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DESIGN AND SYNTHESIS OF NOVEL PYRAZOLO[3,4-*d*]PYRIMIDINES: *IN VITRO* CYTOTOXIC EVALUATION AND FREE RADICAL SCAVENGING ACTIVITY STUDIES

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With evident biological importance, a new series of pyrazolo[3,4-*d*]pyrimidines **3a,3b** and **4a,4b** were synthesized via the formation of pyrazol-3-one **2a** and **2b** substrates. All compounds were evaluated for *in vitro* cytotoxic activity against MCF-7 (breast adenocarcinoma) and A549 (lung cancer) cell lines. The obtained results showed that pyrazolo[3,4-*d*] pyrimidin-4-ol **3a** bearing phenyl group at *N*-1 and *p*-C_6H_4 at *C*-6, and **4b** with dinitrophenyl at *N*-1 and furanyl moiety at *C*-6 had better inhibitory activity against MCF-7 with IC_{50} values in a micromolar range as compared to other substrates. The synthesized compounds can be considered as new candidates for further optimization as anticancer agents.

Keywords: pyrazolo[3,4-*d*]pyrimidines; pharmacophore; cytotoxicity; anticancer; radical scavenging activity.

1. INTRODUCTION

Cancer is the second leading cause of death, and by 2020 its trend is expected to attribute to 15 million death cases per year [1]. Although there have been great developments in the discovery and treatment of cancer, it remains a challenging health concern [2]. Current treatments available for cancer, including surgery, chemotherapy, and radiotherapy [3] are associated with severe side effects. This situation demands for a search of new molecules to improve the selectivity, pharmacokinetic profiles, and *in vivo* efficacy of anticancer treatment [2, 4, 5]. In this context, fused pyrimidine scaffolds gained considerable interests of both research and practical pharmacy as attractive therapeutic targets for many diseases particularly cancer [1, 6, 7]. Pyrazolo[3,4-*d*] pyrimidine, an isostere of purines, is known as a versatile drug-like fragment that has drawn much attention as pharmacophore [8 – 11]. These structures exhibit promising pharmacological properties including anti-proliferative [12] and antitumor activity, highlighting their immense contribution for developing target-specific cancer chemotherapeutics [13 – 15]. Previous findings outlined some potent pyrazolo[3,4-*d*]pyrimidine structures that reduced cell division and/or induced apoptosis in tumor [16] and gastric cancer [17].

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The synthesis and anticancer activity studies of pyrazolo[3,4-*d*]pyrimidine structures inhibiting different key enzymes was described in several reports [18 – 20]. Some examples of pyrazolo[3,4-*d*]pyrimidine derivatives (**A – D)** with potential anticancer activity related to the inhibition of various protein kinases are shown in Fig. 1 [19, 21, 22]. Pyrazolo[3,4-*d*]pyrimidine derivatives **A** and **B** were synthesized bearing chained substrates at various positions and biological evaluation against cyclic dependent kinases (CDKs) were envisaged and reported. Other pyrazolo[3,4-*d*]pyrimidine series **C** and **D** endowed with many functionalities at the fused ring system were evaluated against Ehrlich ascites carcinoma (EAC) cell line and showed mild anticancer activity compared to doxorubicin.

Motivated by these findings and in continuation of our previous studies for identification of new anticancer molecules, the present investigation was directed toward the design of more active anticancer compounds derived from the class of pyrazolo[3,4-*d*]pyrimidines. The synthetic approach was aimed to incorporate various substituents at N-1 and C-6 positions of pyrazolo[3,4-*d*]pyrimidine ring (Fig. 2). The cytotoxic activity of the synthesized pyrazolopyrimidine derivatives was evaluated in two cancer cell lines: MCF-7 breast adenocarcinoma cells and A549 lung cancer cells. The cytotoxic activity results were supported by colony formation inhibition assay.

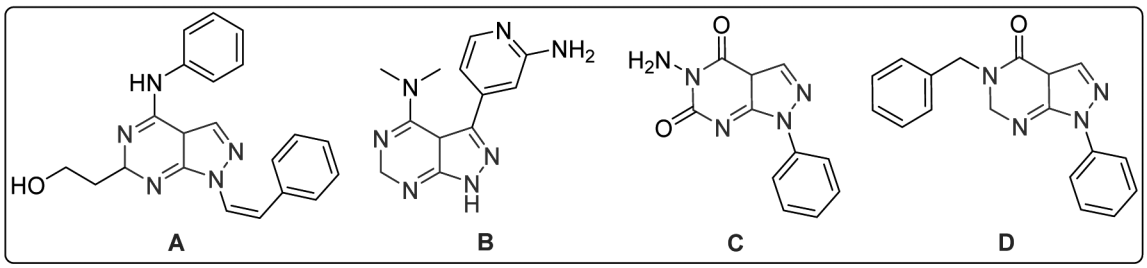


Fig. 1. Chemical structures of pyrazolo[3,4-*d*]pyrimidine derivatives **A – D** as anticancer agents: (**A**) IC_{50} against CDK2, 0.5 μM; [19]**;** (**B**) IC_{50} against CDK9, 17 nM) [21]; (**C**) IC_{50} against EAC, 100 mg/ mL**;** (**D**) IC_{50} against EAC, 90 mg/ mL [22].

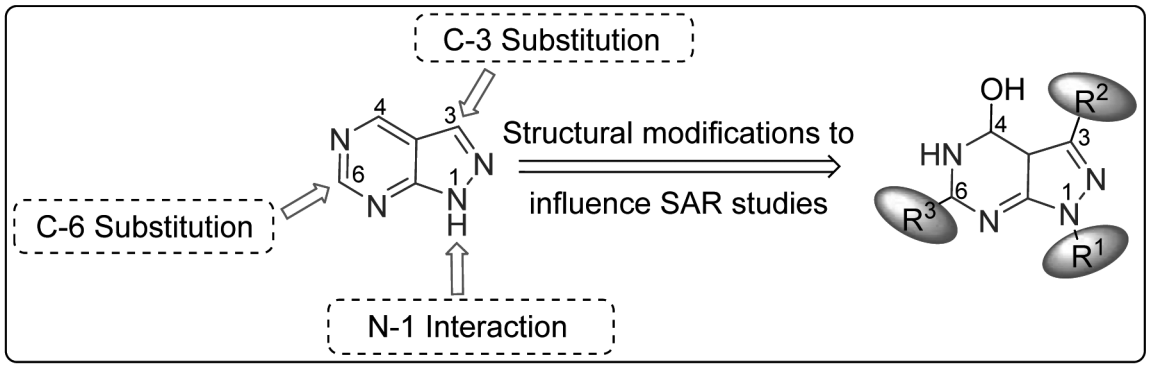


Fig. 2. Structural modifications of pyrazolo[3,4-*d*]pyrimidine scaffold.

2. EXPERIMENTAL

2.1. Materials and methods

Starting materials were obtained from commercial suppliers and used without further purification unless otherwise stated. Melting points were determined with a Kofler block apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out on Merck silica gel pre-coated sheets SIL G/UV254. Patterns were visualized by exposure to UV light (254 nm) followed by staining with basic potassium permanganate and/or vanillin as appropriate. Infrared spectra were recorded on a Perkin-Elmer 1720 FTIR spectrometer, using KBr discs. Elemental analyses were performed on a microanalytical data center at the Faculty of Science, Cairo University, Egypt. NMR spectra were obtained as dilute solutions in the appropriate solvent at 25°C unless otherwise stated. The ^{1}H NMR and ^{13}C spectra were recorded on Brucker DPX 400 MHz spectrometer. The chemical shifts were determined on the δ-scale using residual solvent as an internal standard $(CD_3)_2SO$ (δH, 2.50; δC, 39.43). All coupling constants are reported in Hertz units (Hz) and multiplicities denoted conventionally: s (singlet), d (doublet), and m (multiplet), with prefixes br (broad) and app (apparent). Assignments were made based on the chemical shift and, where appropriate, with the benefit of DEPT sequences. The GC-MS spectra were recorded on Agilent 7000 Triple Quad series gas chromatograph interfaced to a mass spectrometer.

2.2. Chemical Synthesis

General procedure for preparation of pyrazolone derivatives 2a and 2b. Phenyl hydrazine derivatives **1a** and **1b** (0.01 mol) were added to ethyl acetoacetate (0.01 mol) in ethanol (30 mL). The reaction mixture was heated at reflux for 18 h and then cooled, filtered, and recrystallized from ethanol to give target compounds **2a** and **2b.**

3-Methyl-1-phenyl-pyrazol-5-one (2a). Brown powder (90%); m.p. 98 – 100 (C; R_f = 0.72 (10% MeOH in $CHCl_3$); IR spectrum (KBr), ν/cm^{-1}: 3092 (CH aromatic), 1645 (C=O), 1460 and 1375; ^{1}H NMR (400 MH_Z, $(CD_3)_2SO$) 7.72 (2H, d, *J* 2.5, ArC*H*), 7.48 – 7.42 (1H, m, ArC*H*), 7.37 – 7.22 (2H, m, ArC*H*), 3.07 (2H, s, CH_2, pyrazolone) and 1.94 (3H, s, CH_3, pyrazolone); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) 174.5 (C, *C*=O pyrazolone), 162.8 (C, pyrazolone), 138.9 (C, Ar*C*H), 128.9 (*C*H), 128.0 (*C*H), 127.4 (*C*H), 124.6 (*C*H), 123.9 (*C*H) (5*C*, Ar*C*H), 42.5 (*C*H_2, pyrazolone) and 16.7 (*C*H_3, pyrazolone); Anal. Calcd for $C_{10}H_{10}N_2O$: C, 68.95; H, 5.79; N, 16.08. Found C, 69.12; H, 5.54; N, 15.75; HRMS *m/z* (ES$^+$): Found 174.08 (M^+, $C_{10}H_{10}N_2O$ requires 174.08).

1-(2,4-Dinitrophenyl)-3-methyl-pyrazol-5-one (2b). Brown powder (86%); m.p. 88 – 90 (C; R_f = 0.72 (10% MeOH in $CHCl_3$); IR spectrum (KBr), ν/cm^{-1}: 3056 (CH aromatic), 1640 (C=O), 1511, 1336 (NO_2), 1450 and 1375; ^{1}H NMR (400 MH_Z, $(CD_3)_2SO$) 7.90 (1H, d, *J* 2.0, ArC*H*), 7.84

(1H, d, *J* 2.0, ArC*H*), 7.46 (1H, s, ArC*H*), 3.07 (2H, s, CH_2 pyrazolone) and 1.94 (3H, s, CH_3 pyrazolone); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) 174.5 (C, *C*=O pyrazolone), 162.8 (C, pyrazolone), 138.9 (C, Ar*C*H), 128.9 (CH), 128.0 (CH), 124.6 (CH) (3*C*, Ar*C*H), 42.5 (*C*H_2, pyrazolone) and 16.7 (*C*H_3, pyrazolone); Anal. Calcd for $C_{10}H_8N_4O_5$: C, 45.46; H, 3.05; N, 21.21. Found C, 45.87; H, 2.59; N, 22.06; HRMS *m/z* (ES^+): Found 264.05 (M^+, $C_{10}H_8N_4O_5$ requires 264.05).

General procedure for preparation of pyrazolopyrimidine derivatives 3a,3b and 4a,4b.

Pyrazolones **2a** and **2b** (0.01 mol) were dissolved in absolute ethanol (40 mL), corresponding aldehydes (0.01 mol) were added to the reaction mixture followed by urea (0.01 mol). The reaction mixture was heated at reflux for 18 h. The reaction mixture was cooled in refrigerator overnight, filtered, dried and recrystallized from ethanol to afford compounds **3a,3b** and **4a,4b**.

6-(4-Chlorophenyl)-3-methyl-1-phenyl-tetrahydro-1H-pyrazolo[3,4-*d*]pyrimidin-4-ol (3a).

Red powder (73%); m.p. 112 – 114 (C; R_f = 0.45 (7% MeOH in $CHCl_3$); IR spectrum (KBr), ν/cm^{-1}: 3421 (OH), 3283 (NH), 3085 (CH aromatic) and 1367; ^{1}H NMR (400 MH_Z, $(CD_3)_2SO$) 8.23 (1H, s, C*H,* pyrimidine), 7.91 (2H, d, *J* 2.0, ArC*H*), 7.77 (2H, s, ArC*H*), 7.45 (2H, app t, *J* 2.0, ArC*H*), 7.24 – 7.18 (2H, m, ArC*H*), 7.32 (1H, s, ArC*H*), 5.21 (1H, *br* s, O*H*), 4.70 (1H, s, C*H*OH), 1.94 (3H, s, CH_3) 1.88 (1H, s, N*H*) and 1.50 (1H, s, C*H,* pyrazolopyrimidine); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) 166.0 (*C*, pyrazolopyrimidine), 155.6 (*C*, pyrazolopyrimidine), 146.2 (C), 140.2 (C), 132.6 (C), (3*C*, Ar*C*H), 129.5 (CH), 129.3 (CH), 128.6 (CH), 128.0 (CH) 127.7 (CH), 126.9 (CH), 126.0 (CH), 122.4 (CH), 119.3 (CH) (9*C*H, Ar*C*H), 72.9 (*C*H, pyrimidinol), 71.7 (*C*HOH), 44.9 (*C*H$_2$ pyrazole) and 15.3 (*C*H$_3$, pyrazole); Anal. Calcd for $C_{18}H_{17}ClN_4O$: C, 63.44; H, 5.03; N, 16.44. Found C, 63.12; H, 4.85; N, 16.93; HRMS *m/z* (ES^+): Found 340.09 (M^+, $C_{18}H_{17}ClN_4O$ requires 340.09).

6-(4-Chlorophenyl)-1-(2,4-dinitrophenyl)-3-methyl-tetrahydro-1H-pyrazolo[3,4-*d*]pyrimidin-4-ol (3b). Yellow powder (65%); m.p. 78 – 80 (C; R_f = 0.45 (5% MeOH in $CHCl_3$); IR spectrum (KBr), ν/cm^{-1}: 3432 (OH), 3309 (NH), 3102 (CH aromatic), 1512, 1337 (NO_2), 1420; ^{1}H NMR (400 MH_Z, $(CD_3)_2SO$) 8.88 (1H, s, C*H,* pyrimidine), 8.42 (2H, d, *J* 2.0, ArC*H*), 8.38 (2H, d, *J* 2.0, ArC*H*), 7.85 (2H, d, *J* 2.0, ArC*H*), 4.20 (1H, *br* s, O*H*), 4.70 (1H, s, C*H*OH), 1.94 (3H, s, CH_3) 1.89 (1H, s, N*H*) and 1.50 (1H, s, C*H,* pyrazolopyrimidine); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) 166.0 (*C*, pyrazolopyrimidine), 147.9 (*C*, pyrazolopyrimidine), 140.2 (C), 138.8 (C), 132.6 (C), 132.4 (C), 132.2 (C), (5*C*, Ar*C*H), 130.8 (CH), 129.3 (CH), 128.6 (CH), 128.3 (CH), 128.0 (CH), 120.8 (CH), 118.1 (CH), (7*C*H, Ar*C*H), 72.9 (*C*H, pyrimidinol), 71.7 (*C*HOH), 44.9 (*C*H$_2$ pyrazole) and 15.3 (*C*H$_3$, pyrazole); Anal. Calcd for $C_{18}H_{15}ClN_6O_5$: Calcd: C, 50.18; H, 3.51; N, 19.51. Found C, 51.02; H, 3.65; N, 19.68; HRMS *m/z* (ES^+): Found 430.05 (M^+, $C_{18}H_{14}N_6O_6$ requires 430.05).

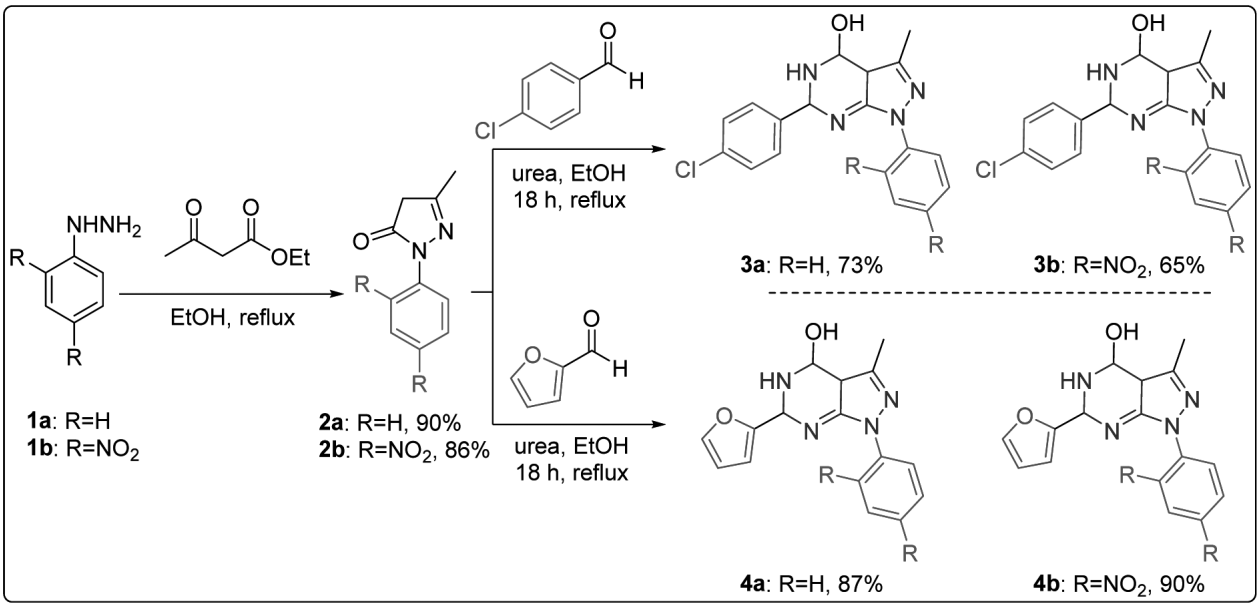
6-(Furan-2-yl)-3-methyl-1-phenyl-tetrahydro-1H-pyrazolo[3,4-*d*]pyrimidin-4-ol (4a). Red crystals (87%); m.p. 136 – 138 (C; R_f = 0.45 (7% MeOH in $CHCl_3$); IR spectrum (KBr), ν/cm^{-1}: 3437 (OH), 3250 (NH), 3095 (CH aromatic), 1375; ^{1}H NMR (400 MH_Z, $(CD_3)_2SO$) 8.86 (1H, s, C*H,* pyrimidine), 7.90 (2H, d, *J* 2.0, ArC*H*), 7.56 (1H, s, ArC*H*), 7.46 (2H, d, *J* 2.0, ArC*H*), 7.32 (1H, s, ArC*H*), 6.39 (1H, s, ArC*H*), 6.29 (1H, s, ArC*H*), 5.31 (1H, *br* s, O*H*), 4.70 (1H, s, C*H*OH), 1.94 (3H, s, CH_3) 1.91 (1H, s, N*H*) and 1.50 (1H, s, C*H,* pyrazolopyrimidine); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) 166.0 (C), 155.6 (C), 152.6 (C), 146.2 (C), (4*C*, Ar*C*H), 142.1 (CH), 129.5 (CH), 128.5 (CH), 128.3 (CH), 128.0 (CH), 122.4 (CH), 110.6 (CH), 106.7 (CH) (8*C*H, Ar*C*H), 74.1 (*C*H, pyrimidinol), 69.3 (*C*HOH), 44.3 (*C*H$_2$ pyrazole) and 15.3 (*C*H$_3$, pyrazole); Anal. Calcd for $C_{16}H_{16}N_4O_2$: Calcd: C, 64.85; H, 5.44; N, 18.91. Found C, 65.10; H, 5.73; N, 16.53; HRMS *m/z* (ES^+): Found 296.09 (M^+, $C_{16}H_{16}N_4O_2$ requires 296.09).

1-(2,4-Dinitrophenyl)-6-(furan-2-yl)-3-methyl-tetrahydro-*1H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (4b). Yellow powder (90%); m.p. 102 – 104(C; R_f = 0.45 (5% MeOH in $CHCl_3$); IR spectrum (KBr), ν/cm^{-1}: 3434 (OH), 3311 (NH), 3101 (CH aromatic), 1512, 1338 (NO_2), 1425; ^{1}H NMR (400 MH_Z, $(CD_3)_2SO$) 8.99 (1H, s, C*H,* pyrimidine), 8.85 (3H, s, ArC*H*), 7.88 – 7.80 (3H, m, ArC*H*), 4.31 (1H, *br* s, O*H*), 4.70 (1H, s, C*H*OH), 1.94 (3H, s, CH_3) 1.91 (1H, s, N*H*) and 1.50 (1H, s, C*H,* pyrazolopyrimidine); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) 166.0 (C), 147.9 (C), 140.2 (C), 140.0 (C), 138.8 (C), 132.6 (C) (6*C*, Ar*C*H), 130.8 (CH), 129.3 (CH), 128.6 (CH), 128.4 (CH), 120.8 (CH), 118.1 (CH) (6*C*H, Ar*C*H), 72.9 (*C*H, pyrimidinol), 71.7 (*C*HOH), 44.9 (*C*H$_2$ pyrazole) and 15.3 (*C*H$_3$, pyrazole); Anal. Calcd for $C_{16}H_{14}N_6O_6$: Calcd: C, 49.74; H, 3.65; N, 21.75. Found C, 50.17; H, 3.42; N, 21.38; HRMS *m/z* (ES^+): Found 386.07 (M^+, $C_{16}H_{14}N_6O_6$ requires 386.07).

2.3. Cell Culture

MCF-7 breast cancer cells and A549 lung cancer cells were purchased from ATCC (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37°C under 5% CO_2. The culture medium was replaced with fresh medium every two days. After reaching 80 – 90% confluence, cells were treated with 0.25% trypsin-EDTA for further passages. In all the experiments, ells were used at passages 4 – 6.

Colony formation inhibition assay. Human cancer cells lines (MCF-7 and A549) at the exponential phase were plated into 24-well culture plates at a single cell density (200 cells/well) and allowed to adhere for 24 h before treatment. Cells were incubated with culture medium containing different concentrations (10, 50, 100 and 200 mg/ mL) of synthesized derivatives. After treatment with extracts for 24 h, the medium was removed and cells were washed with 1X PBS (pH 7.4), fixed with 4% paraformaldehyde, and



Scheme 1. Synthesis of pyrazol-3-ones **2a,2b** and phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol derivatives **3a,3b** and **4a,4b**.

stained with neutral red solutions. Plates were photographed using a Leica DFC420C camera connected to Leica DMI300 B microscope.

3. RESULTS AND DISCUSSION

3.1. Chemistry

The synthesis of phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol derivatives **3a,3b** and **4a,4b** started with the condensation of phenyl hydrazines **1a** and **1b** with ethyl acetoacetate as β-keto ester, resulting in phenyl-3H-pyrazol-3-ones **2a** and **2b** in very good yields 90% and 86%, respectively (Scheme 1). The IR spectra of compounds **2a** and **2b** confirmed the presence of carbonyl group (C=O) at 1645 and 1640 cm^{-1}, and nitro group (NO_2) in compound **2b** at 1511 and 1336 cm^{-1}. Subsequent multicomponent condensation of pyrazol-3-ones **2a** and **2b** with aromatic aldehydes and urea furnished the desired corresponding pyrazolo[3,4-*d*]pyrimidin-4-ol derivatives **3a,3b** and **4a,4b** in good yields, as shown in Scheme 1. The IR spectra indicated the formation of pyrazolo[3,4-*d*]pyrimidin-4-ol derivatives **3a,3b** and **4a,4b** by new peaks corresponding to (-OH) and (-NH) groups.

The ^{1}H and ^{13}C NMR spectra of the synthesized products supported the proposed structures **3a,3b** and **4a,4b**. In the ^{1}H-NMR spectra, the characteristic signals were identified for OH group (at 5.21 and 5.31 ppm of compounds **3a** and **3b**, and at 4.20 and 4.31 ppm of compounds **4a** and **4b,** respectively); same was observed for NH protons (at 1.88 and 1.91 ppm of substrates **3a** and **3b**, and at 1.89 and 1.91 ppm of compounds **4a** and **4b**, respectively).

TABLE 1. Cytotoxic Effect of Pyrazolones **2a**,2**b** and pyrazolopyrimidines **3a,3b** and **4a,4b** against Human Breast Cancer (MCF-7) and Lung Cancer (A549) Cell Lines

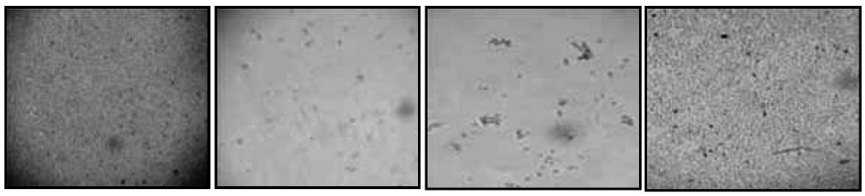
Compound	IC_{50} ± SD (mM)		Log *P**
	MCF-7	A549	
2a	32 ± 3.1	121 ± 2.9	1.282
2b	1257 ± 2.9	233 ± 2.1	1.289
3a	74 ± 4.3	11.5 ± 2.1	1.987
3b	571.5 ± 2.1	460 ± 1.8	2.063
4a	-	179 ± 1.5	0.863
4b	291 ± 5.3	150 ± 2.6	0.969
Doxorubicin	35.2 ± 4.2	9.80 ± 8.7	-

* Log P calculated with MedChem Designer.

3.2. In Vitro Cytotoxic Activity

The synthesized pyrazol-3-ones **2a,2b** and pyrazolopyrimidine derivatives **3a,3b** and **4a,4b** were evaluated for their cytotoxicity against human breast cancer cell line (MCF-7) and lung cancer cell line (A549) using neutral red uptake assay methods. This methodology offers a measurable evaluation of the number of viable cells in any culture and is commonly used in cytotoxicity tests. This test is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. This experiment could allow further direct comparisons between target molecule bearing functionalities at the fused ring system and frag-

(a) MCF-7; positive control, compounds **2a**, **3a** ,**4b**



(b) A549; positive control, compounds **2a, 3a** ,**4b**

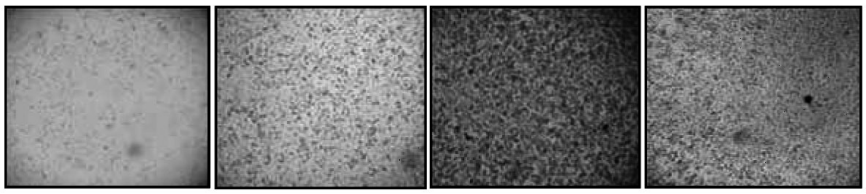


Fig. 3. Colony formation inhibition assay for (a) MCF-7 and (b) A549 cancer cells lines. Cells were treated with pyrazol-3-one **2a** or pyrazolopyrimidine derivatives **3a** and **4b** at final concentration of 200 μg/mL for 24 h. Then, medium was removed, and cells were incubated in fresh medium for 24 h. The viable cells formed colonies, which were stained with neutral red solution.

ment before chemical modification. The results obtained for pyrazol-3-ones **2a**,**2b** and pyrazolopyrimidine derivatives **3a,3b** and **4a,4b** were compared with doxorubicin (standard anticancer agent) used as positive control. The results, as summarized in Table 1 for all examined derivatives, showed variable effects ranging from high to low activity against the MCF-7 and A549 cells tested.

For MCF-7 cells, pyrazol-3-one **2a** and pyrazolopyrimidine derivatives **3a** and **4b** exhibited high to moderate cytotoxic activity. Pyrazol-3-one **2a** showed the highest growth inhibitory activity against MCF-7 cell line with (IC_{50} = 32 ± 3.1 μg/mL) better than that for doxorubicin (IC_{50} = 35.2 ± 4.2 μg/mL), followed by compound **3a** (IC_{50} = 74 ± 4.3 μg/mL) and finally **4b** (IC_{50} = 291 ± 5.3 μg/mL). The remaining compounds including **2b** (IC_{50} = 1257 ± 2.9 μg/mL) and **3b** (IC_{50} = 571.5 ± 2.1 μg/mL) had low growth inhibitory activity compared to the positive control. The structural modifications at *N*-1 and *C*-6 of pyrazol-3-ones **2a,2b** and pyrazolopyrimidine derivatives **3a,3b** and **4a,4b** may play a role for different cytotoxic activities (Fig. 1; Table 1). Probably, the presence of 4-*Cl*-C_6H_4 electron withdrawing group at *C*-6 of **3a**, and 2,4-NO_2 C_6H_3 at *N*-1 together with the furanyl ring at *C*-6 of **4b**, were slightly more active than other structures.

On the other hand, the growth inhibitory activity against the A549 cancer cell line was relatively low for all tested compounds in the following order: **3a** (IC_{50} = 11.5 ± 2.1 μg/mL), **2a** (IC_{50} = 121 ± 2.9 μg/mL), and finally **4b** (IC_{50} = 150 ± 2.6 μg/mL). The IC_{50} of doxorubicin against A549 (9.80 ± 8.7 μg/mL), which is rather low compared to new compounds tested in this study. Distinctive morphological features of cells, including detachment and shrinkage, showed that the number of viable MCF-7 and A549 cells was remarkably reduced by treatment with derivatives **2a** and **3a** as compared to untreated cells and the positive control, as shown in Fig. 3.

The aforementioned findings promote further studies toward the optimum design of pyrazolo[3,4-*d*]pyrimidines as potent anticancer compounds.

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CONFLICT OF INTEREST

Author declares no conflicts of interest**.**

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