **7** can be based on initial condensation of NH_2 group of compound **3** with the intermediate **D** giving the nonisolable intermediate **E**. Then heterocyclization of this intermediate *via* nucleophilic addition of the active CH_2 group at the electrophilic C≡N group gave the intermediate **F** which underwent another heterocyclization through elimination of ethanol molecule as shown in Scheme 5.



Scheme 6

Finally, addition of carbon disulfide to diethyl H-phosphonate in dry dioxane in the presence of a catalytic amount of sodium metal gave the nonisolable (diethoxyphosphoryl)methanedithioic acid **G**[27] which formed *in situ* and then it condensed with compound **3** to afford the nonisolable intermediate **H** through removal of H_2S molecule. The latter intermediate may be rearranged to the tautomeric form **I** which then underwent heterocyclization *via* a nucleophilic addition of SH group at C≡N group to produce the

nonisolable intermediate **J**. Dimroth rearrangement of this intermediate furnished diethyl {3-methyl-1,4-diphenyl-4-thioxo-1,4,5,6-tetrahydropyrazolo[4',3':5,6]pyrano[2,3-*d*]pyrimidin-7-yl}phosphonate (**8**) in 30% yield (Scheme 6). The structure of product **8** was confirmed by mass spectrum which displayed its M^+ peak at *m/z* 508 (48%), while its ^{31}P-NMR spectrum exhibited a singlet at δ 23.8 ppm. Its ^{1}H-NMR spectrum displayed the existence of the characteristic diethoxy protons as two multiplet signals at δ 1.15−1.31 (2 Me) and 3.87−4.34 (2 OCH_2) ppm. Furthermore, the carbon atom of C=S resonated at δ 183.0 ppm while the carbon atom of C−P resonated as a doublet at δ 151.2 ppm with coupling constant J_{PC}=130.7 Hz in the ^{13}C-NMR spectrum.

BIOLOGICAL ACTIVITIES

Antimicrobial activity

The *in vitro* antibacterial activities of the synthesized products were screened towards three organisms namely, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Escherichia coli*. Moreover, all the products were also screened for their *in vitro* antifungal activity towards three organisms namely, *Aspergillus niger*, *Aspergillus clavatus* and *Candida albicans*.[28,29] Minimum inhibitory concentration (MIC) of all synthesized compounds was determined and given in Table 1. MIC is defined as the lowest concentration of inhibitor at which organism growth was not visually apparent. Ketoconazole and Ciprofloxacin as standard drugs were used for the antifungal and antibacterial activities, respectively. Variable antimicrobial activities towards the used microorganisms were recorded for the synthesized compounds. Compounds **4** and **5** did not record any acceptable inhibitory effects towards all bacteria and fungi organisms with comparison with standard drugs. Moreover, compound **6** exhibited relatively moderate effects against all organisms, while compound **8** exhibited moderate effects against only bacterial strains. However, compound **7** displayed excellent antimicrobial activity towards the bacteria organisms and moderate effects against the fungi organisms when compared to that of the standard drugs. The connection of pyranopyrazole ring with acyclic phosphonate group or cyclic phosphonate group (phosphorus heterocycles) exhibited improvements in the antimicrobial effects in comparison with the starting material **3**. The presence of 1,2-azaphosphole ring as cyclic ethyl phosphonate moiety fused with the pyranopyrazole system in one molecular frame **7** exhibited extremely good acceptable antibacterial and antifungal activities.

Table 1. The *in vitro* antimicrobial activities as minimum inhibitory concentration (MIC, μg/mL) for the synthesized compounds

Compound	Bacterial strains			Fungal strains		
	Streptococcus pyogenes	*Staphylococcus aureus*	*Escherichia coli*	*Aspergillus niger*	*Candida albicans*	*Aspergillus clavatus*
3	250	250	250	250	250	500
4	250	250	125	250	250	250
5	250	250	250	250	250	250
6	125	125	125	125	125	125
7	31.25	31.25	31.25	125	125	125
8	125	125	125	250	250	250
Ciprofloxacin	31.25	31.25	31.25	--	--	--
Ketoconazole	--	--	--	31.25	31.25	31.25

Antioxidant activity

The synthesized compounds were investigated for their *in vitro* antioxidant properties by DPPH and H_2O_2 methods.[30-32] The ascorbic acid was used as standard control. The lower IC_{50} value indicated to a higher antioxidant activity. The promising antioxidative properties were observed for the synthesized compounds (Table 2). The results revealed that compounds **4**, **5** and **8** exhibited poor radical scavenging abilities with comparison with substrate **3** and ascorbic acid. However, the remarkable activities were observed with the products **3** and **6**. Among all the investigated compounds by DPPH and H_2O_2 methods, compound **7** displayed promising antioxidative properties more than ascorbic acid. The presence of cyclic ethyl phosphonate skeleton, two acidic NH groups and extended conjugation in compound **7** caused the better antioxidant activity.

Table 2. The *in vitro* antioxidant activities as inhibitory concentration (IC_{50}, μg/mL) for the synthesized compounds by using DPPH and H_2O_2 methods

Compound	Inhibitory Concentration (IC_{50}, μg/mL)	
	DPPH method	H_2O_2 method
3	16.21 ± 0.29	22.53 ± 0.51
4	36.86 ± 0.41	52.12 ± 0.62
5	38.15 ± 0.61	43.29 ± 0.41
6	18.23 ± 0.51	27.68 ± 0.86
7	10.09 ± 0.22	16.63 ± 0.38
8	46.52 ± 0.63	55.63 ± 0.48
Ascorbic acid	10.23 ± 0.23	18.62 ± 0.52

Antiproliferative activity

The *in vitro* cytotoxic activities of the synthesized compounds were determined by using the SRB assay[33] against tumor cell lines MCF-7, HepG-2 and HCT-116 in comparison with doxorubicin as reference drug. The *in vitro* cytotoxicity evaluation was achieved using different concentrations range of 0.01 to 1000 μg/mL. The results were expressed as growth inhibitory concentration (IC_{50}) values, where the necessitated concentration produced a 50% inhibition of cell growth after 72 h of incubation, compared to the untreated cell control. The IC_{50} values are summarized in Table 3. The relation between the surviving cells with different concentrations of tested compounds were plotted to get the survival curve for each type of cancer cell line after 72 h as depicted in Figure 1. The screened synthesized compounds against tumor cell lines (MCF-7, HepG-2 and HCT-116) showed variable cytotoxic activities (Table 3 and Figure 1). Compounds **3** and **4** showed week effects on HepG-2 and HCT-116 cancer cells with IC_{50} values in range 45.4 ± 2.2 to 120.8 ± 6.6 μg/mL, while they have acceptable cell toxicities profile towards MCF-7 tumor cells with IC_{50} values at 23.6 ± 0.5 and 11.1 ± 1.4 μg/mL, respectively, compared to the reference drug.

In addition, compounds **5**, **6** and **7** have moderate cell toxicities towards HepG-2 and HCT-116 cancer cells with IC_{50} values ranging from 20.5 ± 1.1 to 25.7 ± 0.9 μg/mL except **5** and **7** which have acceptable cytotoxicity towards HCT-116 cancer cells. Moreover, the three compounds have good effects against MCF-7 tumor cells. Interestingly, compound **8** recorded the potent antiproliferative properties towards all tumor cells with IC_{50} values in range 5.3 ± 0.9 to 7.5 ± 0.6 μg/mL. (Table 3 and Figure 1). In general, the SAR study revealed that the new molecular frames exhibited cytotoxicity properties more than the starting substrate **3**. The presence of phosphonate group caused increasing in the anticancer properties especially when it was attached to nitrogen heterocycles as in compounds **6**, **7** and **8**. In addition, the presence of phosphonate group attached to pyrimidine moiety as in compound **8** may be more effective than pyrrole and pyridine as in compounds **6** and **7**.

Table 3. The IC_{50} (μg/mL) of the synthesized compounds against different tumor cell lines

Compound	IC_{50} (μg/mL)		
	MCF-7	**HepG-2**	**HCT-116**
3	23.6 ± 0.5	120.8 ± 6.6	46.8 ± 2.4
4	11.1 ± 1.4	51.04 ± 1.2	45.4 ± 2.2
5	7.9 ± 0.8	25.5 ± 2.9	12.5 ± 0.5
6	16.8 ± 0.9	25.7 ± 0.9	20.5 ± 0.8
7	8.8 ± 1.3	20.5 ± 1.1	16.7 ± 3.3
8	6.9 ± 1.6	5.3 ± 0.9	7.5 ± 0.6
Doxorubicin	1.4 ± 0.07	1.6 ± 0.04	2.0 ± 0.03

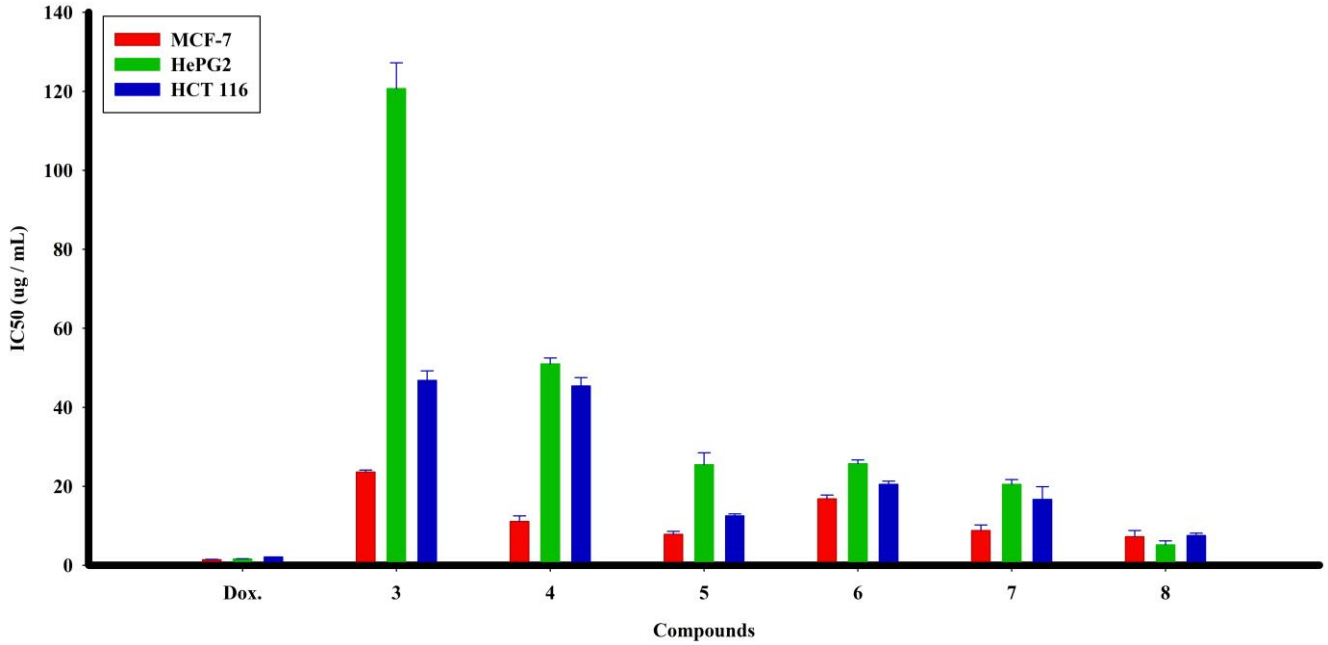


Figure 1. The IC_{50} values for the synthesized compounds against tumor cell lines MCF-7, HepG-2 and HCT-116 in comparison with doxorubicin

Apoptotic effect

The treated tumor cell lines MCF-7, HepG-2 and HCT-116 were stained with acridine orange and ethidium bromide for 48 h. After, their examination under a fluorescent microscope, the cells were appeared in the form of four colors as follows: living cells (normal green nucleus with normal, round, intact nuclei, and cytoplasm that indicates the viability of the cell control), early apoptotic (bright green nucleus with fragmented chromatin), necrotic cells (uniformly bright red- stained cell nuclei) and late apoptotic (red- stained nuclei with chromatin condensation or fragmentation) (Figure 2).[34,35] The highly early apoptotic cell death were observed in all types of treated tumor cells in all compounds. However, compounds **4** and **6** recorded the highest early apoptotic death. The considerable necrotic activities compared with other compounds were displayed in compounds **5**, **7** and **8**, while compounds **3**, **4** and **6** did not cause death of the necrosis pathway in all cancer cells. The late apoptosis was obviously observed with compound **3** especially against HePG-2 cells (Figures 3-5). The obtained results were referenced to the control that absence of any cell death manifestations.

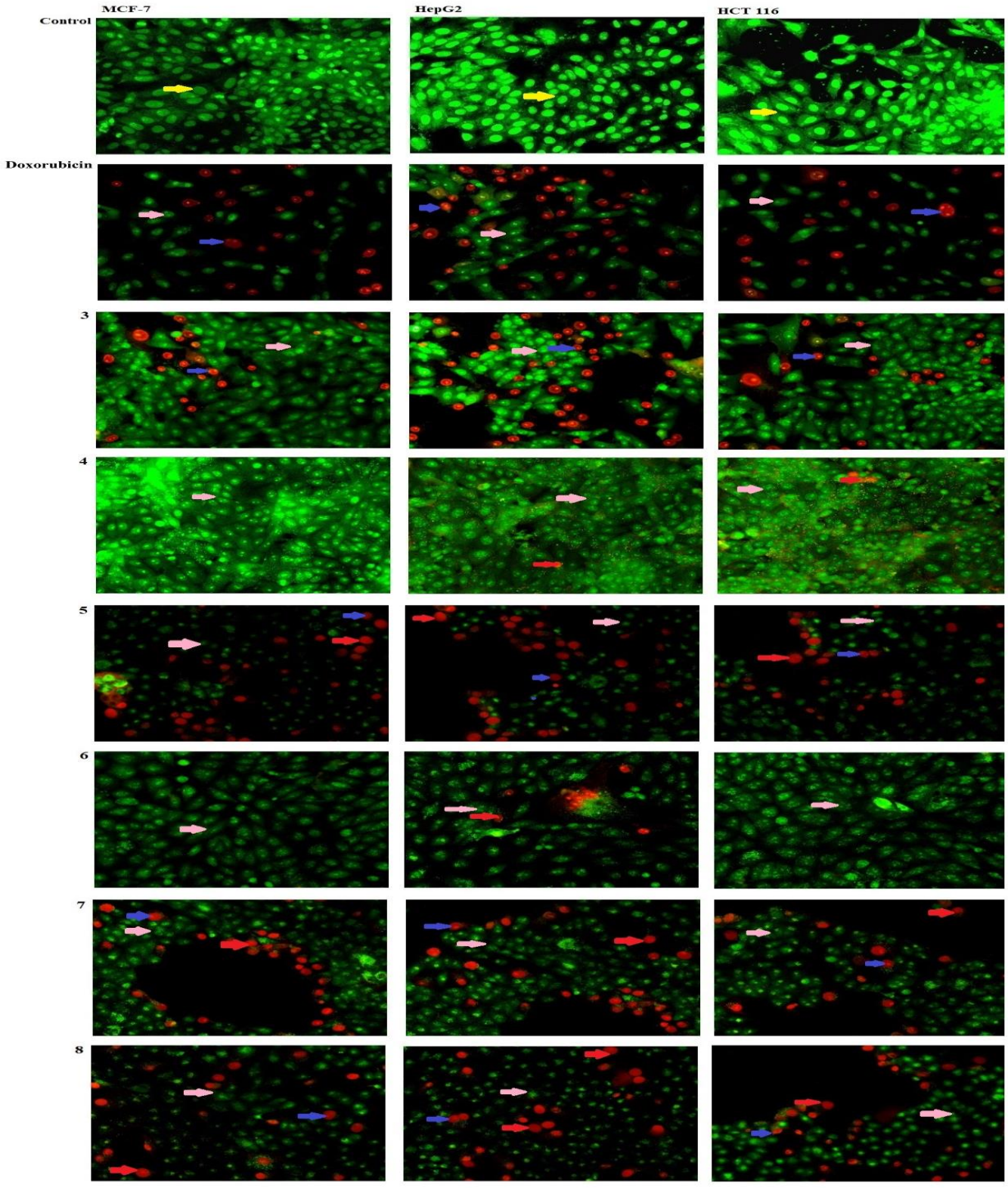


Figure 2. Morphological and nuclear changes using acridine orange (AO) and ethidium bromide (EB) staining that were evaluated by the effect of the synthesized compounds on apoptosis of MCF-7, HepG-2 and HCT-116 human tumor cells after 48 h treatment-induced various nuclear changes such as chromatin fragmented and condensation, nuclei condensation at 200×. Yellow arrows indicate live cell, pink arrows indicate early apoptotic, red arrows indicate necrotic and blue arrows indicate late apoptotic cells.

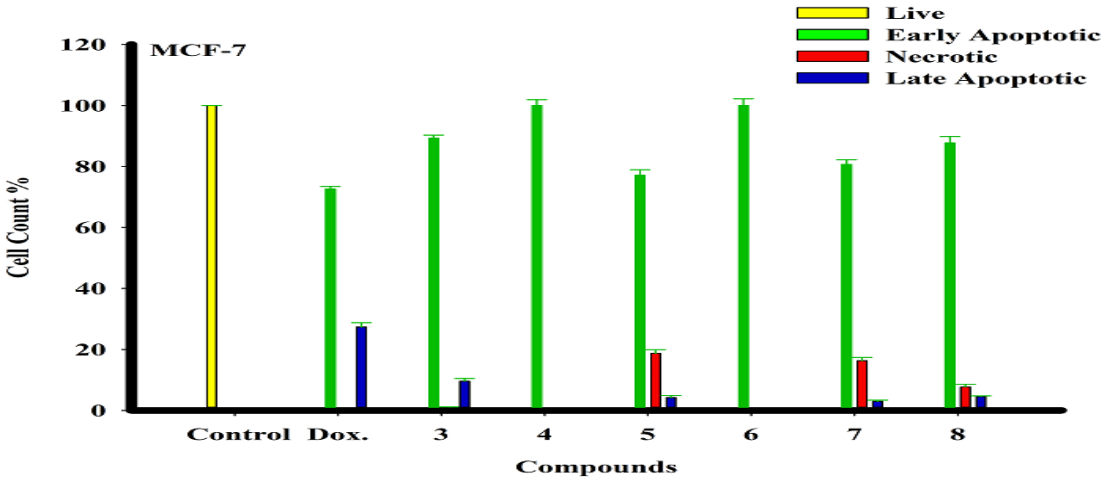


Figure 3. The apoptotic percentage of MCF-7 tumor cells after 48 h treatment with the synthesized compounds

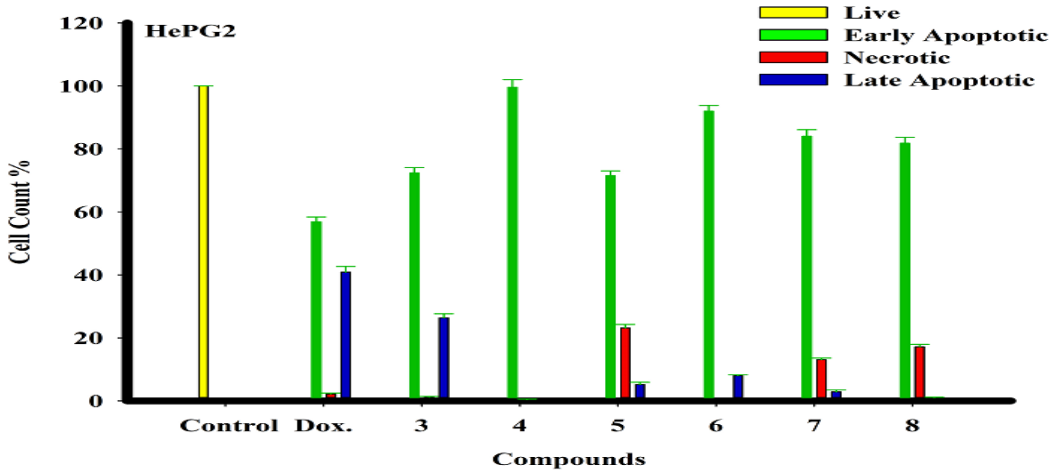


Figure 4. The apoptotic percentage of HePG-2 tumor cells after 48 h treatment with the synthesized compounds

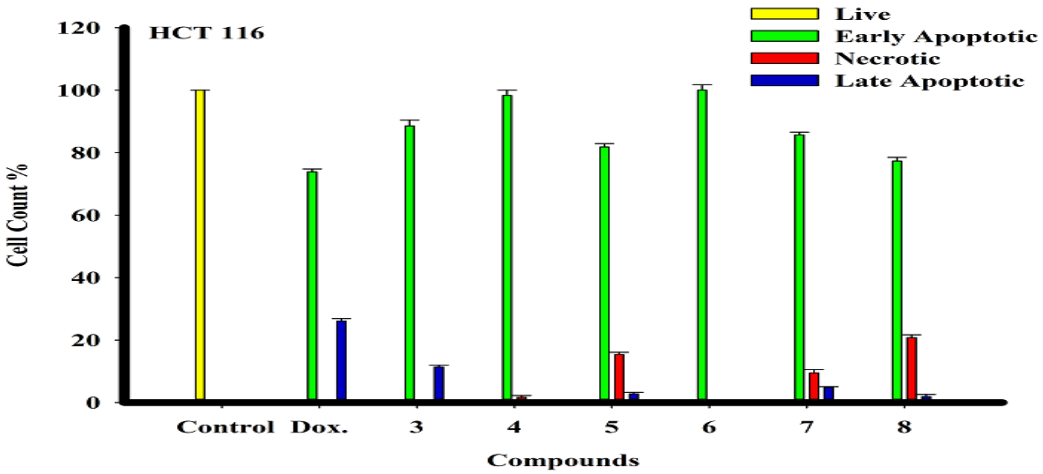


Figure 5. The apoptotic percentage of HCT-116 tumor cells after 48 h treatment with the synthesized compounds

EXPERIMENTAL

The melting points were determined in an open capillary tube on a digital Stuart SMP-3 apparatus. Infrared spectra were measured on FT-IR (Nicolet IS10) spectrophotometer using KBr disks and Perkin-Elmer 293 spectrophotometer using KBr disks. ^{1}H- and ^{13}C-NMR spectra were measured on Gemini-300BB spectrometer (400 and 100 MHz), using DMSO-d_6 as a solvent and TMS (δ) as an internal standard. ^{31}P-NMR spectra were measured on a Bruker (162 MHz) spectrophotometer using DMSO-d_6 as a solvent, TMS as an internal standard and 85% H_3PO_4 as an external reference. Mass spectra were recorded on direct probe controller inlet part to single quadropole mass analyzer in (Thermo Scientific GCMS). Elemental microanalysis was performed on Perkin-Elmer 2400II at the Chemical War department, Ministry of Defense. The purity of the synthesized compounds was checked by thin layer chromatography (TLC) and elemental microanalysis. The biological evaluations were measured in Faculty of Science, King Khalid University, Abha, KSA.

Synthesis of 6-amino-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile (3)

A mixture of 3-methyl-1-phenyl-5-pyrazolone (**1**) (1.74 g, 10 mmol) and 2-benzylidenemalononitrile (**2**) (1.54 g, 10 mmol) in distilled water (40 mL) containing a catalytic amount of sodium benzoate (0.2 g), was stirred for 1 h at 60 ºC. The formed solid was filtered off, washed with water and crystallized from EtOH to give yellow crystalline in 82% yield (2.7 g); mp 179−180 ºC (Lit.[21]169−170 ºC). IR (KBr), (*v* max, cm^{-1}): 3472, 3312 (NH_2), 3062 (C−H_{arom}), 2880 (C−H_{aliph}), 2199 (C≡N), 1660, 1597 (C=C), 1516 (C=N). ^{1}H-NMR (400 MHz, DMSO-d_6): 2.19 (s, 3H, CH_3), 4.66 (s, 1H, H−4), 5.92 (br, 2H, NH_2), 7.18−7.95 (m, 10H, Ph−H).

Synthesis of diethyl {[5-cyano-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazol-6-yl]amino}-phosphoramidate (4)

A mixture of triethyl phosphate (2 mL, 10 mmol) and compound **3** (1.64 g, 5 mmol) was fused on water bath for 10 h. The formed semi-solid was treated with cold water. The precipitated solid was filtered off and crystallized from diluted EtOH to give reddish brown solid in 39% yield (0.87 g); mp 150−152 ºC. IR (KBr), (*v* max, cm^{-1}): 3384 (br, NH), 3060 (C−H_{arom}), 2978, 2930 (C−H_{aliph}), 2209, (C≡N), 1612, 1599 (C=C), 1561 (C=N), 1179 (P=O), 1027 (P−O−C). ^{1}H-NMR (400 MHz, DMSO-d_6): 1.15−1.24 (m, 6H, 2 CH_3), 2.03 (s, 3H, CH_3), 3.88−4.18 (m, 4H, 2 OCH_2), 5.13 (s, 1H, H−4), 5.70 (s, 1H, NH), 7.19−7.73 (m, 10H, Ph−H). ^{13}C-NMR (100 MHz, DMSO-d_6): 11.4 (CH_3), 14.9 (CH_3), 35.1 (C−4), 61.1 (OCH_2), 66.8 (C−5), 110.8 (C≡N), 121.2 (C−3a), 123.2 (C−2',6'$_{phenyl}$), 126.0 (C−4"$_{phenyl}$), 127.6 (C−4'$_{phenyl}$), 127.9 (C−3",5"$_{phenyl}$), 128.4 (C−2",6"$_{phenyl}$), 128.9 (C−3',5'$_{phenyl}$), 135.3 (C−1"$_{phenyl}$), 140.5 (C−1'$_{phenyl}$), 141.5 (C−3), 148.3 (C−7a), 153.9 (C−6). ^{31}P-NMR (162 MHz, DMSO-d_6): 21.6 ppm. MS (*m/z*, I%): 464 (M^+, 18%). Anal. Calcd for $C_{24}H_{25}N_4O_4P$ (464.47): C, 62.06%; H, 5.43%; N, 12.06%. Found: C, 61.86%; H, 5.15%; N, 11.83%.

Synthesis of *N*-(5-cyano-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazol-6-yl)-*N*`-phenyl-phosphenimidous amide (5)

A mixture of hexaethylphosphoramide (1.5 mL, 5 mmol) and compound **3** (1.64 g, 5 mmol) in dry dioxane (30 mL) was heated under reflux for 6 h. Aniline (0.5 mL, 5 mmol) was added to the solution and further heated under reflux for 8 h. The solution was poured on water containing drops of diluted HCl (30%). The formed solid was filtered off, washed with water and crystallized from diluted EtOH to give greyish green solid in 45% yield (1.09 g); mp 149−152 °C. IR (KBr), (*v* max, cm^{-1}): 3423 (br, NH), 3062 (C−H_{arom}), 2981 (C−H_{aliph}), 2193, (C≡N), 1597 (C=C), 1550 (C=N), 1454 (P=N). ^{1}H-NMR (400 MHz, DMSO-d_6): 2.02 (s, 3H, CH_3), 4.95 (s, 1H, H−4), 6.74−7.99 (m, 15H, Ph−H), 8.42 (br, 1H, NH). ^{13}C-NMR (100 MHz, DMSO-d_6): 13.9 (CH_3), 33.1 (C−4), 66.3 (C−5), 96.6 (C≡N), 116.5 (C−3a), 120.4 (C−2',6'$_{phenyl}$), 122.7 (C−4'''$_{phenyl}$), 125.2 (C−3''',5'''$_{phenyl}$), 125.9 (C−4''$_{phenyl}$), 126.3 (C−4'$_{phenyl}$), 127.2 (C−3'',5''$_{phenyl}$), 128.2 (C−2'',6''$_{phenyl}$), 128.6 (C−2''',6'''$_{phenyl}$), 128.8 (C−3',5'$_{phenyl}$), 136.4 (C−1''$_{phenyl}$), 139.2 (C−1'$_{phenyl}$), 142.2 (C−1'''$_{phenyl}$), 144.1 (C−3), 147.3 (C−7a), 158.7 (C−6). MS (*m/z*, I%): 449 (M^+, 20%). Anal. Calcd for $C_{26}H_{20}N_5OP$ (449.46): C, 69.48%; H, 4.49%; N, 15.58%. Found: C, 69.12%; H, 4.21%; N, 15.21%.

Synthesis of diethyl {5-amino-3-methyl-7-oxo-1,4-diphenyl-1,4,7,8-tetrahydropyrazolo[4',3':5,6]-pyrano[2,3-*b*]pyridin-6-yl}phosphonate (6)

***Method A*:** chloroacetyl chloride (0.4 mL, 5 mmol) was added to a solution of compound **3** (1.64 g, 5 mmol) in dry dioxane (30 mL) containing a catalytic amount of triethylamine (0.7 mL, 10 mmol) and further heated under reflux for 30 min. A solution of diethyl phosphite (1.4 mL, 10 mmol) in dry dioxane (10 mL) containing a catalytic amount of sodium metal (0.12 g) was further heated under reflux for 10 h. The solution was concentrated into its half volume then neutralized with drops of diluted HCl (30%). The formed solid was filtered off, washed with water and crystallized with diluted MeOH to give reddish brown solid in 62% yield (1.57 g); mp 100−102 °C.

***Method B*:** a mixture of compound **3** (1.64 g, 5 mmol) and triethyl phosphonoacetate (1.12 mL, 5 mmol) in dry dioxane (30 mL) containing a catalytic amount of triethylamine (0.3 mL, 5 mmol) was heated under reflux for 11 h. The solution was cooled then poured onto water containing drops of diluted HCl (30%). The formed solid was filtered off and crystallized from EtOH to give brown solid in 76% yield (1.92 g); mp 102−104 °C. IR (KBr), (*v* max, cm^{-1}): 3430, 3312 (NH_2), 3262 (NH), 3062 (C−H_{arom}), 2923 (C−H_{aliph}), 1690 (C=O), 1597 (C=C), 1579 (C=N), 1204 (P=O), 1027 (P−O−C). ^{1}H-NMR (400 MHz, DMSO-d_6): 1.16 (t, 3H, *J*=6.8 Hz, CH_3), 1.31 (t, 3H, *J*=6.8 Hz, CH_3), 2.33 (s, 3H, CH_3), 4.02−4.08 (m, 2H, OCH_2), 4.30−4.36 (m, 2H, OCH_2), 4.98 (s, 1H, H−4), 7.18−8.07 (m, 10H, Ph−H), 8.41 (s, 1H, NH), 8.60 (s, 1H, NH), 10.02 (s, 1H, NH). ^{13}C-NMR (100 MHz, DMSO-d_6): 11.5 (CH_3), 13.9 (CH_3), 33.0 (C−4), 62.4 (OCH_2), 118.4 (C−3a), 120.3 (C−4a), 120.7 (C−2',6'$_{phenyl}$), 124.7 (C−4''$_{phenyl}$), 125.9

(C−4'$_{phenyl}$), 127.1 (C−3",5"$_{phenyl}$), 128.1 (C−2",6"$_{phenyl}$), 128.9 (C−3',5'$_{phenyl}$), 130.7 (d, *J*=130.9 Hz, C−6), 133.6 (C−1"$_{phenyl}$), 137.0 (C−5), 142.0 (C−1'$_{phenyl}$), 146.3 (C−3), 148.3 (C−9a), 155.1 (C−8a), 165.4 (C−7). ^{31}P-NMR (162 MHz, DMSO-d_6): 20.0 ppm. MS (*m/z*, I%): 506 (M^+, 25%). Anal. Calcd for $C_{26}H_{27}N_4O_5P$ (506.50): C, 61.66%; H, 5.37%; N, 11.06%. Found: C, 61.43%; H, 5.16%; N, 10.84%.

Synthesis of 2-ethoxy-8-methyl-2-oxido-6,9-diphenyl-1,6,9-trihydropyrazolo[4'',3'':5',6']pyrano-[3',2':4,5]pyrrolo[2,3-*d*][1,2]azaphosphol-3(4*H*)-one (7)

A mixture of diethyl phosphite (0.7 mL, 5 mmol) and chloroacetyl chloride (0.4 mL, 5 mmol) in dry dioxane (30 mL) containing a catalytic amount of triethylamine (0.7 mL, 10 mmol) was heated under reflux for 30 min. A solution of compound **3** (1.64 g, 5 mmol) in dry dioxane (5 mL) was added to the previous mixture and further heated under reflux for 12 h. The solution was concentrated and neutralized with drops of diluted HCl (30%). The formed inorganic salt was removed. The filtrate was treated with MeOH to give pure yellow solid in 35% yield (0.8 g); mp 123−125 °C. IR (KBr), (*v* max, cm^{-1}): 3446 (br, NH), 3163 (br, NH), 3063 (C−H$_{arom}$), 2924 (C−H$_{aliph}$), 1690 (C=O), 1597 (C=C), 1579 (C=N), 1204 (P=O), 1028 (P−O−C). ^{1}H-NMR (400 MHz, DMSO-d_6): 1.31 (t, 3H, *J*=7.2 Hz, CH_3), 2.32 (s, 3H, CH_3), 4.32 (q, 2H, *J*=7.2 Hz, CH_2), 4.96 (s, 1H, H−9), 7.18−8.05 (m, 10H, Ph−H), 8.41 (s, 1H, NH), 10.02 (br, 1H, NH). ^{13}C-NMR (100 MHz, DMSO-d_6): 13.9 (CH_3), 16.1 (CH_3), 33.1 (C−9), 62.4 (OCH_2), 102.6 (C−9a), 118.4 (C−8a), 120.5 (C−2',6'$_{phenyl}$), 125.9 (C−4"$_{phenyl}$), 127.1 (C−3",5"$_{phenyl}$), 127.7 (C−4'$_{phenyl}$), 128.1 (C−2",6"$_{phenyl}$), 128.9 (C−3',5'$_{phenyl}$), 133.6 (C−1"$_{phenyl}$), 138.1 (C−9b), 140.5 (C−1'$_{phenyl}$), 140.9 (C−3a), 142.2 (C−8), 146.3 (C−5a), 161.8 (C−4a), 164.8 (d, *J*=145 Hz, C−3). MS (*m/z*, I%): 460 (M^+, 20%). Anal. Calcd for $C_{24}H_{21}N_4O_4P$ (460.43): C, 62.61%; H, 4.60%; N, 12.17%. Found: C, 62.23%; H, 4.42%; N, 11.80%.

Synthesis of diethyl {3-methyl-1,4-diphenyl-4-thioxo-1,4,5,6-tetrahydropyrazolo[4',3':5,6]pyrano-[2,3-*d*]pyrimidin-7-yl}phosphonate (8)

A mixture of diethyl phosphite (0.7 mL, 5 mmol) and carbon disulfide (0.4 mL, 5 mmol) in dry dioxane (30 mL) containing a catalytic amount of sodium metal (0.12 g) was heated under reflux for 30 min. A solution of compound **3** (1.64 g, 5 mmol) in dry dioxane (5 mL) was added to the solution and further heated under reflux for 10 h. The solution was concentrated, poured onto water and neutralized by drops of diluted HCl (30%). The formed solid was filtered off and crystallized from EtOH to give yellow solid in 30% yield (0.76 g); mp 98−100 °C. IR (KBr), (*v* max, cm^{-1}): 3211 (br, NH), 3068 (C−H$_{arom}$), 2923 (C−H$_{aliph}$), 1650 (C=N$_{pyrimidine}$), 1598 (C=C), 1252 (P=O), 1033 (P−O−C). ^{1}H-NMR (400 MHz, DMSO-d_6): 1.15−1.31 (m, 6H, 2 CH_3), 2.32 (s, 3H, CH_3), 3.87−4.34 (m, 4H, 2 OCH_2), 4.96 (s, 1H, H−4), 7.18−7.97 (m, 10H, Ph−H), 13.98 (br, 1H, NH). ^{13}C-NMR (100 MHz, DMSO-d_6): 13.8 (CH_3), 16.1 (CH_3), 33.2 (C−4), 58.3 (OCH_2), 81.6 (C−4a), 118.4 (C−3a), 120.5 (C−2',6'$_{phenyl}$), 124.6 (C−4"$_{phenyl}$), 125.9 (C−4'$_{phenyl}$), 127.1 (C−3",5"$_{phenyl}$), 128.2 (C−2",6"$_{phenyl}$), 128.9 (C−3',5'$_{phenyl}$), 137.5 (C−1"$_{phenyl}$), 140.5

(C−1'$_{phenyl}$), 142.2 (C−3), 148.4 (C−9a), 151.2 (d, *J*=130.7 Hz, C−7), 159.5 (C−8a), 183.0 (C−5). ^{31}P-NMR (162 MHz, DMSO-d_6): 23.8 ppm. MS (*m/z*, I%): 508 (M^+, 48%). Anal. Calcd for $C_{25}H_{25}N_4O_4PS$ (508.54): C, 59.05%; H, 4.96%; N, 11.02%; S, 6.31%. Found: C, 58.73%; H, 4.63%; N, 10.79%; S, 6.02%.

Evaluation of antimicrobial activity

All the synthesized products were investigated for their *in vitro* antimicrobial activity against bacterial strains namely, *Streptococcus pyogenes, Staphylococcus aureus* and *Escherichia coli* and fungal strains namely, *Aspergillus niger*, *Aspergillus clavatus* and *Candida albicans* by disc diffusion method.[28,29] Ciprofloxacin and Ketoconazole were used as standard drugs for bacteria and fungi, respectively. The inhibitions were recorded by measuring the diameter of the inhibition zone at the end of 24 h for bacteria and 72 h for fungi. Each experiment was repeated twice. Based on the results of zone of inhibition, the minimum inhibitory concentration (MIC) of the synthesized compounds against all bacterial and fungal strains was determined by liquid dilution method. Stock solutions of tested compounds with 500, 250, 125, 62.5, 31.25, 15.62, and 7.84 μg/mL concentrations were prepared with DMSO solvent. The solutions of standard drugs, Ciprofloxacin and Ketoconazole were prepared in the same concentrations. Inoculums of the bacterial and fungal culture were also prepared. To a series of tubes containing 1 mL each of the used compound solution was added with different concentrations and 0.2 mL of the inoculums. Further 3.8 mm^3 of the sterile water was added to each of the test tubes. These tubes were incubated for 24 h at 37 °C and observed for the presence of turbidity. The minimum inhibitory concentration at which no growth was observed was taken as the MIC values (Table 1).

Evaluation of antioxidant activity

DPPH radical scavenging activity

According to the reported method,[30,31] the scavenging activity of the synthesized compounds was performed against DPPH radical. To a medium consisting of different title compounds, 85 μM of DPPH was affixed. Compounds of different concentrations were prepared in distilled EtOH; 1 mL of each compound solution (25, 50, 100, 200, 300 and 400 μmol/L) was taken in different test tubes, and 4 mL of 100 μmol/L EtOH solution of DPPH was added and shaken vigorously. The tubes were then incubated in the dark at rt for 20 min. A DPPH blank was prepared without a compound, and EtOH was used for the baseline correction. Changes (decrease) in the absorbance at 517 nm were measured using an ultraviolet–visible spectrophotometer. The values were articulated in the inhibition of absorbance percentage of DPPH radical with the values of standard without the synthesized compounds (ascorbic acid maximum inhibition was treated 100% of inhibition). The radical scavenging activities were expressed as the inhibition percentage and were calculated using the formula:

% Radical scavenging activity = (*AB*–*AA*)/*AB* x 100, where *AA* indicates the control reaction's

absorbance and *AB* indicates the sample's absorbance. The compound concentration providing 50% inhibition (IC_{50}) was calculated from the graph of percentage against compound concentrations.

Hydrogen peroxide scavenging activity

According to the reported method,[32] the scavenging activity of the synthesized compounds against hydrogen peroxide radical was determined. In PBS (phosphate-buffered saline, pH 7.4), 4 μM solution of hydrogen peroxide was prepared, and its concentration was examined from the absorbance at 230 nm spectrophotometrically using molar absorptivity 81/M/cm. A dissolved solution of 100 μM compounds in 4 mL distilled water was added to H_2O_2–PBS (0.6 mm^3) solution. After 20 min, the absorbance of H_2O_2 at 230 nm was resolved against a blank solution having parent compound with PBS and without H_2O_2. Later, a dissolved solution of ascorbic acid in 4 mL distilled water was added to H_2O_2 solution in PBS (0.6 mm^3). After 10 min, the absorbance was analyzed against a blank solution in the same manner. The hydrogen peroxide scavenging activities were expressed as the inhibition percentage and were calculated using the formula: % H_2O_2 scavenging activity = (*AB*–*AA*)/*AB* x 100, where *AA* indicates the control reaction's absorbance and *AB* indicates the sample's absorbance. The compound concentration providing 50% inhibition (IC_{50}) was calculated from the graph of percentage against compound concentrations.

Evaluation of antiproliferative activity

The cytotoxicity of the new isolated compounds was evaluated against (MCF-7, HepG-2, and HCT- 116) human cancer cells using sulphorhodamine B assay (SRB). This assay was performed as previously published.[33]

Acridine orange/ethidium bromide staining for detection of apoptosis

Acridine orange and ethidium bromide are DNA binding dyes. They have been used for detection of the morphological features of apoptotic and necrotic cells. The staining were performed according to the protocol as previously published.[34,35]

Statistical analysis

Data were presented as mean standard deviation unless otherwise indicated. Significance of the statistical analysis was acceptable to a level of $P < 0.05$. All analyses and graphs were achieved by using GraphPad Prism software, version 6.00 (GraphPad Software, La Jolla, CA, USA).

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