Synthesis, Anti-Microbial, and Cytotoxic Activities Evaluation of Some New Pyrido[2,3-*d*]Pyrimidines

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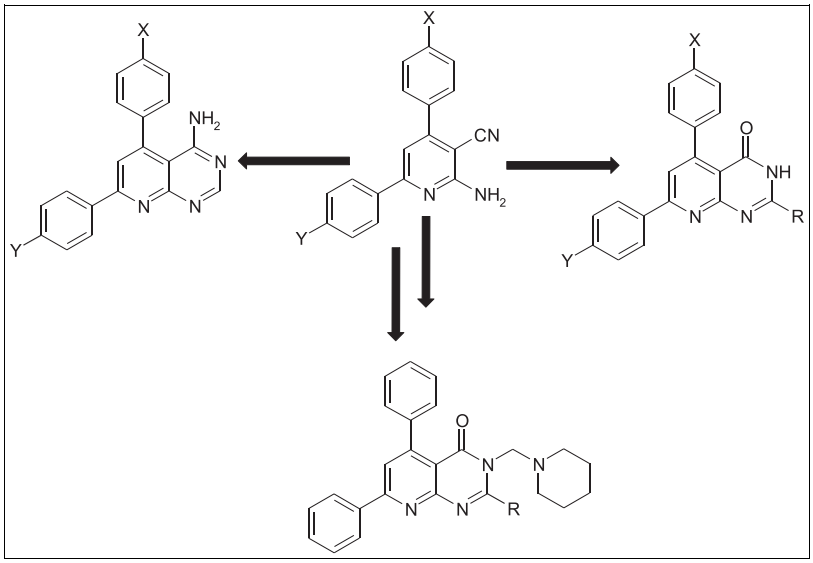


Novel pyridine derivatives **1d** and **7**, pyrido[2,3-*d*]pyrimidine derivatives **2a–d**, **3a–d**, **4a–d**, **5a–d**, **6a–d**, **8a**,**b**, and **9a**,**b** were synthesized and screened for their anti-microbial and cytotoxic activities. Compounds **2b**, **2c**, **3b**, **3c**, **5b**, **5c**, **6b**, **6c**, and **7** showed excellent anti-microbial activities against all the tested bacteria and fungi compared to the reference drugs. Furthermore, they exhibited high safety profile in cytotoxicity test except **2c** and **3c**. The structures of the newly synthesized compounds were confirmed by spectral data and elemental analysis.

INTRODUCTION

For small organic molecules, simple nitrogen-containing heterocycles receive a large amount of attention in the literature; of these heterocycles, the synthesis, reactions, and biological activities of pyridine-containing molecules stand as ever expanding areas of research in hetero aromatic chemistry. Cyano pyridines are well known for their versatile biological activities as anti-tumor [1], protein kinase inhibitors [2], anxietic [3], antimicrobial [4], and antitubercular [4]. Pyrimidine moiety is an important class of *N*-containing heterocyclics, which are widely used as key building blocks for pharmaceutical agents. Pyrimidine moiety exhibits a wide pharmacophores as it acts as antimicrobial [5] and antioxidant [6]. Pyrido[2,3-*d*]pyrimidine heterocycles have received much less attention in the literature, in spite of their structural relationship to pyridines.

Interest in pyrido[2,3-*d*]pyrimidine derivatives has increased dramatically in recent years, based upon a diverse range of biological properties as anti-tumor [7–10], antibacterial [11–18], antifungal [18,19], anti-proliferative CDK2 inhibitor [20], adenosine kinase inhibitors [21], anticonvulsant agents [22], antipyretic [23], analgesic [24], and CNS depressant activity [25]. More specifically pyrido[2,3-*d*]pyrimidines are known to inhibit Pneumocystis carinii(pc) and Toxoplasma gondii(tg) of tumor cell lines in culture [26], and the activity is attributed to inhibition of dihydrofolate reductase (DHFR) [27,28], mTOR kinase inhibitors [29], and cytotoxic agents [30,31]. The synthesis of pyrido[2,3-*d*]pyrimidines is mainly by two methods (annulation of pyrimidine ring over pyridine or vice versa) [32].

Aim of objectives. The therapeutic importance of this scaffolds enthused us to develop selective procedures for the synthesis of novel pyridines and pyrido[2,3-*d*] pyrimidines starting from the corresponding chalcone. These new compounds were tested for their antimicrobial and cytotoxic activities.

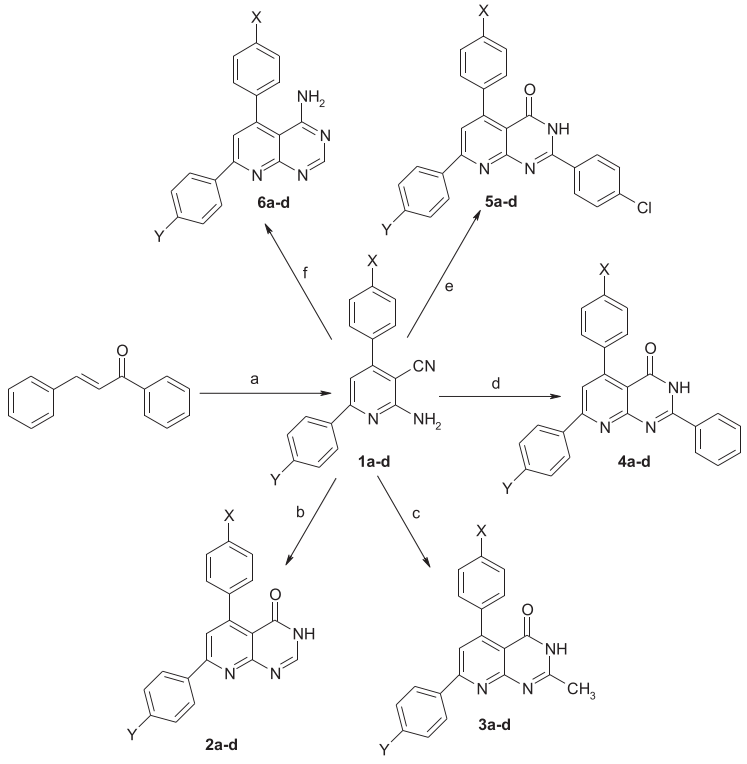
RESULTS AND DISCUSSION

Chemistry. 2-Amino-3-cyano-4,6-diaryl pyridines **1a–d** were synthesized in good yield by the reaction of the

corresponding chalcone with malononitrile in the presence of ammonium acetate with a slight modification of the procedure reported [14], where **1a–c** were known compounds [33,34] while **1d** was a new derivative according to our survey. IR spectrum of compound **1d** showed the presence of absorption bands at 3460, 3354, and 3245 cm^{-1} assigned to (NH_2) group, and strong absorption band at 2216 cm^{-1} for (C≡N stretching); also its ^1H NMR spectrum revealed the presence of singlet signal at 6.9 ppm for (NH_2) group (D_2O exchangeable). Reaction of scaffolds **1a–d** with aliphatic acids such as formic acid (99%) and glacial acetic acid in the presence of conc. H_2SO_4 afforded pyrido[2,3-*d*] pyrimidines **2a–d** and **3a–d**, respectively. The reaction sequence involves acylation of amine, hydrolysis of nitrile to amide, followed by cyclization and dehydration to form the products in single step. IR spectrum of compound **2c** revealed the absence of cyano and amino groups, and the appearance of absorption bands at 3196 cm^{-1} for (NH) and at 1692 cm^{-1} for (C=O). Furthermore, its ^1H NMR spectrum also showed singlet signal at δ 12.29 ppm assigned to (NH) proton (D_2O exchangeable). Mass spectrum of **2c** showed molecular ion peak at *m/z* 333 (M^+, 92.23%) corresponding to its molecular formula $C_{19}H_{12}N_3OCl$; on the other hand, IR spectrum of **3c** showed the presence of bands at 3182 cm^{-1} for (NH) and at 1676 cm^{-1} for (C=O). The ^1H NMR spectrum of **3c** showed singlet signal at δ 2.37 ppm (3H) for methyl proton and singlet at δ 12.19 ppm (1H) for (NH) proton (D_2O exchangeable). Mass spectrum of **3c** showed molecular ion peak at *m/z* 347 (M^+, 100% base peak) corresponding to its molecular formula $C_{20}H_{14}N_3OCl$ (Scheme 1).

Reaction of compounds **1a–d** with acid chloride such as benzoyl chloride and 4-chloro benzoyl chloride in the presence of pyridine afforded **4a–d** and **5a–d**, respectively; the expected products (benzoylation of amino group) were not formed, but pyridopyrimidines were formed. The cause was the use of pyridine without drying; this may proceed by the hydrolysis of cyano group to amide by the used pyridine after benzoylation then cyclization to the final product. IR spectrum of **4c** showed the presence of bands at 3200 cm^{-1} for (NH) and 1669 cm^{-1} for (C=O). ^1H NMR

Scheme 1. Reagents and conditions: (a) malononitrile, amm. acetate, ethanol, reflux, 5–7 h; (b) formic acid (99%), conc. H_2SO_4, reflux, 12–16 h; (c) glacial acetic acid, conc. H_2SO_4, reflux, 12–16 h; (d) benzoyl chloride, pyridine, reflux, 14–17 h; (e) 4-chlorobenzoyl chloride, pyridine, reflux, 12–14 h; and (f) formamide, reflux, 14–16 h.



compound	X	Y	compound	X	Y
1-6a	H	H	1-6c	H	Cl
1-6b	OCH_3	H	1-6d	NO_2	H

spectrum of **4c** showed singlet signal at δ 12.52 ppm (1H) for (NH) proton (D_2O exchangeable). Mass spectrum of **4c** showed molecular ion peak at *m/z* 409 (M^+, 67.1%) corresponding to its molecular formula $C_{25}H_{16}N_3OCl$; on the other hand, IR spectrum of **5a** showed the absence of cyano and amino groups, and the presence of characteristic absorption band at 3190 cm^{-1} for (NH) and strong stretching band at 1666 cm^{-1} for (C=O). ^1H NMR spectrum of **5a** showed singlet signal at δ 12.58 ppm (1H) for (NH) proton (D_2O exchangeable). Mass spectrum of **5a** showed molecular ion peak at *m/z* 409 (M^+, 100% base peak) corresponding to its molecular formula $C_{25}H_{16}N_3OCl$ which confirmed its chemical structure (Scheme 1).

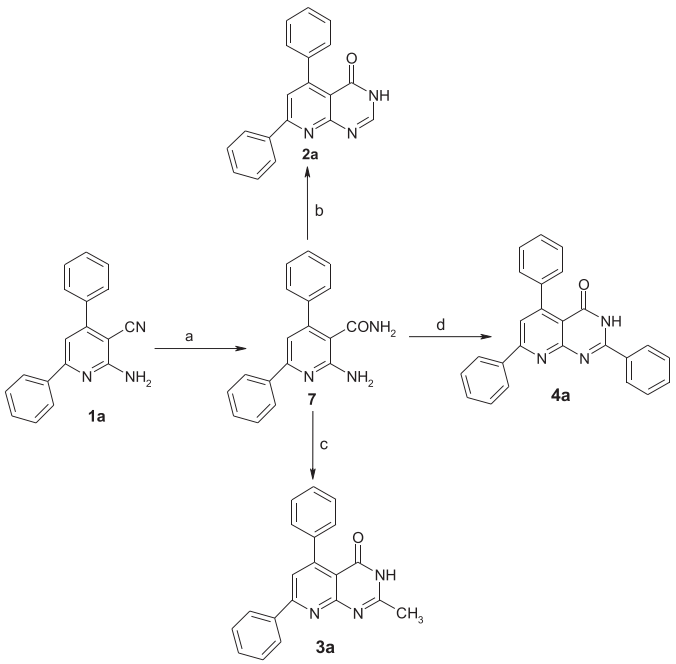
Compounds **1a–d** were refluxed with formamide in oil bath for 14–16 h to afford **6a–d**; IR spectrum of **6b** showed the absence of cyano group and the appearance of absorption bands at 3460 and 3299 cm^{-1} for (NH_2) group. ^1H NMR spectrum of **6b** showed a singlet at δ 7.1–7.5 ppm for (NH_2) protons (D_2O exchangeable) and singlet at δ 8.52 ppm for (C-H pyrimidine) proton. Mass spectrum of **6b** showed molecular ion peak at *m/z* 328 (M^+, 71.6%) corresponding to its molecular formula $C_{20}H_{16}N_4O$ (Scheme 1).

Two-step methods were used to prepare pyrido[2,3-*d*] pyrimidine. The first step was the reaction of **1a** with alcoholic solution of KOH to afford pyridine-carboxamide **7**. IR spectrum of **7** revealed the absence of cyano group and the appearance of absorption bands at 3387 cm^{-1} for (amide NH_2) and at 1658 cm^{-1} for (amide C=O). Its ^1H NMR spectrum showed two singlets at δ 5.08 and 6.5 ppm which indicated the presence of NH_2 and amide NH_2 groups, respectively. The second step was fusion of **7** with triethylorthoformate, acetic anhydride, and benzoyl chloride to afford **2a**, **3a**, and **4a**, respectively (Scheme 2).

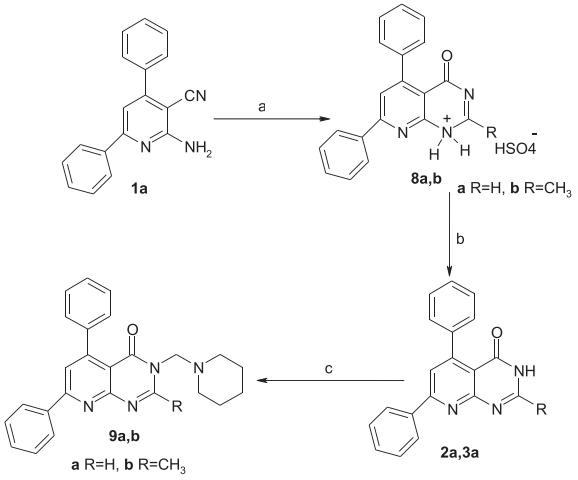
Reaction of compound **1a** with aliphatic acids (formic acid (99%) and glacial acetic acid) in the presence of conc. H_2SO_4 for 18–20 h afforded **8a**,**b** instead of **2a** and **3a**. IR spectrum of **8a** showed the absence of cyano and amino groups, and the appearance of characteristic absorption band at 1726 cm^{-1} for (C=O). ^1H NMR spectrum of **8a** showed a singlet at δ 8.8 ppm for (C—H pyrimidine) proton. Hydrolysis of **8a**,**b** afforded **2a** and **3a**, respectively. The structures of **2a** and **3a** were confirmed by their IR and ^1H NMR spectra; stirring of **2a** and **3a** with formaldehyde and piperidine in the presence of DMF/EtOH for 72 h at room temperature afforded **9a**,**b**. IR spectrum of **9a** showed the absence of NH group and the appearance of absorption band at 2936 cm^{-1} for (C—H aliphatic). ^1H NMR spectrum of **9a** showed the attachment piperidin-1-yl methyl group to imino nitrogen by the presence of a multiplet signals at δ 1.26–1.39, 1.40–1.42, and 2.27 ppm for piperidine protons, and singlet signal at δ ppm 4.7 (2H) corresponding to CH_2 protons (Scheme 3).

Biological studies. ***Anti-microbial activity.*** The antimicrobial screenings of the synthesized compounds were undertaken using agar well diffusion assay [35]. Table 1 lists the screening results of the tested compounds against the gram negative bacteria (*Escherichia coli* and *Pseudomonas sp.*), gram positive bacteria (*Bacillus subtilis*,

Scheme 2. Reagents and conditions: (a) alc. KOH (5%), reflux, 16 h; (b) triethylorthoformate, reflux, 4 h; (c) acetic anhydride, reflux, 5 h; and (d) benzoyl chloride, reflux, 3 h.



Scheme 3. Reagents and conditions: (a) RCOOH (99%), conc. H_2SO_4, reflux, 18–20 h; (b) H_2O; and (c) formaldehyde, piperidine, DMF/EtOH, stirring, 72 h.



Streptococcus pneumoniae, and *Staphylococcus aureus*), and three species of fungi (*Aspergillus niger*, *Penicillium sp*., and *Candida albicans*). The obtained data revealed that most of the tested compounds showed excellent to moderate activities against the microorganisms used at a dose of 1 mg/mL. Compounds showing inhibition of at least 15 mm were considered active and were further evaluated for their minimum inhibitory concentration (MIC). Streptomycin was used as a standard antibacterial, while ketoconazole was used as standard antifungal. DMSO was used as a blank, exhibiting no activity against any of the used organisms.

It is well noticed that compounds **2b**, **2c**, **3b**, **3c**, **5b**, **5c**, **6b**, **6c**, and **7** showed remarkable broad spectrum potency against all bacteria and fungi species compared to MIC of the reference drugs (Table 2); **6b** with MIC value of 25 µg/mL was two times as potent as streptomycin with MIC value of 50 µg/mL against *B. subtilis*, while **2b**, **3b**, and **5b** with MIC value of 50 µg/mL were equipotent against it similar to reference drug. Compound **5b** with MIC value of 25 µg/mL was more than two times as potent as standard drug against *S. pneumoniae* and *E. coli*. Furthermore, compounds **2b**, **5c**, **6b**, **6c**, and **7** were found to be more active than streptomycin against *S. pneumoniae*. Pyrido[2,3-*d*]pyrimidine derivatives **5b** and **6b** were the most active against *S. aureus* and two times as potent as reference drug.

Table 1

Inhibition zones (IZ) in mm of the synthesized compounds at 1 mg/mL.

	Gram positive bacteria			Gram negative bacteria			Fungi	
Compound (mg/mL)	*Bacillus subtilis*	*Streptococcus pneumoniae*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas sp.*	*Aspergillus niger*	*Penicillium* sp.	*Candida albicans*
1d	7	9	11	9	9	9	10	6
2a	3	9	11	14	12	8	11	12
2b	20	20	24	34	28	29	23	31
2c	19	15	18	20	35	17	15	26
2d	4	3	9	11	12	8	8	12
3a	5	8	11	14	10	10	9	11
3b	22	18	23	21	26	15	29	25
3c	15	19	23	19	31	21	25	29
3d	8	8	10	12	14	7	8	11
4a	7	11	13	14	7	11	11	10
4c	10	14	20	20	29	15	18	17
4d	4	13	5	13	14	11	13	12
5a	8	3	20	30	23	17	17	18
5b	24	29	31	39	33	33	36	36
5c	17	20	26	24	27	19	17	19
5d	6	14	9	13	14	8	10	10
6a	6	8	8	14	13	14	11	11
6b	28	22	28	27	30	23	28	30
6c	19	23	26	20	22	20	23	18
6d	10	7	11	12	13	9	10	11
7	16	19	22	28	34	25	32	33
8a	13	14	10	14	—	2	8	—
8b	—	—	—	8	13	—	4	2
9a	10	7	11	14	8	5	11	9
9b	15	13	17	19	21	11	16	18
Streptomycin	18	17	20	22	27	—	—	—
Ketoconazole	—	—	—	—	—	18	21	21

—= no inhibition zone.

Table 2

MIC of the compounds tested against organisms (μg/mL).

	Gram positive bacteria			Gram negative bacteria			Fungi	
Compound (μg/mL)	*Bacillus subtilis*	*Streptococcus pneumoniae*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas sp.*	*Aspergillus niger*	*Penicillium* sp.	*Candida albicans*
2b	50	50	50	50	50	50	50	50
2c	100	100	100	100	25	100	100	50
3b	50	100	100	100	50	100	50	100
3c	100	100	50	100	50	50	50	50
4c	—	—	100	100	100	100	100	100
5a	—	—	100	100	100	100	100	100
5b	50	25	25	25	50	25	25	25
5c	100	50	50	100	50	50	100	100
6b	25	50	25	50	50	50	50	50
6c	100	50	50	100	100	50	50	100
7	100	50	50	50	25	50	25	25
9b	100	—	100	100	100	—	100	100
Streptomycin	50	70	50	70	50	—	—	—
Ketoconazole	—	—	—	—	—	50	50	70

Compounds **2b**, **6b**, and **7** were more potent than streptomycin against *E. coli*. Compounds **2c** and **7** with MIC value of 25 μg/mL were more potent than standard against *Pseudomonas sp*., but **2b**, **3b**, **3c**, **5b**, **5c**, and **6b** were equipotent against *Pseudomonas sp*. similar to reference drug. Compound **5b** with MIC value of 25 μg/mL was twice as potent as ketoconazole against *A. niger*, but **2b**, **3c**, **5c**, **6b**, **6c**, and **7** were equipotent as reference drug against this fungi. Furthermore, compounds **5b** and **7** with MIC value of 25 μg/mL were the most active against *Penicillium sp*. and *C. albicans*. On the other hand, compounds **4c**, **5a**, and **9b** showed moderate activity against bacteria and fungi. The rest of the compounds were found to be inactive against bacteria and fungi.

Cytotoxicity evaluation. The newly synthesized compounds were evaluated for their cytotoxicity by using the previously established methodology [36]. Data obtained in Table 3 showed that all of the tested compounds exhibited hemolysis degree below 5% indicating their lower hemolysis degree; also most of them showed high safety profile (high IC_{50} values) and inhibition of hemolysis degree. The lower IC_{50} values and inhibition of hemolysis degree of compounds **2c**, **3c**, **3d**, **6d**, **8a**, and **8b** indicate their higher cytotoxicity than the other compounds.

Structure activity relationships (SARs). By comparing the experimental anti-microbial data of the compounds reported in this study with their structures, the following structure activity relationships were postulated. (1) Pyrido [2,3-*d*]pyrimidine derivatives in which the pyridine ring is bearing two phenyl groups of compounds **2a**, **3a**, **4a**, **5a**, and **6a**, as well as carrying 5-(4-nitrophenyl),7-phenyl groups of compounds **2d**, **3d**, **4d**, **5d**, and **6d** showed weak antimicrobial activity. (2) Pyrido[2,3-*d*]pyrimidine derivatives in which the pyridine ring is bearing one electron-donating group (4-methoxy phenyl or 4-chlorophenyl) and one phenyl group of compounds **2b**,**c**, **3b**,**c**, **5b**,**c**, and **6b**,**c** showed the best results in biological screening. However, pyrido[2,3-*d*]pyrimidine derivatives in which the pyrimidine ring is bearing phenyl group of compounds **4a–d**, pyrido[2,3-*d*]pyrimidine salts **8a**,**b** as well as compounds **9a**,**b** did not have such an influence on activity. Finally, it was concluded that high electron density increased the anti-microbial activity.

CONCLUSION

In this work, we have synthesized new pyridine derivatives **1d** and **7**, pyrido[2,3-*d*]pyrimidine derivatives **2a–d**, **3a–d**, **4a–d**, **5a–d**, **6a–d**, **8a**,**b**, and **9a**,**b** which were screened for their anti-microbial activity against the gram negative bacteria (*E. coli* and *Pseudomonas sp*), gram positive bacteria (*B. subtilis*, *S. pneumoniae* and *S. aureus*), and three species of fungi (*A. niger*, *Penicillium sp*., and *C. albicans*). The tested compounds **2b**, **2c**, **3b**, **3c**, **5b**, **5c**, **6b**, **6c**, and **7** showed excellent anti-microbial activities against all the tested bacteria and fungi compared to the reference drugs. By comparing the IC_{50} values of the highly active antimicrobial compounds, we concluded that compounds **2b**, **3b**, **5b**, **5c**, **6b**, **6c**, and **7** exhibited higher safety profile (high IC_{50} values) than compounds **2c** and **3c** (low IC_{50} values) so they are the most effective antimicrobial agents.

EXPERIMENTAL

Chemistry. Melting points were determined on MEL_TEMP II apparatus and are uncorrected. IR spectra (KBr) were measured on Perkin-Elmer FT/IR spectrophotometer, ^1H NMR and ^{13}C NMR spectra

Table 3

Cytotoxicity of the tested compounds.

Compound	Inhibition of hemolysis %	Degree of hemolysis %	IC_{50} for normal cells mg/mL
1d	76.389 ± 4.8	2.362 ± 0.08	3.27 ± 0.1
2a	19.444 ± 2	2.553 ± 0.02	12.86 ± 0.2
2b	43.056 ± 3.9	2.702 ± 0.07	5.8 ± 0.5
2c	−122.222 ± 10.8	2.255 ± 0.02	$2.04 \times 10^{-6} \pm 0.1 \times 10^{-6}$
2d	48.611 ± 3.4	2.957 ± 0.006	5.14 ± 0.05
3a	36.806 ± 3.1	3.021 ± 0.08	4.53 ± 0.8
3b	88.194 ± 8.7	0.553 ± 0.005	2.8 ± 0.08
3c	−1.927 ± 0.09	1.979 ± 0.1	$8.6 \times 10^{-15} \pm 0.1 \times 10^{-15}$
3d	−6.944 ± 0.6	2.723 ± 0.03	$24 \times 10^{-9} \pm 0.1 \times 10^{-9}$
4a	90.278 ± 7.4	1.426 ± 0.1	1.38 ± 0.1
4b	36.111 ± 3.8	2.723 ± 0.01	6.92 ± 0.04
4c	72.222 ± 6.2	1.362 ± 0.2	3.46 ± 0.02
4d	31.944 ± 3	2.681 ± 0.03	3.91 ± 0.1
5a	67.361 ± 5.2	1.915 ± 0.01	3.71 ± 0.9
5b	47.917 ± 2.1	2.809 ± 0.02	5.22 ± 0.2
5c	36.111 ± 2.8	1.957 ± 0.01	6.92 ± 0.02
5d	27.778 ± 1.8	0.447 ± 0.002	9 ± 1
6a	76.389 ± 7.1	1.617 ± 0.006	3.27 ± 0.02
6b	46.528 ± 2.8	2.830 ± 0.08	3.58 ± 0.3
6c	38.194 ± 3.8	2.511 ± 0.04	6.54 ± 0.05
6d	−64.583 ± 5.3	2.617 ± 0.02	$3.8 \times 10^{-6} \pm 0.1 \times 10^{-6}$
7	43.75 ± 3.9	2.766 ± 0.04	3.81 ± 0.4
8a	−20.833 ± 2.1	2.106 ± 0.05	$12 \times 10^{-9} \pm 0.2 \times 10^{-9}$
8b	−9.722 ± 1.2	2.532 ± 0.08	$25.7 \times 10^{-9} \pm 0.2 \times 10^{-9}$
9a	20.138 ± 2.1	2.702 ± 0.01	8.27 ± 0.08
9b	41.667 ± 2.8	1.192 ± 0.01	4 ± 0.1

were recorded on JEOL (500 MHz), Varian Mercury VX-300 (300 MHz), and Bruker 500 MHz-avance III in DMSO-d_6 as solvent, using tetramethyl-silane (TMS) as internal reference standard. The chemical shifts values are expressed in ppm. The NMR spectra were performed at Faculty of Science, Alexandria University, Cairo University, and Jordan University. Elemental analysis (C, H, and N) was performed by a Vario III CHN analyzer (Germany) at the Microanalytical Unit of Cairo University. All compounds were within ±0.4% of the theoretical values. Mass spectra were run on DI analysis Shimadzu QP-2010 plus mass spectrometer at the Microanalytical Unit of Cairo University. The progress of the reaction and the purity of the compounds were monitored by TLC analytical silica gel plates $60\,F_{254}$. The chemical reagents used in synthesis were purchased from Fluka, Sigma, and Aldrich.

General procedure for the preparation of (1a–d). Derivatives of 2-amino-3-cyano-4,6-diaryl pyridine **1a–d** were synthesized by refluxing the corresponding chalcone (0.05 mole), malononitrile (0.05 mole), and ammonium acetate (0.36 mole) in absolute ethanol (80 mL) for 5–7 h; after cooling, the formed precipitate was filtered, washed with ethanol, dried, and recrystallized from ethanol.

2-Amino-3-cyano-4(4-nitrophenyl)-6-phenylpyridine (1d). Yield: 79%; mp: 233–235°C; IR (KBr) cm^{-1}: 3460, 3354, 3245 (NH_2), 3067 (C—H aromatic), 2216 (C≡N), 1651 (NH bending), 1573, 1512 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 6.8–8.3 (m, 10H, Ar—H), 6.9 (s, 2H, NH_2 (D_2O exchangeable)); *Anal*. Calcd for $C_{18}H_{12}N_4O_2$ (316): C, 68.35; H, 3.79; N, 17.72. Found: C, 68.01; H, 3.47; N, 17.46.

General procedure for the synthesis of compounds (2a–d). A mixture of **1a–d** (0.01 mol) in formic acid (30 mL) and catalytic amount of conc. H_2SO_4 was heated under reflux for 12–16 h. Then the reaction mixture was cooled and poured into ice cold water, and the separated solid was filtered, washed with water, dried, and recrystallized from the proper solvent, **2a**,**b** from ethanol, while **2c** and **2d** from mixture of DMF/ethanol (1:5).

5,7-Diphenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (2a). Yield: 59%; mp: 301–303°C; IR (KBr) cm^{-1}: 3196 (NH), 3061 (C—H aromatic), 1696 (C=O), 1615, 1537 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.3–8.2 (m, 10H, Ar-H), 7.7 (s, 1H, pyridine-H), 8.28 (s, 1H, pyrimidine–H), 12.27 (s, 1H, NH (D_2O exchangeable)); *Anal*. Calcd for $C_{19}H_{13}N_3O$ (299): C, 76.25; H, 4.3; N, 14.04. Found: C, 75.98; H, 4.20; N, 13.71.

5-(4-Methoxyphenyl)-7-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (2b). Yield: 62%; mp: 250–252°C; IR (KBr) cm^{-1}: 3200 (NH), 2924 (C—H aliphatic), 1660 (C=O), 1605, 1528 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 3.82 (s, 3H, OCH_3), 6.9–7.93 (m, 11H, Ar—H), 10.05 (s, 1H, NH); *Anal*. Calcd for $C_{20}H_{15}N_3O_2$ (329): C, 72.94; H, 4.55; N, 12.76. Found: C, 73.01; H, 4.20; N, 13.11.

7-(4-Chlorophenyl)-5-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (2c). Yield: 66%; mp: 275–277°C; IR (KBr) cm^{-1}: 3196 (NH), 3065 (C—H aromatic), 1692 (C=O), 1616, 1533 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 7.38–8.27 (m, 11H, Ar—H), 12.29 (s, 1H, NH (D_2O exchangeable)); M.S (*m/z*, %): 333 (M^+, 92.23), 335 (M^{+2}, 63.11), 295 (83.5), 185 (100); *Anal*. Calcd for $C_{19}H_{12}N_3OCl$ (333): C, 68.46; H, 3.60; N, 12.61. Found: C, 68.58; H, 3.92; N, 12.41.

5-(4-Nitrophenyl)-7-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (2d). Yield: 64%; mp: 304–306°C; IR (KBr) cm^{-1}: 3200 (NH), 3066 (C—H aromatic), 1687 (C=O), 1610, 1511 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 7.51–8.31 (m, 11H, Ar—H), 12.41 (s, 1H, NH (D_2O exchangeable)); M.S (*m/z*, %): 344 (M^+, 69.61), 317 (93.14), 110 (100), 98 (98), 58 (88.24); *Anal*. Calcd for $C_{19}H_{12}N_4O_3$ (344): C, 66.27; H, 3.48; N, 16.27. Found: C, 66.09; H, 3.8; N, 16.11.

General procedure for the synthesis of compounds (3a–d). A mixture of **1a–d** (0.01 mol) in glacial acetic acid (30 mL) and catalytic amount of conc. H_2SO_4 was heated under reflux for 12–16 h. Then the reaction mixture was cooled and poured into ice cold water, and the separated solid was filtered, washed with water, dried, and recrystallized from the proper solvent, **3b** from ethanol, while **3a**,**c**,**d** from mixture of DMF/ethanol (1:5).

5,7-Diphenyl-2-methyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (3a). Yield: 71%; mp: 321–323°C; IR (KBr) cm^{-1}: 3177 (NH), 3035 (C—H aromatic), 2978 (C—H aliphatic), 1675 (C=O), 1625, 1537 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 2.37 (s, 3H, CH_3), 7.38–8.23 (m, 11H, Ar—H), 12.19 (s, 1H, NH (D_2O exchangeable)); MS (*m/z*, %): 313 (M^+, 80.95), 312 (92.06), 257 (83.33), 120 (80.95), 77 (100), 51 (88.1); *Anal*. Calcd for $C_{20}H_{15}N_3O$ (313): C, 76.67; H, 4.79; N, 13.41. Found: C, 76.90; H, 5.08; N, 13.32.

5-(4-Methoxyphenyl)-2-methyl-7-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (3b). Yield: 69%; mp: 253–255°C; IR (KBr) cm^{-1}: 3171 (NH), 3038 (C—H aromatic), 2971, 2915 (C—H aliphatic), 1675 (C=O), 1621, 1539 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 2.37 (s, 3H, CH_3), 3.78 (s, 3H, OCH_3), 6.9–8.2 (m, 10H, Ar—H), 12.04 (s, 1H, NH (D_2O exchangeable)); ^{13}C NMR (DMSO-d_6) δ ppm: 21.99 (CH_3), 55.68 (OCH_3), 112.17, 113.37, 120.92, 128, 129.39, 130.88, 132, 138, 153.47, 158.56, 159.76, 160.26, 160.89, 161.88; MS (*m/z*, %): 343 (M^+, 78.74), 243 (70.87), 139 (81.1), 77 (100); *Anal*. Calcd for $C_{21}H_{17}N_3O_2$ (343): C, 73.46; H, 4.95; N, 12.24. Found: C, 73.61; H, 5.17; N, 12.46.

7-(4-Chlorophenyl)-2-methyl-5-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (3c). Yield: 73%; mp: 345–347°C; IR (KBr) cm^{-1}: 3182 (NH), 3057 (C—H aromatic), 2922 (C—H aliphatic), 1676 (C=O), 1629, 1534 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 2.37 (s, 3H, CH_3), 7.38–8.26 (m, 10H, Ar—H), 12.19 (s, 1H, NH (D_2O exchangeable)); M.S (*m/z*, %): 347 (M^+, 100), 349 (M^{+2}, 53.92), 346 (74.51), 236 (88.24), 120 (88.24), 79 (80.39); *Anal*. Calcd for $C_{20}H_{14}N_3OCl$ (347): C, 69.16; H, 4.03; N, 12.10. Found: C, 69.10; H, 4.30; N, 12.15.

2-Methyl-5-(4-nitrophenyl)-7-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (3d). Yield: 66%; mp: 338–340°C; IR (KBr) cm^{-1}: 3182 (NH), 3054 (C—H aromatic), 2928 (C—H aliphatic), 1660 (C=O), 1620, 1516 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 2.39 (s, 3H, CH_3), 7.32–8.17 (m, 10H, Ar—H), 12.4 (s, 1H, NH); *Anal*. Calcd for $C_{20}H_{14}N_4O_3$ (358): C, 67.04; H, 3.91; N, 15.64. Found: C, 66.81; H, 4.30; N, 15.25.

General procedure for the synthesis of compounds (4a–d). A mixture of **1a–d** (0.01 mol) in pyridine (10 mL) and benzoyl chloride (20 mL) was heated under reflux for 14–17 h. Then the reaction mixture was cooled, and the formed solid was washed with ethylacetate, filtered, dried, and recrystallized from the proper solvent, **4b** from ethanol, while **4a**,**c**,**d** from mixture of DMF/ethanol (1:5).

2,5,7-Triphenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (4a). Yield: 72%; mp: 316–318°C; IR (KBr) cm^{-1}: 3194 (NH), 3051 (C—H aromatic), 1668 (C=O), 1611, 1540 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 7.43–8.32 (m, 16H, Ar—H), 12.53 (s, 1H, NH (D_2O exchangeable)); MS (*m/z*, %): 375 (M^+, 100), 363 (90.82), 329 (91.84), 254 (88.78), 189 (88.78), 81 (91.84), 61 (90.82); *Anal*. Calcd for $C_{25}H_{17}N_3O$ (375): C, 80.00; H, 4.53; N, 11.2. Found: C, 80.28; H, 4.80; N, 11.50.

2,7-Diphenyl-5-(4-methoxyphenyl)-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (4b). Yield: 44%; mp: 298–300°C; IR (KBr) cm^{-1}: 3181 (NH), 3066 (C—H aromatic), 2933 (C—H aliphatic), 1652 (C=O), 1592, 1509 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 3.3 (s, 3H, OCH_3), 7.5–8.2 (m, 15H, Ar—H), 11.4 (s, 1H, NH); *Anal*. Calcd for $C_{26}H_{19}N_3O_2$ (405): C, 77.03; H, 4.69; N, 10.37. Found: C, 76.81; H, 4.50; N, 10.25.

7-(4-Chlorophenyl)-2,5-diphenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (4c). Yield: 76%; mp: 330–332°C; IR (KBr) cm^{-1}: 3200 (NH), 1669 (C=O), 1610, 1538 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 7.4–8.31 (m, 15H, Ar—H), 12.52 (s, 1H, NH (D_2O exchangeable)); ^{13}C NMR (DMSO-d_6) δ ppm: 113, 121.3, 127.96, 128.46, 128.65, 129.2, 129.3, 129.5, 129.99, 132.5, 132.77, 135.9, 136.7, 139.79, 153.89, 156.4, 159.5, 160.56, 162.3; M.S (*m/z*, %): 409 (M^+, 67.09), 411 (M^{+2}, 36.08), 203 (66.46), 104 (96.84), 77 (100), 57 (50); *Anal*. Calcd for $C_{25}H_{16}N_3OCl$ (409): C, 73.34; H, 3.91; N, 10.26. Found: C, 73.26; H, 4.20; N, 10.01.

2,7-Diphenyl-5-(4-nitrophenyl)-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (4d). Yield: 67%; mp: 318–320°C; IR (KBr) cm^{-1}: 3182 (NH), 3028 (C—H aromatic), 1684 (C=O), 1569, 1509 (C=C, C=N); ^1H NMR (DMSO-d_6) δ ppm: 7.44–8.31 (m, 15H, Ar—H), 12.65 (s, 1H, NH); *Anal*. Calcd for $C_{25}H_{16}N_4O_3$ (420): C, 71.42; H, 3.80; N, 13.33. Found: C, 71.28; H, 4.10; N, 13.01.

General procedure for the synthesis of compounds (5a–d). A mixture of **1a–d** (0.01 mol) in pyridine (10 mL) and 4-chlorobenzoyl chloride (20 mL) was heated under reflux for 12–14 h. Then the reaction mixture was cooled, and the formed solid was washed with ethylacetate, filtered, dried, and recrystallized from the proper solvent, **5b** from ethanol, while **5a**,**c**,**d** from mixture of DMF/ethanol (1:5).

2-(4-Chlorophenyl)-5,7-diphenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (5a). Yield: 64%; mp: 352–354°C; IR (KBr) cm^{-1}: 3190 (NH), 3057 (C—H aromatic), 1666 (C=O), 1607, 1539 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.4–8.3 (m, 15H, Ar—H), 12.58 (s, 1H, NH (D_2O exchangeable)); MS (*m/z*, %): 409 (M^+, 100), 411 (M^{+2}, 63.27), 217 (91.84), 179 (88.78), 99 (91.84); *Anal*. Calcd for $C_{25}H_{16}N_3OCl$ (409): C, 73.34; H, 3.91; N, 10.26. Found: C, 73.06; H, 4.11; N, 10.3.

2-(4-Chlorophenyl)-5-(4-methoxyphenyl)-7-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (5b). Yield: 67%; mp: 220–222°C; IR (KBr) cm^{-1}: 3093 (NH), 3052 (C—H aromatic), 2923 (C—H aliphatic), 1685 (C=O), 1592, 1492 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 3.81 (s, 3H, OCH_3), 7.5–7.92 (m, 14H, Ar—H), 13.16 (s, 1H, NH); *Anal*. Calcd for $C_{26}H_{18}N_3O_2Cl$ (439): C, 71.01; H, 4.10; N, 9.56. Found: C, 71.06; H, 4.11; N, 9.3.

2,7-Di(4-chlorophenyl)-5-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (5c). Yield: 61%; mp: 354–356°C; IR (KBr) cm^{-1}: 3189 (NH), 3048 (C—H aromatic), 1666 (C=O), 1607, 1536 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.4–8.3 (m, 14H, Ar—H), 12.56 (s, 1H, NH); *Anal*. Calcd for $C_{25}H_{15}N_3OCl_2$ (443): C, 67.72; H, 3.38; N, 9.48. Found: C, 67.51; H, 3.41; N, 9.12.

2-(4-Chlorophenyl)-5-(4-nitrophenyl)-7-phenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (5d). Yield: 53%; mp: 358–360°C; IR (KBr) cm^{-1}: 3094 (NH), 1685 (C=O), 1592, 1491 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.52–7.91 (m, 14H, Ar—H), 13.2 (s, 1H, NH); MS (*m/z*, %): 454 (M^+, 9.45), 456 (M^{+2}, 6.1), 50 (100); *Anal*. Calcd for $C_{25}H_{15}N_4O_3Cl$ (454): C, 66.07; H, 3.30; N, 12.33. Found: C, 66.31; H, 3.22; N, 12.11.

General procedure for the synthesis of compounds (6a–d). A mixture of **1a–d** (0.01 mol) and formamide (30 mL) was refluxed on an oil bath for 14–16 h. Then the reaction mixture was cooled and poured into ice cold water, and the separated solid was filtered, washed with water, dried, and recrystallized from the proper solvent **6b** from ethanol and **6a**,**c**,**d** from mixture of DMF/ethanol (1:5).

5,7-Diphenyl-pyrido[2,3-d]pyrimidin-4-amine (6a). Yield: 69%; mp: 308–310°C; IR (KBr) cm^{-1}: 3480, 3289 (NH_2), 3058 (C—H aromatic), 1577, 1548 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.5–8.3 (m, 12H, Ar—H + NH_2), 7.83 (s, 1H, CH pyridine), 8.54 (s, 1H, H at C_2); MS (*m/z*, %): 298 (M^+, 63.22), 297 (100), 77 (64.65), 51 (65.92); *Anal*. Calcd for $C_{19}H_{14}N_4$ (298): C, 76.51; H, 4.69; N, 18.79. Found: C, 76.72; H, 4.91; N, 18.62.

5-(4-Methoxyphenyl)-7-phenyl-pyrido[2,3-d]pyrimidin-4-amine (6b). Yield: 71%; mp: 245–247°C; IR (KBr) cm^{-1}: 3460 and 3299 (NH_2), 3057 (C—H aromatic), 2929 (C—H aliphatic), 1554,1507 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 3.82 (s, 3H, OCH_3), 7.1–8.3 (m, 11H, Ar—H + NH_2), 7.72 (s, 1H, CH pyridine), 8.52 (s, 1H, H at C_2); ^{13}C NMR (DMSO-d_6) δ ppm: 55.91 (OCH_3), 106.3, 115.1, 120.96, 128.17, 129.4, 130.63, 130.69, 131.03, 138.07, 150.26, 158.8, 160.24, 160.58, 160.7, 162.89; MS (*m/z*, %): 328 (M^+, 71.60), 327 (100), 284 (32.91), 77 (15.94), 51 (11.97); *Anal*. Calcd for $C_{20}H_{16}N_4O$ (328): C, 73.17; H, 4.87; N, 17.07. Found: C, 73.40; H, 5.10; N, 17.22.

7-(4-Chlorophenyl)-5-phenyl-pyrido[2,3-d]pyrimidin-4-amine (6c). Yield: 68%; mp: 315–317°C; IR (KBr) cm^{-1}: 3399 and 3345 (NH_2), 3047 (C—H aromatic), 1564, 1487 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.1–8.1 (m, 13H, Ar—H + NH_2); *Anal*. Calcd for $C_{19}H_{13}N_4Cl$ (332): C, 68.67; H, 3.91; N, 16.86. Found: C, 68.51; H, 4.11; N, 16.62.

5-(4-Nitrophenyl)-7-phenyl-pyrido[2,3-d]pyrimidin-4-amine (6d). Yield: 53%; mp: 312–314°C; IR (KBr) cm^{-1}: 3476, 3382, 3307 and 3208 (NH_2), 1610, 1521 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.1–8.1 (m, 13H, Ar—H + NH_2); *Anal*. Calcd for $C_{19}H_{13}N_5O_2$ (343): C, 66.47; H, 3.79; N, 20.4. Found: C, 66.39; H, 3.52; N, 20.17.

2-Amino-4,6-diphenyl pyridine-3-carboxamide (7). To 100 mL of alcoholic solution of KOH (5%), 0.01 mole of compound **1a** was added, and the reaction mixture was refluxed for 16 h. After cooling, the reaction mixture was diluted with water, and the formed solid was filtered off, washed with water, dried, and recrystallized from DMF to afford **7** as pale yellow solid. Yield: 37%; mp: 294–296°C; IR (KBr) cm^{-1}: 3387 (amide NH_2), 3033 (C—H aromatic), 1658 (amide C=O), 1629, 1598 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 5.08 (s, 2H, NH_2), 6.5 (s, 2H, amide NH_2), 7.33–7.46 (m, 11H, Ar—H); MS (*m/z*, %): 289 (M^+, 3.7), 286 (80.5), 285 (76), 240 (81), 215(56.15), 157(54.9), 134 (49.7), 121 (100), 107 (37.1), 77 (30.43); *Anal*. Calcd for $C_{18}H_{15}N_3O$ (289): C, 74.74; H, 5.19; N, 14.53. Found: C, 74.53; H, 5.01; N, 14.18.

5,7-Diphenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (2a). A mixture of **7** (0.01 mole) was fused with triethylorthoformate (0.01 mole) for 4 h. After cooling the formed solid was washed with ethanol, dried, and recrystallized from ethanol to give **2a**. Yield: 53%.

5,7-Diphenyl-2-methyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (3a). A mixture of **7** (0.01 mol) and acetic anhydride (20 mL) was fused for 5 h. The reaction mixture was allowed to cool and added to ice cold water. The solid obtained was filtered, washed with water, dried, and recrystallized from mixture of DMF/ethanol (1:5) to afford **3a** as yellow crystals. Yield: 78%.

2,5,7-Triphenyl-3,4-dihydropyrido[2,3-d]pyrimidin-4-one (4a). A mixture of **7** (0.01 mol) and benzoyl chloride (20 mL) was fused for 3 h. After cooling the formed solid was

filtered, washed with ethanol, dried, and recrystallized from DMF/ethanol (1:5) to afford **4a** as pale yellow solid. Yield: 73%.

General procedure for the synthesis of compounds (8a,b). A mixture of **1a** (0.01 mol), aliphatic acid (formic acid or acetic acid) (30 mL), and (1 mL) of conc. H_2SO_4 was refluxed for 18–20 h. Then the reaction mixture was cooled, the separated solid was filtered, dried, and recrystallized from methanol.

5,7-Diphenylpyrido[2,3-d]pyrimidin-4(1H)-one sulfate (8a). Yield: 58%; mp: 353–355°C; IR (KBr) cm^{-1}: 3110 (C—H aromatic), 1726 (C=O), 1584, 1531 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 7.4–8.3 (m, 10H, Ar—H), 7.9 (s, 1H, CH pyridine), 8.8 (s, 1H, CH pyrimidine); ^{13}C NMR (DMSO-d_6) δ ppm: 113.98, 122.82, 128.16, 128.35, 128.88, 129.28, 129.64, 131.69, 136.91, 138.8, 151.14, 154.43, 155.57, 159.96, 160.72; *Anal*. Calcd for $C_{19}H_{15}N_3O_5S$ (397): C, 57.43; H, 3.78; N, 10.58. Found: C, 57.18; H, 3.42; N, 10.27.

5,7-Diphenyl-2-methylpyrido[2,3-d]pyrimidin-4(1H)-one sulfate (8b). Yield: 60%; mp: 300–302°C; IR (KBr) cm^{-1}: 3050 (C—H aromatic), 2927 (C—H aliphatic), 1726 (C=O), 1606, 1544 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.6 (s, 3H, CH_3), 7.4–8.3 (m, 10H, Ar—H), 8.01 (s, 1H, CH pyridine); *Anal*. Calcd for $C_{20}H_{17}N_3O_5S$ (411): C, 58.39; H, 4.14; N, 10.21. Found: C, 58.01; H, 3.94; N, 10.09.

General procedure for the synthesis of (compounds (9a,b)). Formaldehyde (1.0 mL, 40%) was added to compound **2a** or **3a** (0.01 mole) in (30 mL) 1:1 anhydrous ethanol/DMF and heated on steam bath for 5 min, then piperidine (0.01 mole) was added to the cold solution; the reaction mixture was stirred for 72 h at room temperature. The formed solid was filtered off and recrystallized from mixture of DMF/EtOH (1:1).

5,7-Diphenyl-3-(piperidin-1-ylmethyl)pyrido[2,3-d]pyrimidin-4-one (9a). Yield: 48%; mp: 230–232°C; IR (KBr) cm^{-1}: 3058 (C—H aromatic), 2936 (C—H aliphatic) 1683 (C=O), 1607, 1539 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 1.26–1.39 (m, 4H, piperidine-H), 1.40–1.42 (m, 4H, piperidine-H), 2.27 (m, 2H, piperidine-H), 4.7 (s, 2H, CH_2 protons), 7.3–8.3 (m, 10H, Ar—H), 7.8 (s, 1H, CH pyridine), 8.6 (s, 1H, CH pyrimidine); ^{13}C NMR (DMSO-d_6) δ ppm: 23.8, 26, 51.2 (piperidine Carbons), 67.55 (CH_2 aliphatic), 121.9, 127.9, 128.15, 128.29, 129.09, 129.2, 129.4, 131, 137.77, 140.15, 152.1, 154.17, 159.8, 160.6, 160.87; *Anal*. Calcd for $C_{25}H_{24}N_4O$ (396): C, 75.75; H, 6.06; N, 14.14. Found: C, 75.42; H, 5.84; N, 13.95.

5,7-Diphenyl-2-methyl-3-(piperidin-1-ylmethyl)pyrido[2,3-d] pyrimidin-4-one (9b). Yield: 50%; mp: 297–299°C; IR (KBr) cm^{-1}: 3058 (C—H aromatic), 2928 (C—H aliphatic), 1680 (C=O), 1577, 1537 (C=C, C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 1.2–2.8 (m, 13H, piperidine-H + CH_3), 3.9 (s, 2H, aliphatic CH_2), 7.3–8.3 (m, 11H, Ar—H); *Anal*. Calcd for $C_{26}H_{26}N_4O$ (410): C, 76.09; H, 6.34; N, 13.65. Found: C, 75.86; H, 6.02; N, 13.27.

Anti-microbial. Determination of the minimum inhibitory concentration (MIC) of the tested compounds was measured by the broth dilution method [37]. Each of the test compounds and standards (Streptomycin, Ketoconazole) was dissolved in DMSO, at concentration of 1 mg/mL. Further dilutions of the compounds and standards in the test medium were prepared at required quantities 0.5, 0.1, 0.05, and 0.025 mg/mL.

Culture of microorganisms. Bacteria and fungi species used were obtained from the Microbiology Department of Alexandria University, Faculty of Pharmacy, Alexandria, Egypt, namely *B. subtilis* (NCTC-3610), *S. pneumoniae* (ATCC-70669), *S. aureus* (NCTC-6751), *E. coli* (NCTC-10416), *Pseudomonas sp*. (NCTC-1656), *A. niger* (NCTC-3781) *Penicillium sp*. (ATCC-1059), and *C. albicans* (ATCC-10231). The bacterial strains were maintained on MHA (Mueller-Hinton Agar) medium for 24 h at 37 ± 1°C, and fungi were maintained on SDS (Sabouraud Dextrose Agar) for 48 h at 25 ± 1°C. The bacteria and fungi inocula were prepared by suspension in 5 mL of sterile saline for colonies from culture on MHA and SDA medium.

Cytotoxicity evaluation. Human blood: The blood was collected from a healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment. The blood was washed three times with 0.9% saline and centrifuged simultaneously for 10 min at 3000 rpm. The packed cells were washed with 0.9% saline and a 40% *v/v* suspension made using isotonic phosphate buffer which was composed of 154 mM NaCl in 10 mM sodium phosphate buffer at pH 7.4 used as stock erythrocyte or RBC suspension.

Different concentrations (from 0.1 to 10 mg/mL) of different compounds were mixed with 7 mL of phosphate buffer saline solution (PBS, g/l, NaCl, 8.7; $K_2HPO_4.3H_2O$; 1.82; KH_2PO_4, 0.23, pH 7.4) and 100-μL blood; mixture was maintained at 37°C for 3 h. Positive and negative controls were prepared by adding the same amount of blood to 7 mL of water and PBS, respectively. Each tube was gently inverted twice each 30 min to maintain contact of the blood with the material. After incubation, each fluid was transferred to a suitable tube and centrifuged at 2000 rpm for 15 min. The hemoglobin released by hemolysis was measured at 540 nm. The percentage of hemolysis was calculated as follows.

$$\text{Haemolysis}\ (\%) = (\text{OD sample - OD negative control}) / (\text{OD positive control} - \text{OD negative control}) \times 100$$

Materials can be classified into three different categories according to their hemolytic index (hemolysis %). Materials with percentages of hemolysis over 5% are considered hemolytic and considered a non-hemolytic below 2%, while the ones with hemolytic index between 5% and 2% are classified as slightly hemolytic.

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