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FACILE AND CONVENIENT SYNTHESIS OF NEW ISOXAZOLO[5,4-*b*]PYRIDINE, PYRROLO[3,2-*d*]ISOXAZOLE, ISOXAZOLO[5,4-*b*]-AZEPINE-4,7-DIONE AND ISOXAZOLE DERIVATIVES WITH POTENTIAL ANTICANCER ACTIVITY

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Abstract – The readily available 3-methylisoxazol-5-amine (**1**) has been used as a key intermediate for the synthesis of isoxazolo[5,4-*b*]pyridine, pyrrolo[3,2-*d*]isoxazole, isoxazolo[5,4-*b*]azepine-4,7-dione and isoxazole derivatives *via* its reactions with some chemical reagents. Sixteen compounds were designed to study their cytotoxic properties against three human cell lines: colorectal carcinoma (HCT-116), prostate cancer (PC3) and normal lung fibroblast (WI-38), using the MTT colorimetric assay. 5-Fluorouracil, a well-known anticancer drug, was used for comparison. Among the tested candidates, pyrrolo[3,2-*d*]isoxazole **5** and isoxazole derivatives **11**-**14** showed the highest activity against the tested cancer cell lines with good selectivity by their lower toxicity against normal cells.

INTRODUCTION

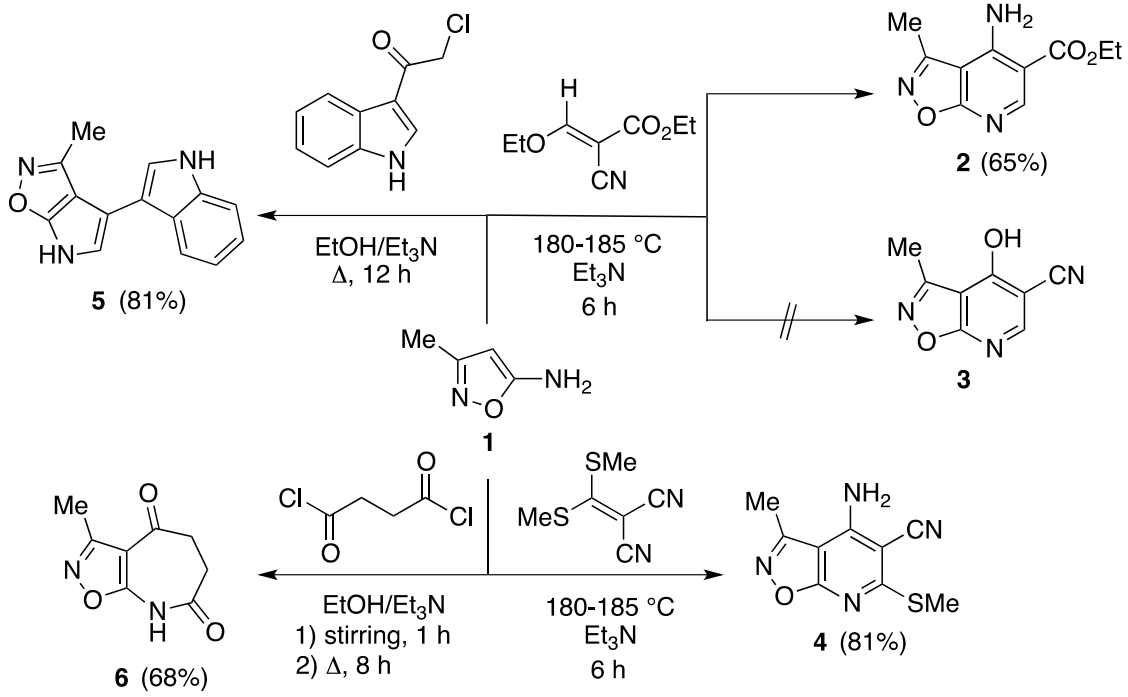
The biological activity spectrum of isoxazoles and their fused heterocyclic derivatives is quite wide. They have remarkable antitumor,[1] antiviral,[2] anti-inflammatory,[3] antifungal,[4] antimicrobial,[5] anticonvulsant,[6] anti-hyperglycemic,[7] antithrombotic,[8] anti-nociceptive,[9] analgesic,[10] antioxidant,[11] herbicidal, insecticidal and anti-parasitic[12] activities. In addition, they show $GABA_A$ antagonist[13] and T-type Ca^{2+} channel blocking activities,[14] protein tyrosine phosphatase 1B, Aβ precursor protein inhibitors,[15,16] and act as regulators of immune functions.[17] Methylene bis-isoxazoles have shown to be 100% effective against PMA-stimulated neutrophils.[18] Compounds containing the isoxazole ring are in the center of medicinal chemists because of their effectiveness in the treatment of cancer. For example, Luminespib

(NVP-AUY922)[19] is active against a variety of tumor xenografts and has been examined in phase II clinical trials,[20] Leflunomide has been identified as a potential anticancer drug,[21] and a naturally occurring diarylisoxazole derivative that was active against AR-expressing breast cancer cells.[22] The marketed drugs of isoxazole, such as Sulfamethoxazole, Sulfisoxazole, Oxacillin, Cycloserine, Isoxaflutole, Drazoxol, Acetylsulfisoxazole, Zonisamide and Acivicin have great medicinal value. Given the immense biological potential associated with isoxazoles, and as a continuation of our interest on the synthesis of bioactive molecules,[23-25] it would be worthwhile to focus on the synthesis of these derivatives and evaluating their anticancer activities. In the present study, 3-methylisoxazol-5-amine (**1**)[26] serves as a good intermediate to achieve this goal.

RESULTS AND DISCUSSION

The solvent-free reaction of 3-methylisoxazol-5-amine (**1**) with ethyl 2-cyano-3-ethoxyacrylate at 180-185 °C in the presence of three drops of triethylamine gave a brown product for which two possible structures, **2** and **3**, could be formulated (Scheme 1). The spectroscopic data were in perfect agreement with ethyl 4-amino-3-methylisoxazolo[5,4-*b*]pyridine-5-carboxylate (**2**) as its IR spectrum showed amino and carbonyl of ester stretching frequencies at 3455-3380 and 1691 cm^{-1}, respectively. The ^{1}H-NMR spectrum of **2** presented a set of signals centered at δ 4.21 and 1.26 ppm typical of the ethyl ester group, in addition to a broad singlet signal at δ 5.87 ppm specific for NH_2 protons. Under the same experimental conditions, fusion of isoxazole **1** with 2-(bis(methylthio)methylene)malononitrile gave directly the isoxazolo[5,4-*b*]pyridine derivative **4**, which provided satisfactory elemental analyses and spectroscopic data. Compound **4** was assumed to be formed through the nucleophilic addition of the amino group of isoxazole **1** to the activated double bond of 2-(bis(methylthio)methylene)malononitrile, followed by elimination of methylthiol, and subsequent cyclization and aromatization *via* the nucleophilic addition of isoxazole-C4 to the nitrile function. Many 3-substituted indole derivatives exhibit strong inhibitory effects toward diverse types of tumor cell lines, including ovarian cancer, renal cancer, colon cancer, breast cancer, non-small cell lung cancer, and leukemia.[27] Based on this finding, 4-(1*H*-indol-3-yl)-3-methyl-6*H*-pyrrolo[3,2-*d*]isoxazole (**5**) was obtained in 81% yield by refluxing a mixture of isoxazole **1** with 2-chloro-1-(1*H*-indol-3-yl)ethanone[28] in EtOH/Et_3N. The reaction proceeds *via* nucleophilic displacement of the chlorine atom from 2-chloro-1-(1*H*-indol-3-yl)ethanone, followed by intramolecular cyclization with the elimination of water. The ^{1}H-NMR spectrum of **5** exhibited indole-H2 and pyrrole-H2 protons as singlet signals at δ 8.31 and 6.43 ppm, respectively, in addition to two singlet signals at δ 12.20 and 11.47 ppm attributable to two NH protons. Its mass spectrum showed the parent molecular ion peak *m/z* = 237 (M^+, 28.86%), confirming the supposed structure. Stirring **1** with succinyl

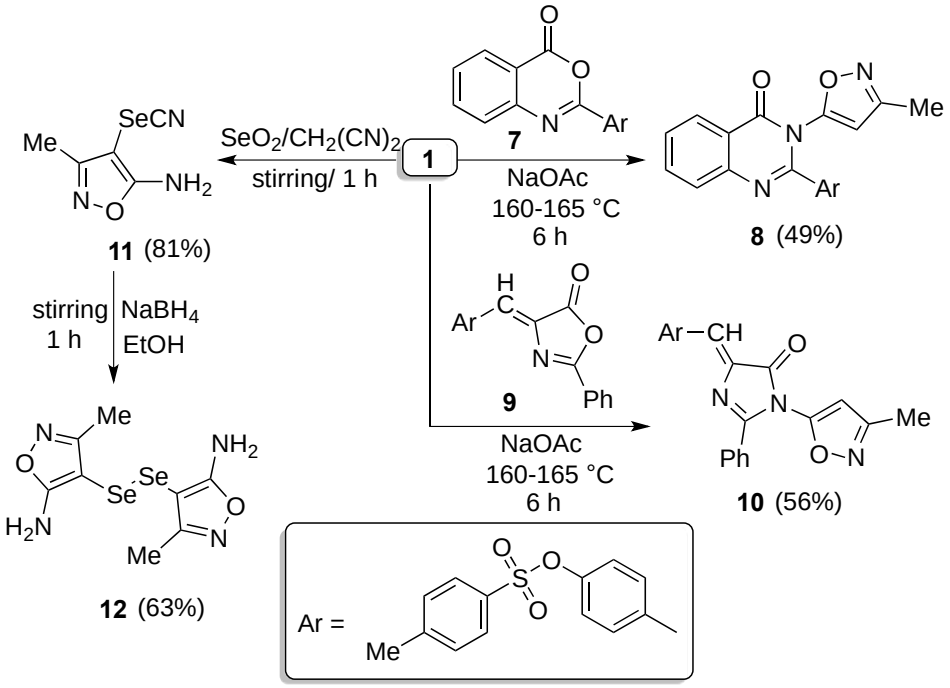
dichloride in ethanol containing four drops of triethylamine, followed by refluxing at 80 °C yielded exclusively the isoxazolo[5,4-*b*]azepine-4,7-dione **6**.



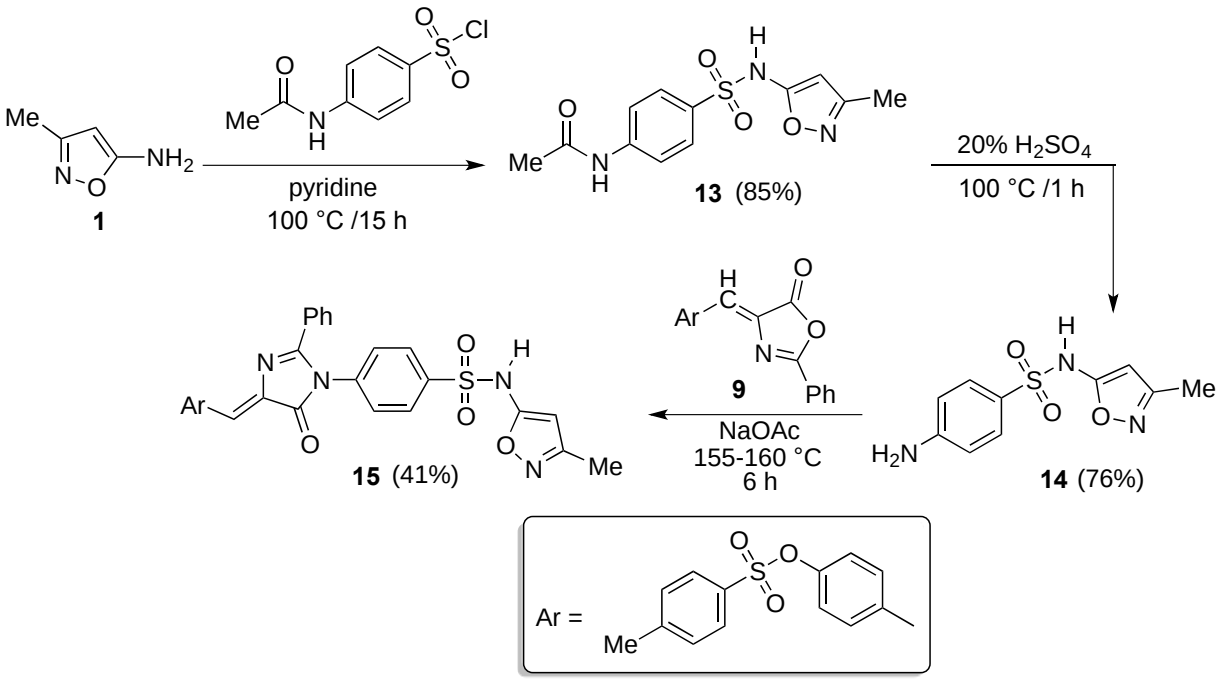
Scheme 1

Quinazolines and imidazolines are considered to be important heterocycles because of their pharmacological activities especially with anticancer.[29] Herein, we decided to introduce the isoxazole pharmacophore into quinazoline and imidazoline to obtain new scaffolds as cytotoxic agents. Fusion of **1** with each of benzo[*d*][1,3]oxazin-4-one **7**[30] and oxazol-5-one **9**[31] at 160-165 °C in the presence of sodium acetate provided directly quinazolinone **8** and imidazolinone **10**, respectively (Scheme 2). There is a growing interest in the synthesis of organoselenium compounds which are less toxic and could be used as chemo-preventive agents for cancer.[32] In this context, addition of isoxazole **1** to triselenium dicyanide [selenocyanating reagent formed by the interaction of selenium dioxide and malononitrile in dimethyl sulfoxide] gave the corresponding selenocyanate **11** in one-pot. The latter **11** was supported on the basis of IR spectrum which exhibited a nitrile stretching frequency at 2217 cm^{-1}. Besides, ^{1}H-NMR spectrum displayed a lack of the isoxazole-H4 proton. Selenocyanates are important in seleno-organic chemistry; that can be transformed into various seleno-organic derivatives.[32] Thus, stirring **11** with an excess amount of reducing agent such as sodium borohydride ($NaBH_4$) in ethanol gave the diselenide **12**. Its mass spectrum showed the parent molecular ion peak *m/z* = 353 (M^++1, 37.65), confirming the supposed structure. Shifting to Scheme 3, sulfonamide derivatives have shown wonderful anticancer activity toward various cancer cell lines.[33] Prompted by this fact, we emphasize that the introduction of a sulfonamide

moiety into the isoxazole skeleton may be potentially useful for the development of new anticancer active compounds. So, isoxazole **1** reacted with 4-acetamidobenzenesulfonyl chloride in pyridine at 100 °C to afford the sulfonamide derivative **13** with 85% yield. Acid hydrolysis of the latter **13** gave 4-amino-*N*-(3-methylisoxazol-5-yl)benzenesulfonamide (**14**), which could be condensed with oxazol-5-one **9** at 155-160 °C in the presence of sodium acetate to afford the imidazolinone **15**.

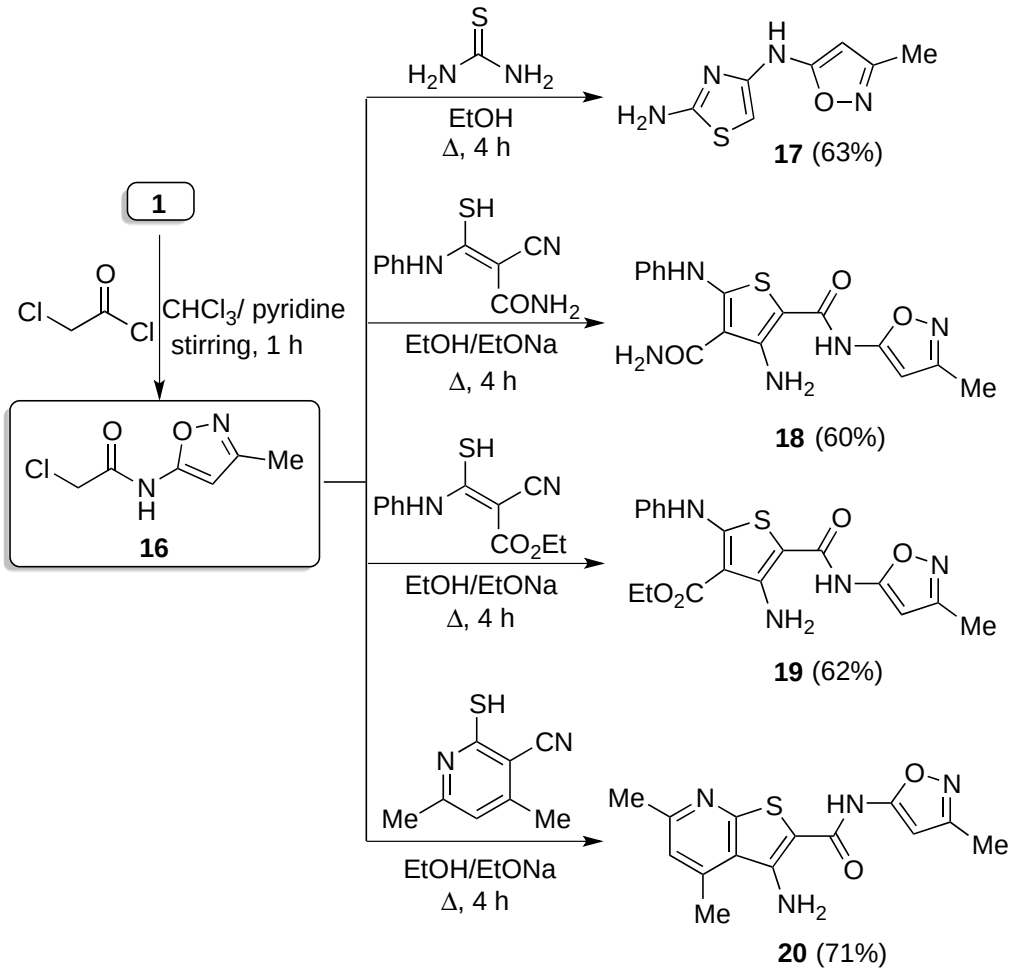


Scheme 2



Scheme 3

Previously, the reaction of isoxazole **1** with 2-chloroacetyl chloride in $CHCl_3$/pyridine to afford 2-chloro-*N*-(3-methylisoxazol-5-yl)acetamide (**16**) has been investigated (Scheme 4).[1] As an extension of our study, we utilized **16** as a convenient precursor for the construction of some heterocyclic ring systems incorporating the isoxazole backbone. Thus, when **16** was subjected to react with thiourea in ethanol, it provided exclusively the thiazole **17**. Besides, the reaction of **16** with each of 2-cyano-3-mercapto-3-(phenylamino)acrylamide and ethyl 2-cyano-3-mercapto-3-(phenylamino)acrylate[34] in an ethanolic solution of sodium ethoxide provided the corresponding thiophenes **18** and **19**, respectively. Moreover, the reflux of an equimolar amounts of **16** and 2-mercapto-4,6-dimethylnicotinonitrile under the same experimental conditions furnished the thieno[2,3-*b*]pyridine **20**. The IR spectrum of **20** revealed the presence of NH_2 bands at 3424-3320 cm^{-1} region and the absence of any absorption due to the nitrile function. Besides, the ^{1}H-NMR spectrum displayed a singlet signal at δ 6.50 ppm for the protons of amino function, confirming the cyclized structure. The formation of thiophenes **18** and **19** and thieno[2,3-*b*]pyridine **20** proceeds *via* nucleophilic displacement of the chlorine atom from **16**, followed by intramolecular cyclization through the nucleophilic addition of active methylene to the nitrile function.



Scheme 4

IN VITRO CYTOTOXIC ACTIVITY

The cytotoxicity of sixteen compounds was screened using the MTT assay[35,36] at the National Institute of Cancer, Cairo, Egypt against three human cell lines: HCT-116, PC3 and WI-38. 5-Fluorouracil (5-Fu), a well-known anticancer drug, was used for comparison and the data are listed in Table 1. In general, most of the compounds displayed good cytotoxicity which was more pronounced in HCT-116 cells than in PC3 cells. As for activity against HCT-116, most of the compounds are able to inhibit the growth of the cancer cells, and in some cases display better inhibition than the known anticancer medication 5-fluorouracil. The data reveal that compounds **5**, **8**, **10**-**14** are very strong active agents (IC_{50} = 6.3, 9.7, 8.2, 8.8, 5.0, 4.4, and 5.1 µM, respectively). Also, compounds **4** and **20** have remarkable activity with an IC_{50} values at 18.1 and 14.1 µM, respectively. The rest of tested compounds **2** and **15**-**19** show moderate activity range of 21.8-49.1 µM. Only one compound **6** have no cytotoxic effect. In the case of PC3 cells, it was obvious that compounds **5**, **11**-**15** and **17** were found to be the most potent derivatives with an IC_{50} values range of 8.0-18.0 µM. Besides, compounds **4**, **6**, **8**, **10**, **19** and **20** possessed moderate activities, while compounds **2**, **16** and **18** appeared to be weak or inactive. To further examine if our compounds have a cytotoxic effect, they were evaluated against non-tumorigenic cells (WI-38). The results indicated that most of the compounds exhibited moderate to weak cytotoxic effect range of 31.1-92.5 µM. Only one compound **2** have strong cytotoxic effect. Interestingly, a promising selectivity was observed in compounds **5** and **11**-**14**, since they showed weak cytotoxic effects on normal cells (WI-38), compared to the investigated cancer cells. So, these active candidates may serve as lead compounds in search for selective and powerful anticancer agents. By observing the results, we were able to deduce valuable data on structure-activity relationships. (1) We explored the effect of substitution on 6-position of the isoxazolo[5,4-*b*]pyridine moiety. The decrease in the IC_{50} value of compound **4** against both cancer cell lines with incorporated the SMe group compared to the corresponding **2** indicated that substitution is more advantageous than unsubstituted one. (2) As an expected, the introduction of a heterocyclic substituent such as pyrrolo[3,2-*d*]isoxazole into position 3 of the indole nucleus (compound **5**) led to a significant cytotoxic effect. (3) Quinazolinone **8** and imidazolinone **10** exhibited wonderful anticancer activity, which was more pronounced in HCT-116 cells, while imidazolinone **15** was more pronounced in PC3 cells. (4) The observed cytotoxic activity of selenocyanate **11** and diselenide **12** confirms the significant effect of organoselenium compounds as chemo-preventive agents for cancer. (5) The synthesized sulfonamides **13** and **14** manifested good anticancer activities comparable to 5-fluorouracil. Further, the results not only confirmed the selectivity of these compounds, but also indicated their lower toxicity against normal cells. (6) The introduction of some heterocycles such as thiazole, thiophene and thieno[2,3-*b*]pyridine to the 2-chloroacetamide derivative **16** enhanced the anticancer activities.

Table 1. *In vitro* cytotoxic activity of the synthesized compounds using the MTT colorimetric technique

Compounds	IC_{50} (µM)[a]		
	HCT-116	**PC3**	**WI-38**
2	34.8±2.0	54.3±3.6	12.1±1.4
4	18.1±2.6	29.1±1.3	35.1±2.6
5	6.3±3.2	18.0±0.5	85.4±3.5
6	>100	28.1±1.3	31.1±0.5
8	9.7±1.5	35.4±2.8	61.8±3.6
10	8.2±2.7	27.9±3.6	68.9±3.8
11	8.8±1.1	10.8±0.2	75.4±3.6
12	5.0±0.8	8.3±1.7	80.8±4.4
13	4.4±1.9	14.5±0.4	92.5±4.6
14	5.1±3.2	8.0±3.6	88.1±2.6
15	38.8±2.8	16.7±3.9	74.2±3.3
16	49.1±2.3	>100	46.8±1.6
17	32.4±0.5	15.5±2.7	43.5±1.0
18	37.0±2.0	60.7±4.1	59.7±2.5
19	21.8±3.3	41.0±2.0	31.7±1.9
20	14.1±1.7	33.8±1.8	43.3±2.8
5-Fu	5.2±0.2	8.3±0.3	4.3±0.6

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

3-Methylisoxazol-5-amine (**1**) has been used as a key intermediate for the synthesis of isoxazolo[5,4-*b*]pyridine, pyrrolo[3,2-*d*]isoxazole, isoxazolo[5,4-*b*]azepine-4,7-dione and isoxazole derivatives. Sixteen compounds were evaluated for their cytotoxic properties against two cancer cell lines: HCT-116 and PC3. The selective cytotoxicity was also tested against non-tumorigenic cells (WI-38). Most of the compounds displayed good cytotoxicity which was more pronounced in HCT-116 cells than in PC3 cells. Also, pyrrolo[3,2-*d*]isoxazole **5** and isoxazole derivatives **11**-**14** showed the highest activity against the tested cancer cell lines with good selectivity by their lower toxicity against normal cells. So, these active candidates may serve as lead compounds in search for selective and powerful anticancer agents.

EXPERIMENTAL

Melting points were determined on an electrothermal Gallenkamp apparatus (Germany) and are uncorrected. The IR spectra were measured on a Mattson 5000 FTIR Spectrometer (USA) in potassium bromide discs. The ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker Avance III spectrometer (Germany) at 400 and 100 MHz, respectively. The mass spectra were recorded on Kratos MS (Kratos Analytical Instrument, Ramsey, NJ) apparatus (USA) and the ionizing voltage was 70 ev. Elemental analyses have been achieved by the Micro-analytical unit of Faculty of Science, Cairo University, Egypt. All reactions in the present consideration have been followed by TLC (silica gel, aluminum sheets 60 F254, Merck).

Synthesis of isoxazolo[5,4-*b*]pyridine derivatives (2) and (4). A mixture of 3-methylisoxazol-5-amine (**1**) (0.98 g, 0.01 mol) and ethyl 2-cyano-3-ethoxyacrylate or 2-(bis(methylthio)methylene)malononitrile (0.01 mol) was heated at 180-185 °C in the presence of a base such as triethylamine (three drops) for 6 h in an oil bath. After cooling, the solid formed was washed with petroleum ether, dried well, and recrystallized from EtOH.

Ethyl 4-amino-3-methylisoxazolo[5,4-*b*]pyridine-5-carboxylate (2): yield 1.44 g (65%); brown crystals; mp 238-240 °C; IR (KBr, cm^{-1}) ν_{max}: 3455-3380 (NH_2), 2984, 2934 (C-H, aliphatic), 1691 (C=O, ester), 1646 (C=N), 1590 (C=C); ^{1}H-NMR (DMSO) δ (ppm): 8.18 (s, 1H, pyridine-H2), 5.87 (br, s, 2H, NH_2), 4.21 (q, 2H, CH_2-ester, J = 7.2 Hz), 2.20 (s, 3H, CH_3), 1.26 (t, 3H, CH_3-ester, J = 7.2 Hz); ^{13}C-NMR (DMSO) δ (ppm): 163.91, 162.17 (C=O), 161.94, 152.05, 150.80, 88.77, 79.56 (Ar-C), 61.51 (CH_2-ester), 14.66 (CH_3-ester), 11.82 (CH_3); MS (*m/z*, %): 221.18 (M^+, 25.78), 209.01 (33.19), 191.72 (76.83), 113.88 (67.10), 105.64 (100.00), 91.55 (33.67), 81.00 (57.58), 74.79 (51.60), 43.18 (35.30). Anal. Calcd for $C_{10}H_{11}N_3O_3$ (221.22): C, 54.30; H, 5.01; N, 19.00%. Found: C, 53.96; H, 4.87; N, 18.86%.

4-Amino-3-methyl-6-(methylthio)isoxazolo[5,4-*b*]pyridine-5-carbonitrile (4): yield 1.78 g (81%); brown powder; mp 275-277 °C; IR (KBr, cm^{-1}) ν_{max}: 3449-3370 (NH_2), 2965, 2927 (C-H, aliphatic), 2196 (CN), 1646 (C=N), 1559 (C=C); ^{1}H-NMR (DMSO) δ (ppm): 6.49 (br, s, 2H, NH_2), 2.65 (s, 3H, CH_3S), 2.35 (s, 3H, CH_3); MS (*m/z*, %): 221.81 (M^++1, 25.05), 220.81 (M^+, 42.32), 209.24 (42.57), 199.82 (27.50), 177.15 (66.04), 140.49 (46.06), 111.23 (48.40), 81.09 (61.86), 58.37 (100.00), 42.41 (25.27). Anal. Calcd for $C_9H_8N_4OS$ (220.25): C, 49.08; H, 3.66; N, 25.44%. Found: C, 48.78; H, 3.36; N, 25.19%.

Synthesis of 4-(1*H*-indol-3-yl)-3-methyl-6*H*-pyrrolo[3,2-*d*]isoxazole (5). To a solution of 3-methylisoxazol-5-amine (**1**) (0.98 g, 0.01 mol) in 30 mL EtOH and Et_3N (four drops), 2-chloro-1-(1*H*-indol-3-yl)ethanone (1.93 g, 0.01 mol) was added. The mixture was heated under reflux for 12 h, and then poured onto ice-water. The pyrrolo[3,2-*d*]isoxazole that formed was isolated by filtration and purified by recrystallization from EtOH; yield 1.92 g (81%); yellow crystals; mp 210-212 °C; IR (KBr, cm^{-1}) ν_{max}: 3369, 3214 (2 NH), 2959, 2897 (C-H, aliphatic), 1662 (C=N), 1606 (C=C);

^{1}H-NMR (DMSO) δ (ppm): 12.20 (s, 1H, NH), 11.47 (s, 1H, NH), 8.31 (s, 1H, indole-H2), 8.01-7.13 (m, 4H, Ar-H), 6.43 (s, 1H, pyrrole-H2), 2.30 (s, 3H, CH_3); ^{13}C-NMR (DMSO) δ (ppm): 156.63, 151.19, 149.93, 142.58, 137.03, 126.35, 125.41, 122.20, 120.20, 112.14, 109.04, 95.55, 85.30 (Ar-C), 19.06 (CH_3); MS (*m/z*, %): 237.63 (M^+, 28.86), 236.71 (M^+-1, 68.07), 214.56 (30.17), 192.68 (41.58), 170.33 (58.65), 123.55 (50.94), 116.09 (35.86), 98.38 (100.00), 63.88 (48.02), 56.90 (57.85), 50.16 (66.01). Anal. Calcd for $C_{14}H_{11}N_3O$ (237.26): C, 70.87; H, 4.67; N, 17.71%. Found: C, 70.67; H, 4.37; N, 17.45%.

Synthesis of 3-methyl-5,6-dihydro-4*H*-isoxazolo[5,4-*b*]azepine-4,7(8*H*)-dione (6). To a solution of 3-methylisoxazol-5-amine (**1**) (0.98 g, 0.01 mol) in 30 mL EtOH containing Et_3N (four drops), succinyl dichloride (1.54 g, 0.01 mol) was added with the temperature maintained at 0-10 °C. The reaction mixture was stirred for 1 h, and then heated under reflux for 8 h. The isoxazolo[5,4-*b*]azepinone which formed after pouring onto ice-water was filtered off and purified by recrystallization from EtOH; yield 1.23 g (68%); white crystals; mp 178-180 °C; IR (KBr, cm^{-1}) ν_{max}: 3448 (NH), 2935, 2857 (C-H, aliphatic), 1689 (br, 2 C=O), 1609 (C=N), 1554 (C=C); ^{1}H-NMR (DMSO) δ (ppm): 11.62 (s, 1H, NH), 2.59 (m, 2H, CH_2), 2.51 (m, 2H, CH_2), 2.34 (s, 3H, CH_3); ^{13}C-NMR (DMSO) δ (ppm): 174.05 (C=O), 170.64 (C=O), 160.69, 159.11, 78.29 (Ar-C), 32.42 (CH_2), 29.26 (CH_2), 11.76 (CH_3); MS (*m/z*, %): 180.38 (M^+, 17.67), 155.79 (66.92), 121.02 (90.25), 101.28 (47.56), 84.54 (52.10), 74.21 (73.56), 68.06 (48.37), 44.67 (100.00), 43.16 (44.98). Anal. Calcd for $C_8H_8N_2O_3$ (180.16): C, 53.33; H, 4.48; N, 15.55%. Found: C, 53.21; H, 4.31; N, 15.27%.

Synthesis of quinazolin-4-one (**8**) **and imidazol-5-one (10).** A mixture of 3-methylisoxazol-5-amine (**1**) (0.98 g, 0.01 mol), fused sodium acetate (1.64 g, 0.02 mol) and benzo[*d*][1,3]oxazin-4-one **7** or oxazol-5-one **9** (0.01 mol) was heated at 160-165 °C for 6 h in an oil bath. After cooling, the solid formed was washed with petroleum ether and recrystallized from EtOH.

4-(3-(3-Methylisoxazol-5-yl)-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 4-methylbenzenesulfonate (8): yield 2.32 g (49%); brown powder; mp 211-213 °C; IR (KBr, cm^{-1}) ν_{max}: 2921, 2840 (C-H, aliphatic), 1656 (CO, amidic), 1604 (C=N), 1548 (C=C), 1376 (SO_3); ^{1}H-NMR (DMSO) δ (ppm): 8.20-7.26 (m, 12H, Ar-H), 6.50 (s, 1H, isoxazole-H4), 2.43 (s, 3H, CH_3), 2.42 (s, 3H, CH_3); ^{13}C-NMR (DMSO) δ (ppm): 170.64, 160.71 (C=O), 159.17, 155.86, 152.36, 146.64, 137.37, 131.53, 130.83, 130.30, 129.64, 129.26, 128.77, 128.57, 127.42, 127.01, 123.20, 117.45, 78.28 (Ar-C), 21.66 (CH_3), 11.75 (CH_3); MS (*m/z*, %): 473.53 (M^+, 43.76), 409.33 (25.67), 390.54 (53.88), 334.89 (36.26), 302.28 (42.81), 260.54 (49.86), 232.58 (100.00), 163.25 (35.11), 130.64 (40.14), 79.35 (19.10). Anal. Calcd for $C_{25}H_{19}N_3O_5S$ (473.50): C, 63.42; H, 4.04; N, 8.87%. Found: C, 63.25; H, 3.79; N, 8.57%.

4-((1-(3-Methylisoxazol-5-yl)-5-oxo-2-phenyl-1,5-dihydro-4*H*-imidazol-4-ylidene)methyl)phenyl 4-methylbenzenesulfonate (10): yield 2.80 g (56%); brown powder; mp 254-256 °C; IR (KBr, cm^{-1}) ν_{max}: 2932, 2850 (C-H, aliphatic), 1680 (CO, amidic), 1646 (C=N), 1597 (C=C), 1373 (SO_3); ^{1}H-NMR

(DMSO) δ (ppm): 8.34-7.20 (m, 13H, Ar-H), 7.34 (s, 1H, CH=), 6.50 (s, 1H, isoxazole-H4), 2.43 (s, 3H, CH_3), 2.41 (s, 3H, CH_3); ^{13}C-NMR (DMSO) δ (ppm): 170.64, 167.20 (C=O), 164.02, 160.70, 150.89, 146.54, 134.40, 133.02, 131.69, 130.82, 129.82, 129.19, 128.75, 128.62, 125.46, 124.54, 123.10, 118.32 (CH=), 78.27 (Ar-C), 21.66 (CH_3), 11.75 (CH_3); MS (*m/z*, %): 498.99 (M^+, 13.22), 485.65 (30.17), 445.61 (20.72), 400.07 (21.12), 368.27 (41.59), 293.01 (20.20), 261.79 (35.86), 226.62 (100.00), 185.95 (33.70), 178.42 (60.97), 161.83 (70.99), 152.85 (56.54), 113.40 (36.95), 68.43 (24.66), 46.36 (42.24). Anal. Calcd for $C_{27}H_{21}N_3O_5S$ (499.54): C, 64.92; H, 4.24; N, 8.41%. Found: C, 64.65; H, 3.99; N, 8.22%.

Synthesis of 3-methyl-4-selenocyanatoisoxazol-5-amine (11). To a solution of malononitrile (0.10 g, 1.5 mmol) in 3 mL DMSO, selenium dioxide (0.33 g, 3 mmol) was added with stirring. After 10 min., the reaction mixture became reddish and 3-methylisoxazol-5-amine (**1**) (0.20 g, 2 mmol) was added with stirring. After 1 h, the homogeneous solution was diluted with 10 mL water. The selenocyanate which formed was filtered off and recrystallized from EtOH; yield 0.37 g (81%); orange crystals; mp 166-168 ^{o}C; IR (KBr, cm^{-1}) ν_{max}: 3453-3370 (NH_2), 2976, 2924 (C-H, aliphatic), 2217 (CN), 1623 (C=N), 1580 (C=C); ^{1}H-NMR (DMSO) δ (ppm): 7.14 (s, 2H, NH_2), 2.11 (s, 3H, CH_3); MS (*m/z*, %): 204.34 (M^++2, 6.47), 202.85 (M^+, 20.55), 174.92 (49.95), 157.31 (68.80), 137.40 (50.39), 117.55 (59.90), 91.87 (67.67), 81.22 (29.27), 78.04 (68.06), 76.37 (45.89), 60.45 (52.96), 42.68 (100.00). Anal. Calcd for $C_5H_5N_3OSe$ (202.09): C, 29.72; H, 2.49; N, 20.79%. Found: C, 29.55; H, 2.35; N, 20.43%.

Synthesis of 4,4'-diselanediylbis(3-methylisoxazol-5-amine) (12). Sodium borohydride (0.12 g, 3 mmol) was added portionwise to a solution of selenocyanate **11** (0.20 g, 1 mmol) in 15 mL EtOH with stirring. Stirring was continued for 1 h, and then poured onto cold water. The diselenide which formed was filtered off and recrystallized from EtOH; yield 0.44 g (63%); brown crystals; mp 201-203 ^{o}C; IR (KBr, cm^{-1}) ν_{max}: 3450-3360 (2 NH_2), 2969, 2850 (C-H, aliphatic), 1650 (C=N), 1551 (C=C), 830 (Se-Se); ^{1}H-NMR (DMSO) δ (ppm): 7.14 (s, 4H, 2 NH_2), 2.10 (s, 6H, 2 CH_3); ^{13}C-NMR (DMSO) δ (ppm): 172.29, 162.82, 75.30 (Ar-C), 11.72 (2 CH_3); MS (*m/z*, %): 353.46 (M^++1, 37.65), 352.14 (M^+, 50.46), 306.13 (46.82), 293.21 (78.59), 220.55 (51.33), 180.93 (82.99), 143.02 (42.42), 117.13 (51.81), 114.11 (100.00), 74.12 (27.92), 71.66 (45.37), 64.74 (75.82). Anal. Calcd for $C_8H_{10}N_4O_2Se_2$ (352.14): C, 27.29; H, 2.86; N, 15.91%. Found: C, 26.95; H, 2.72; N, 15.73%.

Synthesis of *N*-(4-(*N*-(3-methylisoxazol-5-yl)sulfamoyl)phenyl)acetamide (13). To a mixture of 3-methylisoxazol-5-amine (**1**) (0.98 g, 0.01 mol) in pyridine (15 mL), 4-acetamidobenzenesulfonyl chloride (2.34 g, 0.01 mol) was added. The reaction mixture was heated on water bath for 15 h, then stand to cool at 25 ^{o}C. The solid that formed after dilution with cold water has been collected by filtration and recrystallized from EtOH; yield 2.51 g (85%); yellow crystals; mp 174-176 ^{o}C; IR (KBr, cm^{-1}) ν_{max}: 3249, 3238 (2 NH), 2921, 2848 (C-H, aliphatic), 1680 (C=O), 1609 (C=N), 1537 (C=C), 1369, 1160 (SO_2); ^{1}H-NMR (DMSO) δ (ppm): 13.63 (s, 1H, NH), 11.48 (s, 1H, NH), 8.30 (d, 2H, Ar-H, *J* = 8.8 Hz), 8.16 (d,

2H, Ar-H, *J* = 8.8 Hz), 6.09 (s, 1H, isoxazole-H4), 2.16 (s, 3H, CH_3), 2.08 (s, 3H, CH_3); ^{13}C-NMR (DMSO) δ (ppm): 167.26 (C=O), 161.63, 161.03, 150.46, 136.91, 131.13, 124.15, 88.83 (Ar-C), 23.47 (CH_3), 11.75 (CH_3); MS (*m/z*, %): 296.55 (M^++1, 21.65), 271.31 (55.64), 270.78 (100.00), 256.71 (33.88), 252.06 (82.25), 247.24 (34.26), 226.01 (35.54), 210.49 (48.53), 190.08 (95.21), 178.35 (44.88), 150.98 (55.33), 113.92 (97.07), 98.28 (26.65), 66.61 (83.42). Anal. Calcd for $C_{12}H_{13}N_3O_4S$ (295.31): C, 48.81; H, 4.44; N, 14.23%. Found: C, 48.53; H, 4.19; N, 13.96%.

Synthesis of 4-amino-*N*-(3-methylisoxazol-5-yl)benzenesulfonamide (14). 4-Acetamidosulfonamide **13** (2.95 g, 0.01 mol) was suspended in 20% H_2SO_4 (50 mL), and then heated on water bath for 1 h. The product that formed upon cooling was collected by filtration and recrystallized from EtOH; yield 1.92 g (76%); white crystals; mp 151-153 ºC; IR (KBr, cm^{-1}) ν_{max}: 3440-3341 (NH_2), 3239 (NH), 2927, 2850 (C-H, aliphatic), 1641 (C=N), 1596 (C=C), 1383, 1145 (SO_2); ^{1}H-NMR (DMSO) δ (ppm): 13.67 (s, 1H, NH), 8.32 (d, 2H, Ar-H, *J* = 8.8 Hz), 8.17 (d, 2H, Ar-H, *J* = 8.8 Hz), 6.49 (s, 1H, isoxazole-H4), 4.79 (s, 2H, NH_2), 2.00 (s, 3H, CH_3); ^{13}C-NMR (DMSO) δ (ppm): 166.31, 160.70, 150.49, 136.94, 131.17, 124.20, 78.27 (Ar-C), 11.75 (CH_3); MS (*m/z*, %): 255.21 (M^++2, 32.46), 253.20 (M^+, 30.75), 245.61 (48.48), 224.71 (52.69), 198.07 (19.56), 134.69 (33.45), 97.75 (37.03), 86.75 (28.96), 64.33 (38.13), 62.96 (100.00), 42.01 (86.24). Anal. Calcd for $C_{10}H_{11}N_3O_3S$ (253.28): C, 47.42; H, 4.38; N, 16.59%. Found: C, 47.26; H, 4.28; N, 16.41%.

Synthesis of 4-((1-(4-(*N*-(3-methylisoxazol-5-yl)sulfamoyl)phenyl)-5-oxo-2-phenyl-1,5-dihydro-4*H*-imidazol-4-ylidene)methyl)phenyl 4-methylbenzenesulfonate (15). A mixture of sulfonamide **14** (2.53 g, 0.01 mol), fused sodium acetate (1.64 g, 0.02 mol) and oxazol-5-one **9** (4.19 g, 0.01 mol) was heated at 155-160 °C for 6 h in an oil bath. After cooling, the solid formed was washed with petroleum ether and recrystallized from EtOH; yield 2.68 g (41%); brown crystals; mp 248-250 ºC; IR (KBr, cm^{-1}) ν_{max}: 3335 (NH), 2922, 2850 (C-H, aliphatic), 1700 (C=O), 1642 (C=N), 1594 (C=C), 1383, 1163 (SO_2); ^{1}H-NMR (DMSO) δ (ppm): 9.86 (s, 1H, NH), 8.38-6.83 (m, 18H, Ar-H, CH=), 6.50 (s, 1H, isoxazole-H4), 2.42 (s, 3H, CH_3), 2.41 (s, 3H, CH_3); MS (*m/z*, %): 654.67 (M^+, 60.11), 651.19 (62.91), 619.73 (61.51), 596.98 (33.82), 500.07 (42.11), 462.60 (69.92), 397.80 (50.29), 356.41 (72.66), 336.21 (71.14), 310.15 (100.00), 188.75 (68.28), 114.22 (52.69), 88.22 (53.15), 55.30 (52.98), 44.16 (54.03). Anal. Calcd for $C_{33}H_{26}N_4O_7S_2$ (654.71): C, 60.54; H, 4.00; N, 8.56%. Found: C, 60.35; H, 3.78; N, 8.26%.

Synthesis of *N*-(3-methylisoxazol-5-yl)thiazole-2,4-diamine (17). To a solution of 2-chloro-*N*-(3-methylisoxazol-5-yl)acetamide (**16**) (1.74 g, 0.01 mol) in 30 mL EtOH, thiourea (0.76 g, 0.01 mol) was added and refluxed for 4 h. The thiazole that formed after cooling was isolated by filtration and purified by recrystallization from EtOH; yield 1.24 g (63%); yellow crystals; mp 168-170 ºC; IR (KBr, cm^{-1}) ν_{max}: 3453-3370 (NH_2), 3161 (NH), 2923 (C-H, aliphatic), 1653 (C=N), 1517 (C=C);

^{1}H-NMR (DMSO) δ (ppm): 9.21 (s, 1H, NH), 7.52 (s, 2H, NH_2), 7.40 (s, 1H, isoxazole-H4), 7.27 (s, 1H, thiazole-H5), 2.42 (s, 3H, CH_3); ^{13}C-NMR (DMSO) δ (ppm): 170.25, 160.18, 153.81, 146.41, 108.32, 79.81 (Ar-C), 11.73 (CH_3); MS (*m/z*, %): 198.10 (M^++2, 49.94), 197.32 (M^++1, 17.23), 196.77 (M^+, 48.47), 182.22 (20.66), 169.31 (44.40), 163.69 (46.83), 148.18 (35.02), 134.81 (70.84), 130.72 (40.79), 114.42 (15.53), 91.09 (33.24), 71.87 (34.41), 68.60 (37.14), 59.41 (100.00), 48.77 (52.14). Anal. Calcd for $C_7H_8N_4OS$ (196.23): C, 42.85; H, 4.11; N, 28.55%. Found: C, 42.62; H, 3.91; N, 28.38%.

General procedure for the synthesis of isoxazole derivatives 18-20.

2-Chloro-*N*-(3-methylisoxazol-5-yl)acetamide (**16**) (1.74 g, 0.01 mol) was stirred for 15 min. in sodium ethoxide solution (prepared by dissolving 0.23 g sodium in 30 mL EtOH), and then 2-cyano-3-mercapto-3-(phenylamino)acrylamide, ethyl 2-cyano-3-mercapto-3-(phenylamino)acrylate or 2-mercapto-4,6-dimethylnicotinonitrile (0.01 mol) was added. The reaction mixture was refluxed for 4 h and then allowed to pour onto ice water. The isoxazole that formed after neutralization by dilute HCl was filtered off and recrystallized from EtOH.

3-Amino-*N*-(3-methylisoxazol-5-yl)-5-(phenylamino)thiophene-2,4-dicarboxamide (18): yield 2.14 g (60%); buff powder; mp 198-200 ºC; IR (KBr, cm^{-1}) ν_{max}: 3446-3193 (2 NH_2 and 2 NH), 2925 (C-H, aliphatic), 1647 (br, 2 CO), 1607 (C=N), 1511 (C=C); ^{1}H-NMR (DMSO) δ (ppm): 10.15 (s, 1H, NH), 9.87 (s, 1H, NH), 9.00 (s, 2H, NH_2), 7.43-7.11 (m, 5H, Ar-H), 6.23 (s, 1H, isoxazole-H4), 4.15 (s, 2H, NH_2), 2.55 (s, 3H, CH_3); MS (*m/z*, %): 357.82 (M^+, 24.30), 356.05 (M^+-1, 45.98), 315.24 (55.31), 307.86 (75.43), 284.47 (36.88), 280.05 (56.68), 248.95 (100.00), 229.59 (69.73), 205.09 (48.24), 188.22 (56.68), 163.63 (51.37), 142.39 (47.66), 83.24 (82.70), 62.25 (56.95), 50.78 (35.39). Anal. Calcd for $C_{16}H_{15}N_5O_3S$ (357.39): C, 53.77; H, 4.23; N, 19.60%. Found: C, 53.62; H, 3.98; N, 19.47%.

Ethyl 4-amino-5-((3-methylisoxazol-5-yl)carbamoyl)-2-(phenylamino)thiophene-3-carboxylate (19): yield 2.40 g (62%); orange powder; mp 211-213 ºC; IR (KBr, cm^{-1}) ν_{max}: 3443-3370, 3329, 3209 (NH_2 and 2 NH), 2921 (C-H, aliphatic), 1652, 1649 (2 CO), 1569 (C=N), 1506 (C=C); ^{1}H-NMR (DMSO) δ (ppm): 10.47 (s, 1H, NH), 10.01 (s, 1H, NH), 7.65-7.16 (m, 5H, Ar-H), 6.20 (s, 1H, isoxazole-H4), 4.87 (s, 2H, NH_2), 4.18 (q, 2H, CH_2-ester, J = 7.2 Hz), 2.42 (s, 3H, CH_3), 1.17 (t, 3H, CH_3-ester, J = 7.2 Hz); MS (*m/z*, %): 386.13 (M^+, 34.67), 381.99 (34.54), 353.64 (61.90), 331.10 (56.56), 317.88 (32.80), 249.90 (35.71), 237.89 (100.00), 190.07 (54.52), 126.66 (60.77), 119.12 (87.40), 93.03 (39.49), 63.11 (52.22), 46.29 (75.07). Anal. Calcd for $C_{18}H_{18}N_4O_4S$ (386.43): C, 55.95; H, 4.70; N, 14.50%. Found: C, 55.65; H, 4.50; N, 14.35%.

3-Amino-4,6-dimethyl-*N*-(3-methylisoxazol-5-yl)thieno[2,3-*b*]pyridine-2-carboxamide (20): yield 2.15 g (71%); orange powder; mp 223-225 ºC; IR (KBr, cm^{-1}) ν_{max}: 3424-3320 (NH_2), 3259 (NH), 2956, 2925 (C-H, aliphatic), 1696 (CO), 1621 (C=N), 1551 (C=C); ^{1}H-NMR (DMSO) δ (ppm): 10.90 (s, 1H, NH), 7.11 (s, 1H, pyridine-H5), 6.50 (s, 2H, NH_2), 6.08 (s, 1H, isoxazole-H4), 2.41 (s, 3H, CH_3), 2.37 (s,

3H, CH_3), 2.17 (s, 3H, CH_3); MS (*m/z*, %): 302.87 (M^+, 18.96), 301.55 (M^+-1, 36.15), 279.66 (22.13), 260.34 (26.14), 210.17 (28.21), 196.87 (35.22), 183.43 (46.10), 144.92 (38.36), 120.95 (100.00), 118.81 (47.48), 75.97 (35.57), 52.02 (29.42), 40.49 (39.57). Anal. Calcd for $C_{14}H_{14}N_4O_2S$ (302.35): C, 55.62; H, 4.67; N, 18.53%. Found: C, 55.39; H, 4.44; N, 18.35%.

MTT CYTOTOXICITY ASSAY

The cells used in cytotoxicity assay were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells suspended in the medium (2×10^4 mL) were placed in 96-well culture plates and incubated at 37 °C in a 5% CO_2 incubator. After 12 h, the test sample (2 μL) was added to the cells (2×10^4) in 96-well plates and cultured at 37 °C for 3 days. The cultured cells were mixed with 20 μL of MTT solution and incubated for 4 h at 37 °C. The supernatant was carefully removed from each well and DMSO (100 μL) was added to each well to dissolve formazan crystals that were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, the absorbance of each well was measured by a microplate reader at 570 nm. The relative cell viability was expressed as the mean percentage of viable cells compared to the untreated control cells. All experiments were conducted in triplicate and repeated on three different days. All the values were represented as mean ± SD. IC_{50} values were determined by probit analysis using the SPSS software program (version 20, SPSS Inc., Chicago, IL, USA).

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