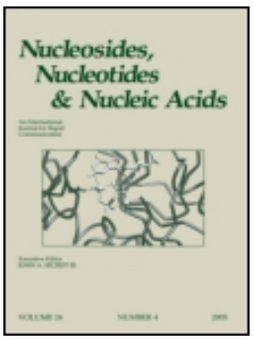

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NOVEL NUCLEOSIDE ANALOGUES: FIRST SYNTHESIS OF PYRIDINE-4-THIOGLYCOSIDES AND THEIR CYTOTOXIC EVALUATION

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□ *The reaction of sodium 2,2-dicyanoethene-1,1-bis(thiolate) with 2-cyano-N-arylacetamides afforded sodium pyridine-4-thiolates, coupling of the latters with 2,3,4,6-tetra-O-acetyl-D-gluco- and D-galactopyranosyl bromides, respectively, afforded new pyridine-4-thioglycosides. Ammonolysis of the latter compounds afforded the free thioglycosides. The antitumor activities of the synthesized compounds were tested against human tumor cell lines; lung (A549), colon (HCT116), liver (HEPG2), and prostate (PC3).*

Keywords Ketene dithioacetals; sodium pyridine-4-thiolates; pyridine-4-thioglycosides; activated nitriles

1. INTRODUCTION

Thiosugars are well known for their diverse array of bioactivities.[1] Thus, the new developments in the synthetic and medicinal chemistry of thiosugars are important for carbohydrate drug design.[2] Till date various methods have been developed for the synthesis of these very interesting molecules.[3]

Recently, deazanucleoside analogues have been known to exhibit antitumor activity.[4] During our studies of nucleoside analogues with novel H-bonding patterns, a route for the synthesis of S-glycosides bearing a substituted pyridine ring as the heterocyclic aglycone was desired.[5–8] Such a route could provide access to a variety of analogues of pyrimidine nucleosides with novel H-bonding patterns.[9–11] Such molecules might display pharmaceutically useful antimetabolite activity.[12–14] In recent reports from

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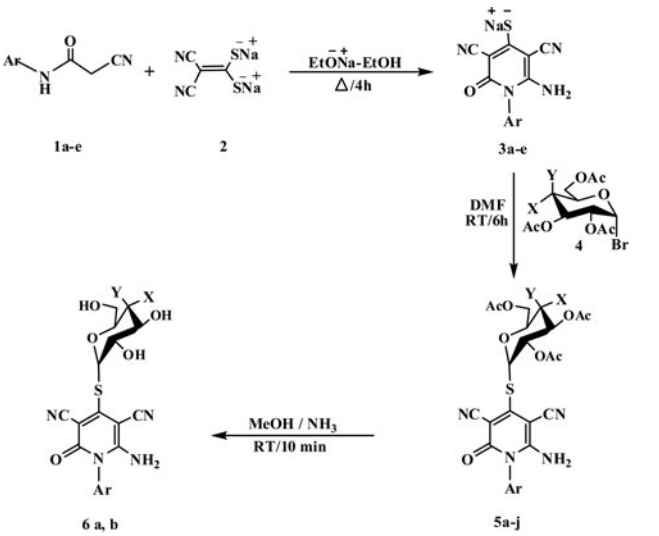
our laboratory, we described the preparation of different novel functionalized pyridine thioglycosides, which revealed antagonistic activity.[15] In an earlier communication, we had already reported the use of dihydropyridine thioglycosides as substrates or inhibitors of protein glycosylation.[16] These common features encouraged us to develop a new, straightforward route for the synthesis of pyridine thioglycosides. Continuing our efforts for the development of simple, eco-friendly, and cost-effective methodologies, we report here a novel synthesis of pyridine-4-thioglycosides. This new protocol has been applied to a variety of substituted pyridine-4-thioglycosides with excellent yield. As far as we know, this is the first report of a pyridine-4-thioglycoside.

2. RESULTS AND DISCUSSION

2.1. Chemistry

It has been found that reaction of malononitrile with carbon disulfide in the presence of sodium ethoxide gives the sodium 2,2-dicyanoethene-1,1-bis(thiolate) **2**. Compounds **2** are readily reacted with one equivalent of 2-cyano-*N*-arylacetamides **1a–e** in the presence of sodium ethoxide to give the corresponding sodium 6-amino-3,5-dicyano-2-oxo-1-aryl-1,2-dihydropyridine-4-thiolate derivatives **3a–e** in good yields. Compounds **3** reacted with 2,3,4,6-tetra-*O*-acetyl-α–*D*-gluco- and galacto-pyranosyl bromides **4a**,**b** in dimethylformamide at room temperature to give in high yield the corresponding *S*-glycosides **5a–j** (Scheme 1). It is suggested that the *cis*-(α)sugars donors react with **3** by a simple SN_2 reaction to give the β-glycoside products.[17] The structures of **5** were established on the basis of its elemental analysis and spectral data (IR, ^{1}H NMR, and ^{13}C NMR). For example, the analytical data for **5a** revealed a molecular formula $C_{27}H_{26}N_4O_{10}S$, its ^{1}H NMR spectrum showing the anomeric proton as a doublet at δ 5.21–5.23 ppm with a spin–spin coupling constant of 10.50 Hz indicating the β-configuration. The other six glucose protons resonated at δ 3.78–5.01 ppm. When glycosides **5** were treated with methanolic ammonia at room temperature for 10 minutes, the free glycoside derivatives **6a**,**b** were obtained in almost quantitative yields (Scheme 2), the structures of which were established on the basis of elemental analysis and spectral data. Thus, the analytical data for **6a** reveal the molecular formula $C_{19}H_{18}N_4O_6S$. The ^{1}H NMR spectrum shows the anomeric proton as a doublet at δ 5.79–5.81 ($J_{1'\text{-}2'}$ 10.0 Hz), indicating the presence of only the β-D-configuration. Encouraged by these results, we decided to synthesize the pyridine-4-thioglycosides **5** using the reaction of **1a–e** with [bis(mercapto)methylene]malononitrile **7** and comparing the resulting products for stereochemical considerations. Thus, in a simple experimental procedure, treatment of compounds **1a–e** with **7** in

sodium ethoxide, followed by acidification afforded the corresponding 4-mercaptopyridines **9**. The latter compounds were treated with peracetylated sugars **4** in sodium ethoxide-dimethylformamide (DMF) at room temperature to afford the *S*-glycosyl compounds **5**. The latter were shown to be the same as those obtained from the reaction of **3** with **4** by comparison of their melting points and spectral data. Upon methylation of compounds **9** with methyl iodide in sodium ethoxide gave the 4-methylsulfanylpyridine products **10a–e**. Compounds **10** can also be prepared by reaction of **1a-e** with [bis(methylthio)methylene]malononitrile **8** in sodium ethoxide. When compounds **5** and **10** were subjected to the reaction with hydrazine, the pyrazolo[4,3-*c*]pyridines **11** were obtained[18] (Scheme 2).

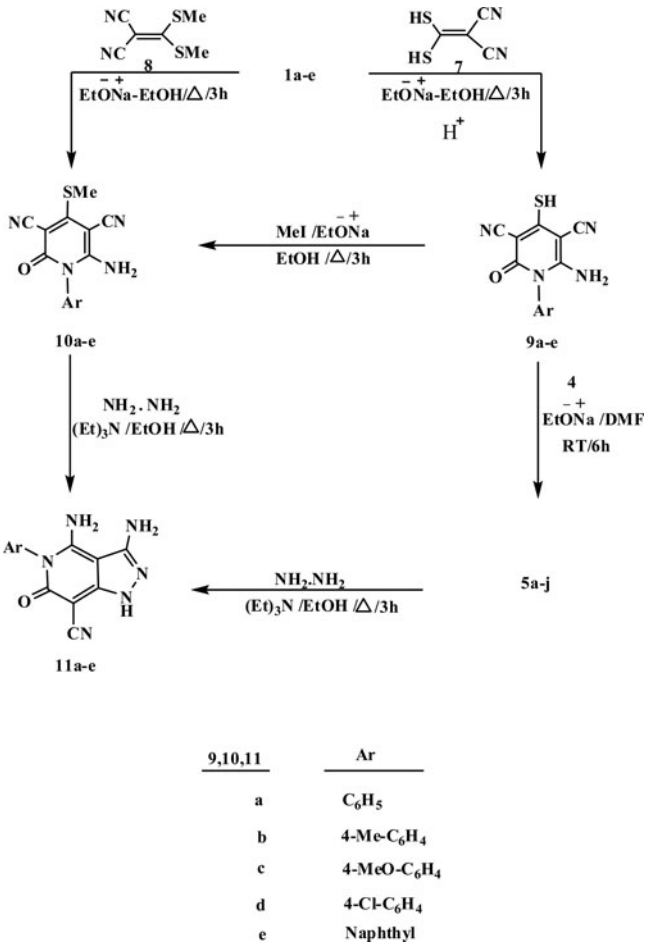


5	Ar	X	Y
a	C_6H_5	OAc	H
b	4-Me-C_6H_4	OAc	H
c	4-MeO-C_6H_4	OAc	H
d	4-Cl-C_6H_4	OAc	H
e	Naphthyl	OAc	H

5	Ar	X	Y
f	C_6H_5	H	OAc
g	4-Me-C_6H_4	H	OAc
h	4-MeO-C_6H_4	H	OAc
i	4-Cl-C_6H_4	H	OAc
j	Naphthyl	H	OAc

6	Ar	X	Y
a	C_6H_5	OH	H
b	4-MeO-C_6H_4	H	OH

SCHEME 1 Synthetic pathway for pyridine-4-thioglycosides 5a-j, 6a,b.



9,10,11	Ar
a	C_6H_5
b	4-Me-C_6H_4
c	4-MeO-C_6H_4
d	4-Cl-C_6H_4
e	Naphthyl

SCHEME 2 Synthesized derivatives 9a-e, 10a-e, 11a-e.

2.2. Antitumor Activity

The 10 of newly synthesized pyridine and pyridine-4-glucopyranosylthio **5a–d**, **5h**, **6b**, **9a**,**c**,**d**, **10a** were selected and evaluated for in vitro anticancer activity against human cancer cells according to the standard procedures described in experimental part. The tumor cell lines panel consisted of four human tumor cell lines, namely, hepatocellular carcinoma HEPG2, colon carcinoma HCT116, prostate carcinoma PC3, and lung carcinoma A549, which were incubated with 10 concentrations (0.19–100 μM) for each compound. Cell viability was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan. A probit analysis was carried for LC_{50} determination using SPSS 11 program. The antitumor drug doxorubicin was used

TABLE 1 Cytotoxicity of the synthesized candidates on A549, HCT116, HEPG2, and PC3 cancer cell lines

	LC_{50}[a] (μM)			
Compound no.	(A549)	(HCT116)	(HEPG2)	(PC3)
5h	—	—	73.3	83.3
6b	—	—	45.6	67.6
9c	—	—	82.5	—
Doxorubicin	48.8	65.1	37.8	41.1

LC_{50}[a] is lethal concentration of the sample, which causes the death of 50% of cells in 48 hours. Cancer cell lines A549 (lung cell line), HCT116 (colon cell line), HEPG2 (liver cell line), and PC3 (prostate cell line).

as positive control. The results are presented in Table 1. LC_{50} (cytostatic activity) values were based on dose–response curves (LC_{50} value, defined as the concentration corresponding to 50% growth inhibition). The tested compounds **5a–d**, **9a**,**d**, and **10a** exhibited weak antitumor activity toward both the four cell lines (at 100 μM the values of mortality of cancer cell lines range between 15% and 58%). While compounds **5h**, **6b**, **9c** proved to be selective toward hepatocellular carcinoma HEPG2 cancer cell line with LC_{50} values of 73.3, 45.6, 82.5, respectively (Figure 1). On the other hand, compounds **5h** and **6b** displayed a considerable antitumor activity toward prostate carcinoma PC3 with LC_{50} values of 83.3 and 67.6, respectively (Figure 2). This means that these compounds displayed selectivity on particular cell lines

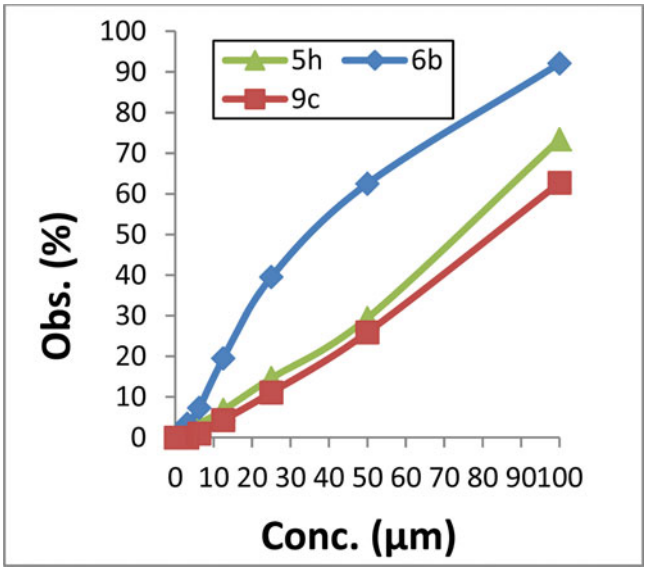


FIGURE 1 Representative graph showing observed responses of HEPG2 cell in the presence of increasing concentrations of compounds **5h**, **6b**, and **9c**.

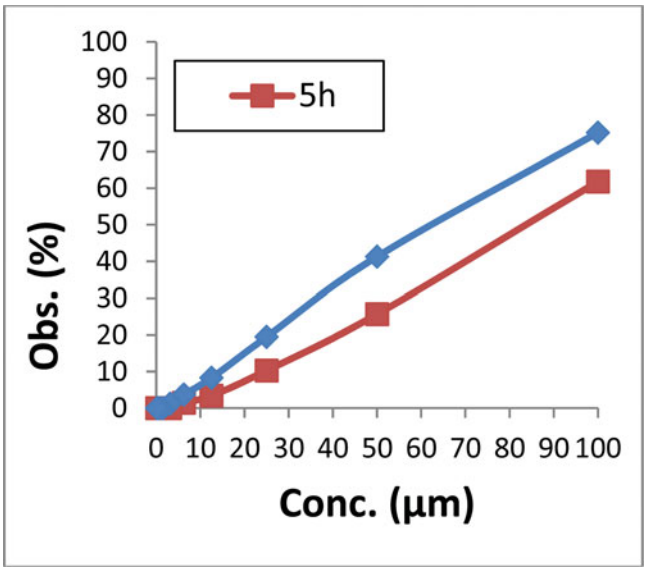


FIGURE 2 Representative graph showing observed responses of PC3 cell in the presence of increasing concentrations of compound **5h** and **6b**.

(hepatocellular carcinoma HEPG2 and prostate carcinoma PC3). Pyridine carrying two substituents (**5h** and **6b**) especially when contain thioglycosidic linkage at C4 and *Para* methoxy phenyl at *N* showed more activity toward HEPG2 and prostate carcinoma cell lines PC3. Substitution of electron releasing groups such as methoxy on the benzene ring present at 4-position increased the anticancer activity and has shown good activity, whereas the introduction of electron attracting chloro, group on benzene ring decreased the activity and has shown weak activity. Compound **6b** has more cytotoxic activity. The results suggested that, pyridine-*N*-Para methoxyphenyl with deprotected thiogalactosidic linkage at C4 played a vital role in the modulation of cytotoxic activity.

3. CONCLUSION

In conclusion, we have developed a new and simple method for the synthesis of the first pyridine-4-thioglycosides. The mild reaction conditions, clean reaction profiles, zero side product, and cost efficiency render this approach as useful and innovative to the existing methods for glycoside formation. Further studies on the application of this method for the synthesis of other highly functionalized biologically active glycosides are underway. The antitumor activities of the synthesized compounds were tested against human tumor cell lines; lung (A549), colon (HCT116), liver (HEPG2), and prostate (PC3).

4. EXPERIMENTAL PROTOCOLS

4.1. Chemistry

All melting points were measured on a Gallenkamp melting point apparatus. The ^{1}H NMR spectra were measured on a Jeol-500 MHz spectrometer for solutions DMSO-d_6 and $CDCl_3$ using $Si(CH_3)_4$ as an internal standard at National Research Center, Cairo, Egypt. Progress of the reactions was monitored by thin-layer chromatography (TLC) using aluminum sheets coated with silica gel F254 (Merck). Viewing under a short-wavelength UV lamp (Spectronics Corporation, USA) effected detection.

4.1.1. General Procedure for the Synthesis of 3a–e

A mixture of 2-cyano-*N*-arylacetamide **1a–e** (10 mmol) and sodium 2,2-dicyanoethene-1,1-bis(thiolate) **2** (10 mmol) in EtOH (30 ml), sodium ethoxide (10 mmol) was refluxed for 4 hours. The reaction mixtures were cooled to room temperature and triturated with MeOH to afford compounds **3a–e,** which were collected by filtration and recrystallized from the appropriate solvent. Yield (90%).

4.1.1.1. Sodium 6-amino-3,5-dicyano-2-oxo-1-phenyl-1,2-dihydropyridine-4-thiolate (3a). Brown solid; yield (90%), mp > 300°C; IR (KBr, cm^{-1}) υ 3434, 3396 (NH_2), 3089 (CH), 2207 (CN), 1692 (CO). $C_{13}H_7N_4NaOS$.

4.1.1.2. Sodium 6-amino-3,5-dicyano-2-oxo-1-p-tolyl-1,2-dihydropyridine-4-thiolate (3b). Brown solid; yield (85%), mp > 300°C; IR (KBr, cm^{-1}) υ 3427, 3368 (NH_2), 3008 (CH), 2213 (CN), 1689 (CO). $C_{14}H_9N_4NaOS$.

4.1.1.3. Sodium 6-amino-3,5-dicyano-1-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-4-thiolate (3c). Brown solid; yield (85%), mp > 300°C; IR (KBr, cm^{-1}) υ 3436, 3373 (NH_2), 1387 (CH_3), 2215 (CN), 1690 (CO). $C_{14}H_9N_4NaO_2S$.

4.1.1.4. Sodium 6-amino-1-(4-chlorophenyl)-3,5-dicyano-2-oxo-1,2-dihydropyridine-4-thiolate (3d). Brown solid; yield (85%), mp > 300°C. $C_{13}H_6ClN_4$ NaOS.

4.1.1.5. Sodium 6-amino-3,5-dicyano-1-(4-(naphthalen-2-yl)phenyl)-2-oxo-1,2 dihydropyridine-4-thiolate (3e). Brown solid; yield (83%), mp > 300°C. $C_{17}H_9$
N_4NaOS.

4.1.2. General Procedures for the Synthesis of 5a–j

A solution of compound **3a–e** (10 mmol) in dry DMF (20 ml) was stirred at room temperature, then a solution of 2,3,4,6-tetra-*O*-acetyl-α-*D*-gluco- or galactopyranosyl bromide **4** in DMF (10 ml) was dropped within 30 minutes and the reaction mixture was stirred at room temperature until compilation (TLC, 6–8 hours). After completion, the reaction mixture was poured onto

water. The resulting solid was filtered off, purified by column chromatography (petroleum ether 40–60/ethyl acetate 3:1) and recrystallized from ethanol to give the compounds (**5a–j**).

4.1.2.1. 6-Amino-4-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosylthio)-2-oxo-1-phenyl-1,2-dihydropyridine-3,5-dicarbonitrile (5a). Yellow solid; (EtOH), yield (86%), mp 210°C, ^{1}H NMR (500 MHz, DMSO): δ 1.95–2.46 (4s, 12H, 4xOAc), 3.87 (m, 2H, 2H-6'), 4.20 (m, 1H, H-5'), 4.53 (t, 1H, J = 9.5 Hz, H-4'), 4.78 (t, 1H, J = 9.3 Hz, H-3'), 5.01(t, 1H, J = 9.5 Hz H-2'), 5.21–5.23 (d, 1H, $J_{1'\text{-}2'}$ = 10.50 Hz, H-1'), 6.32 (s, 2H, NH_2), 7.47–7.70 (m, 5H, C_6H_5). ^{13}C NMR: δ 21.25 ($4CH_3CO$), 62.12 (C-6'), 68.64 (C-4'), 70.22 (C-2'), 73.22 (C-3'), 79.13 (C-5'), 81.23 (C-1'), 113.26 (CN), 116.11 (CN), 126.25 (Ar-C), 128.43 (2C, Ar-C), 129.75 (2C, Ar-C), 132.51 (Ar-C), 150.25 (C-5), 158.10 (C-3), 160.34 (pyridine C-4), 168.34 (CO), 171.52 (C-6), 176.52 (4CO). UV (EtOH): 265, 311, 390 nm. Anal. Calcd. for $C_{27}H_{26}N_4O_{10}S$ (598.58): C, 54.18; H, 4.38; N, 9.36; S, 5.36%. Found: C, 54.2; H, 4.2; N, 9.2; S, 5.4%.

4.1.2.2. 6-Amino-1-(4-methylphenyl)-4-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (5b). Yellow solid; (EtOH), yield (82%), mp 198°C, ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.93–1.95 (4s, 12H, 4xOAc), 2.60 (s, 3H, CH_3), 3.89–3.92 (m, 2H, H-6' H-6"), 4.11 (m, H, H-5'), 4.90 (t, 1H, J = 9.5 Hz, H-4'), 5.14 (t, 1H, J = 9.3 Hz, H-3'), 5.31 (t, 1H, J = 9.5 Hz, H-2'), 5.58–5.60 (d, 1H, $J_{1'\text{-}2'}$ = 6.90 Hz, H-1'), 6.88 (s, 2H, NH_2) 7.36–7.51 (m, 4H, C_6H_4). ^{13}C NMR: δ 17.55 (CH_3), 20.00 ($4CH_3CO$), 60.10 (C-6'), 66.00 (C-4'), 69.15 (C-2'), 71.00 (C-3'), 76.10 (C-5'), 80.00 (C-1'), 110.20 (CN), 113.00 (CN), 122.20 (Ar-C), 125.21 (2C, Ar-C), 126.23 (2C, Ar-C), 130.99 (Ar-C), 149.20 (C-5), 156.00 (C-3), 162.00 (pyridine C-4), 166.99 (CO), 170.77 (C-6), 178.92 (4CO). UV (EtOH): 260, 300, 399 nm. Anal. Calcd. for $C_{28}H_{28}N_4O_{10}S$ (612.61): C, 54.90; H, 4.61; N, 9.15; S, 5.23%. Found: C, 54.9; H, 4.5; N, 9.2; S, 5.3%.

4.1.2.3. 6-Amino-1-(4-methoxyphenyl)-4-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (5c). Yellow solid; (EtOH), yield (82%), mp 182°C, ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.14 (4s, 12H, 4xOAc), 2.99 (s, 3H, OCH_3), 3.94 (m, 2H, 2H-6'), 4.14 (m, 1H, H-5'), 4.37 (t, 1H, J = 9.2 Hz, H-4'), 4.65 (t, 1H, J = 9.1 Hz, H-3'), 4.79 (t, 1H, J = 9.3 Hz, H-2'), 5.09–5.10 (d, 1H, $J_{1'\text{-}2'}$ = 5.70 Hz, H-1'), 6.42–6.51 (s, 2H, NH_2), 7.25–7.33 (m, 4H, C_6H_4). Anal. Calcd. for $C_{28}H_{28}N_4O_{11}S$ (628.61): C, 53.50; H, 4.49; N, 8.91; S, 5.10%. Found: C, 53.4; H, 4.4; N, 8.8; S, 5.1%.

4.1.2.4. 6-Amino-1-(4-chlorophenyl)-4-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (5d). Yellow solid; (EtOH), yield (81%), mp 175°C, ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.84–1.96 (4s, 12H, 4xOAc), 3.91 (m, 2H, 2H-6'), 4.21 (m, 1H, H-5'), 5.22–5.30 (m, 2H, H-4' and H-3'), 5.43 (t, 1H, J = 9.5 Hz, H-2'), 6.18–6.19 (d, 1H, $J_{1'\text{-}2'}$ = 5.00 Hz, H-1'), 6.66 (s, 2H, NH_2), 7.50–7.81 (m, 4H, C_6H_4). ^{13}C NMR: δ 19.99 ($4CH_3CO$), 61.77 (C-6'), 67.68 (C-4'), 69.99 (C-2'), 72.56 (C-3'), 78.89 (C-5'), 80.94 (C-1'), 112.73 (CN), 115.87 (CN), 124.89 (Ar-C),

127.93 (2C, Ar-C), 129.00 (2C, Ar-C), 131.93 (Ar-C), 151.84 (C-5), 156.83 (C-3), 161.74 (pyridine C-4), 169.93 (CO), 171.52 (C-6), 176.52 (4CO). UV (EtOH): 270, 320, 390 nm. Anal. Calcd. for $C_{27}H_{25}ClN_4O_{10}S$ (633.03): C, 51.23; H, 3.98; Cl, 5.60; N, 8.85; S, 5.07%. Found: C, 51.3; H, 3.9; N, 8.7; S, 5.1%.

4.1.2.5. 6-Amino-4-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosylthio)-1-(naphthalen-2-yl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (5e). Yellow solid; (EtOH), yield (80%), mp 206°C ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.03–2.19 (4s, 12H, 4xOAc), 3.92 (m, 1H, H-5'), 4.25–4.26 (t, 1H, H-4'), 4.46–4.52 (m, 2H, 2H-6'), 4.72 (t, 1H, J = 9.3 Hz, H-3'), 5.02 (t, 1H, J = 9.5 Hz, H-2'), 5.37–5.38 (d, 1H, $J_{1'\text{-}2'}$ = 7.00 Hz, H-1'), 6.24 (s, 2H, NH_2), 7.37–7.69 (m, 7H, $C_{10}H_7$). UV (EtOH): 270, 319, 385 nm. UV (EtOH): 266, 317, 392 nm. Anal. Calcd. for $C_{31}H_{28}N_4O_{10}S$ (648.64): C, 57.40; H, 4.35; N, 8.64; S, 4.94%. Found: C, 57.5; H, 4.3; N, 8.5; S, 4.8%.

4.1.2.6. 6-Amino-4-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosylthio)-2-oxo-1-phenyl-1,2-dihydropyridine-3,5-dicarbonitrile (5f). Yellow solid; (EtOH), yield (83%), mp 212°C, IR (KBr, cm^{-1}) υ 3065, 3033 (NH_2), 2956 (CH), 2225 (CN), 1601 (CO). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.96–2.12 (4s, 12H, 4xOAc), 3.73–3.81 (m, 1H, H-5'), 4.04 (t, 1H, J = 9.2 Hz, H-4'), 4.25 (t, 1H, J = 9.5 Hz, H-3'), 4.46–4.50 (t, 1H, J = 9.3 Hz, H-2'), 4.82 (m, 2H, 2H-6'), 5.29–5.32 (d, 1H, $J_{1'\text{-}2'}$ = 11.25 Hz, H-1'), 6.26–6.31 (s, 2H, NH_2) 7.13–7.66 (m, 5H, C_6H_5). ^{13}C NMR: δ 19.00 ($4CH_3CO$), 61.10 (C-6'), 67.00 (C-4'), 71.56 (C-2'), 73.00 (C-3'), 78.95 (C-5'), 80.83 (C-1'), 114.00 (CN), 117.34 (CN), 123.99 (Ar-C), 126.00 (2C, Ar-C), 128.76 (2C, Ar-C), 131.67 (Ar-C), 151.00 (C-5), 156.43 (C-3), 161.78 (pyridine C-4), 165.98 (CO), 170.26 (C-6), 175.00 (4CO). Anal. Calcd. for $C_{27}H_{26}N_4O_{10}S$ (598.58): C, 54.18; H, 4.38; N, 9.36; S, 5.36%. Found: C, 54.2; H, 4.3; N, 9.3; S, 5.2%.

4.1.2.7. 6-Amino-4-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosylthio)-2-oxo-1-p-tolyl-1,2-dihydropyridine-3,5-dicarbonitrile (5g). Yellow solid; (EtOH), yield (85%), mp 201°C, ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.21–2.34 (4s, 12H, 4xOAc), 2.41 (s, 3H, CH_3), 3.90 (m, 1H, H-5'), 4.23 (m, 2H, 2H-6'), 4.72 (t, 1H, J = 9.6 Hz, H-4'), 4.92 (t, 1H, J = 9.4 Hz, H-3'), 5.12 (t, 1H, J = 9.5 Hz, H-2'), 5.33–5.35 (d, 1H, $J_{1'\text{-}2'}$ = 10.50 Hz, H-1'), 6.52 (s, 2H, NH_2) 7.51–7.61 (m, 4H, C_6H_4). Anal. Calcd. for $C_{28}H_{28}N_4O_{10}S$ (612.61): C, 54.90; H, 4.61; N, 9.15; S, 5.23%. Found: C, 54.9; H, 4.6; N, 9.2; S, 5.1%.

4.1.2.8. 6-Amino-1-(4-methoxyphenyl)-4-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (5h). Yellow solid; (EtOH), yield (84%), mp 195°C, ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.91–2.01 (4s, 12H, 4xOAc), 3.45 (s, 3H, OCH_3), 3.96 (m, 1H, H-5'), 4.13–4.14 (m, 2H, 2H-6'), 4.60 (t, 1H, J = 9.5 Hz, H-4'), 4.89 (t, 1H, J = 9.4 Hz, H-3'), 5.12 (t, 1H, J = 9.5 Hz, H-2'), 5.41–5.43 (d, 1H, $J_{1'\text{-}2'}$ = 10.00 Hz, H-1'), 6.34 (s, 2H, NH_2), 7.37–7.64 (m, 4H, C_6H_4). Anal. Calcd. for $C_{28}H_{28}N_4O_{11}S$ (628.61): C, 53.50; H, 4.49; N, 8.91; S, 5.10%. Found: C, 53.4; H, 4.3; N, 8.7; S, 5.1%.

4.1.2.9. 6-Amino-1-(4-chlorophenyl)-4-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (5i). Yellow solid; (EtOH), yield (86%), mp 188°C purified by using column chromatography (petroleum ether/ethyl acetate 3:1); ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.97–2.13 (4s, 12H, 4xOAc), 3.93–4.08 (m, H, H-5'), 4.12–4.19 (m, 2H, 2H-6'), 4.72–4.82 (t, 1H, J = 9.5 Hz, H-4'), 4.85–5.16 (t, 1H, J = 9.4 Hz, H-3'), 5.22 (t, 1H, J = 9.5 Hz, H-2'), 5.40–5.42 (d, 1H, $J_{1'\text{-}2'}$ = 9.70 Hz, H-1'), 6.27 (s, 2H, NH_2), 7.25–7.50 (m, 4H, C_6H_4). Anal. Calcd. for $C_{27}H_{25}ClN_4O_{10}S$ (633.03): C, 51.23; H, 3.98; Cl, 5.60; N, 8.85; S, 5.07%. Found: C, 51.1; H, 3.7; Cl, 5.6; N, 8.7; S, 5.2%.

4.1.2.10. 6-Amino-4-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosylthio)-1-(naphthalen-2-yl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (5j). Yellow solid; (EtOH), yield (85%), mp 205°C, ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.93–2.05 (4s, 12H, 4xOAc), 3.80–4.25 (m, 3H, 2H-6' and H-5'), 4.79–5.28 (m, 2H, H-4' and H-3'), 5.35 (t, 1H, J = 9.2 Hz, H-2'), 5.51–5.53 (d, 1H, $J_{1'\text{-}2'}$ = 10.00 Hz, H-1'), 6.18–6.20 (s, 2H, NH_2), 7.16–8.11 (m, 7H, $C_{10}H_7$). Anal. Calcd. for $C_{31}H_{28}N_4O_{10}S$ (648.64): C, 57.40; H, 4.35; N, 8.64; S, 4.94%. Found: C, 57.5; H, 4.2; N, 8.5; S, 4.8%.

4.1.3. Ammonolysis of 5a and 5h

To a solution of an individual nucleoside **5a** or **5h** (1 mmol) in anhydrous MeOH (10 ml), the dry gaseous ammonia was passed at room temperature for 10 minutes. The reaction mixture was stirred until the reaction was judged complete by TLC using (petroleum ether/ethyl acetate) (R_f, 0.72–0.74). The resulting mixture was then concentrated under reduced pressure to afford a solid residue that was crystallized from appropriate solvent.

4.1.3.1. 6-Amino-4-(2',3',4',6'-tetrahydroxy-β-D-glucopyranosylthio)-2-oxo-1-phenyl-1,2-dihydropyridine-3,5-dicarbonitrile (6a). Yellow solid; (MeOH), yield (86%), mp 195°C; IR (KBr, cm^{-1}) υ 3502 (OH), 3302–3219 (NH_2), 2221 (CN), 1657 (CO). ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.69–3.71 (m, 3H, 2H-6' and H-5'), 3.90 (t, 1H, H-4'), 4.19 (s, 1H, OH-2'), 4.25–4.35 (m, 2H, H-3' and H-2'), 4.45 (s, 1H, OH-3'), 5.11–5.34 (m, 2H, OH-4' and OH-6'), 5.79–5.81 (d, 1H, $J_{1'\text{-}2'}$ = 9.90 Hz, H-1'), 6.76 (s, 2H, NH_2), 7.12–7.56 (m, 5H, C_6H_5). ^{13}C NMR: δ 61.10 (C-6'), 67.73 (C-4'), 71.00 (C-2'), 74.09 (C-3'), 77.67 (C-5'), 80.63 (C-1'), 114.78 (CN), 116.78 (CN), 125.78 (Ar-C), 127.73 (2C, Ar-C), 130.00 (2C,Ar-C), 133.91 (Ar-C), 151.75 (C-5), 156.19 (C-3), 161.73 (pyridine C-4), 167.34 (CO), 172.00 (C-6). UV (EtOH): 255, 320, 380 nm. Anal. Calcd. for $C_{19}H_{18}N_4O_6S$ (430.43): C, 53.02; H, 4.22; N, 13.02; S, 7.45%. Found: C, 53.1; H, 4.1; N, 13.1; S, 7.3%.

4.1.3.2. 6-Amino-1-(4-methoxyphenyl)-4-(2',3',4',6'-hydroxy-β-D-glucopyranosylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (6b). Yellow solid; (MeOH), yield (84%), mp 185°C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.18 (s, 3H, OCH_3), 4.02–4.13 (m, 2H, 2H-6'), 4.34 (m, 2H, H-5' and H-4'), 4.60 (s, 1H, OH-2'), 4.80 (s, 1H, OH-3'), 4.91–4.95 (m, 2H, H-3' and H-2'),

5.15–5.38 (m, 2H, OH-4' and OH-6') 5.76–5.78 (d, 1H, $J_{1'\text{-}2'}$ = 10.30 Hz, H-1'), 6.58–6.60 (s, 2H, NH_2), 7.40–7.64 (m, 5H, C_6H_5). Anal. Calcd. for $C_{20}H_{20}N_4O_7S$ (460.46): C, 52.17; H, 4.38; N, 12.17; S, 6.96%. Found: C, 52.1; H, 4.2; N, 12.1; S, 6.8%.

4.1.4. General Procedure for the Synthesis of 9a–e

A mixture of each of **1a–e** (10 mmol), 2-(dimercaptomethylene) malononitrile) **7** (10 mmol) and sodium ethoxide (10 mmol) was dissolved in EtOH (30 ml). The reaction mixture was refluxed for 3 hours. The progress of the reaction was monitored by TLC until the reactants disappeared, then the solution was concentrated, acidified with dilute HCl, and the remaining residue was triturated with MeOH to afford **9a–e**, which was purified by column chromatography (petroleum ether 40–60/ethyl acetate 3:1) and recrystallized from the appropriate solvent.

4.1.4.1. 6-Amino-4-mercapto-2-oxo-1-phenyl-1,2-dihydropyridine-3,5-dicarbonitrile (9a). Brown solid; (MeOH), yield (89%), mp 185–186°C, IR (KBr, cm^{-1}) υ 3437,3378 (NH_2), 3049 (CH), 2217 (CN), 1640 (CO). ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.67 (s, 2H, NH_2), 7.24–7.49 (m, 5H, C_6H_5), 11.67 (s, 1H, SH). ^{13}C NMR: δ 61.00 (C-5), 113.09 (C-3), 115.87 (2C, Ar-c), 117.00 (2CN), 123.99 (Ar-C), 127.54 (2C, Ar-C), 158.00 (C-6), 160.00 (C-4), 162.33 (Ar-C), 166.00 (CO). Anal. Calcd. for $C_{13}H_8N_4OS$ (268.29): C, 58.20; H, 3.01; N, 20.88; S, 11.95%. Found: C, 58.4; H, 3.2; N, 20.8; S, 11.8%.

4.1.4.2. 6-Amino-4-mercapto-1-(4-methylphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (9b). Brown solid; (MeOH), yield (83%), mp 198°C, ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.36 (s, 3H, CH_3), 6.32 (s, 2H, NH_2), 7.20–7.41 (m, 4H, C_6H_4), 11.04 (s, 1H, SH); Anal. Calcd. for $C_{14}H_{10}N_4OS$ (282.32): C, 59.56; H, 3.57; N, 19.85; S, 11.36%. Found: C, 59.5; H, 3.6; N, 19.7; S, 11.2%.

4.1.4.3. 6-Amino-4-mercapto-1-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (9c). Brown solid; (MeOH), yield (82%), mp 182°C, ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.81 (s, 3H, OCH_3), 6.60 (s, 2H, NH_2), 7.33–7.90 (m, 4H, C_6H_4), 11.61 (s, 1H, SH). ^{13}C NMR: δ 57.31 (CH_3), 62.24 (C-5), 110.12 (C-3), 113.25 (2C, Ar-c), 115.22 (2CN), 122.43 (Ar-C), 129.14 (2C, Ar-C), 159.12 (C-6), 159.85 (C-4), 160.10 (Ar-C), 165.21 (CO). Anal. Calcd. for $C_{14}H_{10}N_4O_2S$ (298.32): C, 56.37; H, 3.38; N, 18.78; S, 10.75%. Found: C, 56.4; H, 3.3; N, 18.6; S, 10.6%.

4.1.4.4. 6-Amino-1-(4-chlorophenyl)-4-mercapto-2-oxo-1,2-dihydropyridine-3,5 dicarbonitrile (9d). Brown solid; (MeOH), yield (80%), mp 179°C, ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.81(s, 2H, NH_2), 7.15–7.30 (m, 4H, C_6H_4), 13.15 (s, 1H, SH). Anal. Calcd. for $C_{13}H_7ClN_4OS$ (302.74): C, 51.58; H, 2.33; Cl, 11.71; N, 18.51; S, 10.59%. Found: C, 56.3; H, 2.2; Cl, 11.7; N, 18.6; S, 10.5%.

4.1.4.5. 6-Amino-4-mercapto-1-(naphthalen-2-yl)-2-oxo-1,2-dihydropyridine-3, 5 dicarbonitrile (9e). Brown solid; (MeOH), yield (79%), mp 200°C, IR (KBr, cm^{-1}) υ 3446, 3386 (NH_2), 2963 (CH), 2189 (CN), 1626 (CO); ^{1}H

NMR (500 MHz, DMSO-d_6): δ 6.25 (s, 2H, NH_2), 7.01–7.47 (m, 7H, $C_{10}H_7$), 11.82 (s, 1H, SH). Anal. Calcd. for $C_{17}H_{10}N_4OS$ (318.35): C, 64.14; H, 3.17; N, 17.60; S, 10.07%. Found: C, 64.2; H, 3.2; N, 17.5; S, 10.1%.

4.1.5. General Procedure for the Synthesis of (10a–e)

4.1.5.1. Method A. A solution of compounds **1a–e** (10 mmol), 2-(bis(methylthio)methylene)malononitrile **8** (10 mmol), and sodium ethoxide (10 mmol) was dissolved in EtOH (20 ml). The reaction was refluxed for 3 hours, monitored by TLC (petroleum ether 40–60–ethyl acetate 3:1) until the reactants disappeared. The solvent was then evaporated and the remaining residue was triturated with MeOH to afford compounds **10a–e**, which was separated by column chromatography and recrystallized from the appropriate solvent.

4.1.5.2. Method B. A solution of compounds **9a–e** (10 mmol) in sodium ethoxide (10 mmol) was stirred at 50°C for 30 minutes and allowed to cool to room temperature and then methyl iodide (10 mmol) was slowly added to the mixture. Then the reaction mixture was refluxed with stirring for 3 hours. The formed solid product was collected by filtration and recrystallized from appropriate solvent.

4.1.5.3. 6-Amino-4-(methylthio)-2-oxo-1-phenyl-1,2-dihydropyridine-3,5-dicarbonitrile (10a). Brown solid; (MeOH), yield (80%), mp 290–291°C, IR (KBr, cm^{-1}) υ 3405, 3322 (NH_2), 3044 (CH), 2947 (CH), 2207 (CN), 1650 (CO). ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.69–2.71 (S, 3H, SCH_3), 6.52 (s, 2H, NH_2), 7.29–7.66 (m, 5H, C_6H_5). Anal. Calcd. for $C_{14}H_{10}N_4OS$ (282.32): C, 59.56; H, 3.57; N, 19.85; S, 11.36%. Found: C, 59.5; H, 3.4; N, 19.7; S, 11.4%.

4.1.5.4. 6-Amino-4-(methylthio)-2-oxo-1-p-tolyl-1,2-dihydropyridine-3,5-dicarbonitrile (10b). Brown solid; (MeOH), yield (80%), mp 260°C, IR (KBr, cm^{-1}) υ 3401, 3312 (NH_2), 3034 (CH), 2947 (CH), 2207 (CN), 1645 (CO). ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.38 (s, 3H, CH_3), 2.78 (s, 3H, SCH_3), 6.45 (s, 2H, NH_2), 7.16–7.44 (m, 5H, C_6H_5). Anal. Calcd. for $C_{15}H_{12}N_4OS$ (296.35): C, 60.79; H, 4.08; N, 18.91; S, 10.82%. Found: C, 60.8; H, 4.2; N, 18.8; S, 10.7%.

4.1.5.5. 6-Amino-1-(4-methoxyphenyl)-4-(methylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (10c). Brown solid; (MeOH), yield (78%), mp 257°C, ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.35–2.43 (s, 3H, SCH_3), 3.84 (s, 3H, OCH_3), 6.43 (s, 2H, NH_2), 7.14–7.53 (m, 5H, C_6H_5). Anal. Calcd. for $C_{15}H_{12}N_4O_2S$ (312.35): C, 57.68; H, 3.87; N, 17.94; S, 10.27%. Found: C, 57.8; H, 3.7; N, 17.8; S, 10.1%.

4.1.5.6. 6-Amino-1-(4-chlorophenyl)-4-(methylthio)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (10d). Brown solid; (MeOH), yield (79%), mp 282°C (MeOH); IR (KBr, cm^{-1}) υ 3421, 3332 (NH_2), 3044 (CH), 2949 (CH), 2219 (CN), 1696 (CO). Anal. Calcd. for $C_{14}H_9ClN_4OS$ (316.77): C, 53.08;

H, 2.86; Cl, 11.19; N, 17.69; S, 10.12%. Found: C, 53.1; H, 2.7; Cl, 11.2; N, 17.7; S, 10.1%.

4.1.5.7. 6-Amino-4-(methylthio)-1-(naphthalen-2-yl)-2-oxo-1,2-dihydropyridine-3,5-dicarbonitrile (10e). Brown solid; (MeOH), yield (80%), mp 255°C IR (KBr, cm^{-1}) υ 3407, 3314 (NH_2), 3042 (CH), 2942 (CH), 2221 (CN), 1697 (CO). Anal. Calcd. for $C_{18}H_{12}N_4OS$ (332.38): C, 65.04; H, 3.64; N, 16.86; S, 9.65%. Found: C, 65.1; H, 3.5; N, 16.9; S, 9.6%.

4.1.6. General Procedure for the Synthesis of Pyrazolo[3,4-c]pyridines 11a–e

A mixture of **5a–j** or **10a–e** (10 mmol) and hydrazine hydrate (10 mmol) was dissolved in ethanol (20 ml), and then a few drops of triethylamine were added. The mixture was refluxed for 3 hours. The resulting precipitated solid was filtered off and recrystallized from ethanol.

4.1.6.1. 3,5-Diamino-7-oxo-6-phenyl-6,7-dihydro-1H-pyrazolo[3,4-c]pyridine-4-carbonitrile (11a). Yellow solid, mp > 300°C; IR (KBr, cm^{-1}) υ 3214 (NH, NH_2), 2198 (CN), and 1660 (CO). Anal. Calcd. for $C_{13}H_{10}N_6O$ (266.26): C, 58.7; H, 3.8; N, 31.6%. Found: C, 58.9; H, 4.0; N, 31.2%. M^+, 266.

4.1.6.2. 3,5-Diamino-6-(4-methylphenyl)-7-oxo-6,7-dihydro-1H-pyrazolo[3,4-c]pyridine-4-carbonitrile (11b). Yellow solid, mp 253°C; IR (KBr, cm^{-1}) υ 3500–3211 (NH, NH_2), 2205 (CN), and 1690–1660 (CO). Anal. Calcd. for $C_{14}H_{12}N_6O$ (280.28): C, 60.0; H, 4.3; N, 30.0%. Found: C, 59.6; H, 4.5; N, 30.4%. M^+, 280.

4.1.6.3. 3,5-Diamino-6-(4-methoxyphenyl)-7-oxo-6,7-dihydro-1H-pyrazolo[3,4-c]pyridine-4-carbonitrile (11c). Yellow solid, mp > 300°C; IR (KBr, cm^{-1}) υ 3411, 3228 (NH, NH_2), 2197 (CN), and 1680 (CN). Anal. Calcd. for $C_{14}H_{12}N_6O_2$ (296.28): C, 56.8; H, 4.1; N, 28.4%. Found: C, 56.5; H, 4.3; N, 28.1%. M^+, 296.

4.1.6.4. 3,5-Diamino-6-(4-chlorophenyl)-7-oxo-6,7-dihydro-1H-pyrazolo[3,4-c]pyridine-4-carbonitrile (11d). Yellow solid, mp > 300°C; IR (KBr, cm^{-1}) υ 3401, 3328, 3230 (NH, NH_2), 2202 (CN), and 1644 (CO). Anal. Calcd. for $C_{13}H_9ClN_6O$ (300.70): C, 51.92; H, 3.02; N, 27.95%. Found: C, 51.8; H, 3.1; N, 27.8%; M^+, 300.

4.1.6.5. 3,4-Diamino-5-(naphthalen-2-yl)-6-oxo-5,6-dihydro-1H-pyrazolo[4,3-c]pyridine-7-carbonitrile (11e). Yellow solid, mp > 300°C; IR (KBr, cm^{-1}) υ 3418, 3336, 3241 (NH, NH_2), 2217 (CN), 1639 (CO). Anal. Calcd for $C_{17}H_{12}N_6O$ (300.70): C, 64.55; H, 3.82; N, 26.57%. Found: C, 64.4; H, 3.7; N, 26.5%.

4.2. Antitumor Screening

Cell viability was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan.[19]

Procedure: All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, and Sanford, ME, USA). Cells were suspended in RPMI 1640 medium for HEPG2-PC3 and HCT116–DMEM for A549. The media are supplemented with 1% antibiotic–antimycotic mixture (10,000 U/ml Potassium Penicillin, 10,000 μg/ml Streptomycin Sulfate, and 25 μg/ml Amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37°C under 5% CO_2. Cells were batch cultured for 10 days, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37°C for 24 hours under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added, and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100-50-25-12.5-6.25-3.125-0.78 and 1.56 ug/ml). After 48 hours of incubation, medium was aspirated, 40 ul MTT salt (2.5 μg/ml) were added to each well and incubated for further 4 hours at 37°C under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200 μl of 10% sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37°C. A positive control composed of 100 μg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent *t*-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula: ((Reading of extract/Reading of negative control) − 1) × 100. A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

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