Middle East Journal of Applied Sciences **Volume : 09 | Issue :04 |Oct.-Dec.| 2019**
EISSN: 2706 -7947 ISSN: 2077- 4613 **Pages: 1215-1224**
DOI: 10.36632/mejas/2019.9.4.33

Design, Synthesis and Evaluation Antitumor Activity of Some Novel fused Nitrogenous Rings Containing Pyrazolo [3, 4-b] pyridine Moiety

Nermin A. M. El -Seadawy, Sadia A. Hessein and Abeer M. Ali

Department of Chemistry, Faculty of Science (Girl's), Al-Azhar University, Nasr City, 11884, Cairo, Egypt.

Received: 10 Oct. 2019 / Accepted 05 Dec. 2019/ Publication date: 30 Dec. 2019

ABSTRACT
A simple route was performed for the synthesis of variety of some novel heterocyclic compounds bearing pyrazole moiety based on pyrazolo [3, 4-b] pyridine-5-carbonitrile (2a-c). Also, the compound of N-(4-(4-chlorophenyl)-5-cyano-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b] pyridin-6-yl)formimidate (6a) was considered the second intermediate through the interaction with different reagents to afford more than one fused heterocyclic ring 7-11. All the newly synthesized compounds have been characterized on the basis of IR, ^{1}H-NMR, ^{13}C-NMR spectral data and mass spectroscopy as well as physical data. The synthesized compounds were evaluated for their the antitumor activity against Hepatocellular carcinoma (HePG-2) and Colorectal adeno carcinoma (Caco-2) via the standard MTT method. The investigation of cytotoxicity screening data showed that compounds 6c, 6b and 5 promising strong cytotoxic activity against the tested human cancer cell lines.

***Keywords*:** Pyrazolo [3, 4-b] pyridine, [1, 2, 4]triazolo [1, 5-c]pyrimidine, cytotoxic activity.

Introduction

Literature survey appeared that pyrazole derivatives were found to be pharmacologically more potent and hence their design and synthesis are the potential area of research. It was found that modification on pyrazole moiety displayed valuable biological activities (Naim *et al.,* 2016). It will be interesting to observe that these modifications can be utilized as potent therapeutic agents in future.

Many pyrazole derivatives are reported to have a broad spectrum of biological activities, such as anticancer (Esvan *et al.,* 2016), anti-inflammatory (Hassan *et al.,* (2019), antifungal, antibacterial (Abdellatif and Bakr, 2018)**,** anti-proliferative agents (Salem and Ali, 2016) and antioxidant activity (Abd El-All *et al.,* 2016; Hessein and Sorror, 2014). Heterocyclic compounds containing nitrogen as (pyrazole, pyrazoline, and pyrazolo [3, 4-d] [1, 2, 3] triazole) are very important classes of compounds owing to their wide spectrum of biological activities (El-Naggar *et al.,* 2018; Hassan *et al.,* 2018; Hassan *et al.,* 2018). In particular, pyrazolo [3, 4-b] pyridines, thieno [2, 3-b] pyridines and (1, 2, 4-triazin-3-yl)-pyrazolo [3, 4-b] pyridine derivatives showed antibacterial activity with good inhibitions against Staphylococcus aureus and Staphylococcus epidermidis (Mohi-El-Deen *et al.,* 2019; Ali, 2009). Also, pyrazolo [3, 4-b] pyridine derivative showed potent and selective Fibroblast growth factor receptor (FGFR) kinase inhibitors (Zhao *et al.,* 2016), anticonvulsant and antidepressant activity (Ahsan, 2013). Pyridine-2-carboxamide showed potent cytotoxicity against human cancer cells lines, as breast adenocarcinoma (MCF7), hepatocellular carcinoma (HepG2), colon adenocarcinoma (HCT116), non-small lung (A549), and prostate (PC3) (Naguib and El-Nassan, 2016).

In view of these facts, we reported here the synthesis of some new heterocyclic compounds incorporated Pyrazolo [3, 4-b] pyridine moiety, to study the effect of these compounds toward the anti-tumor activity.

Cytotoxicity assay (Mosmann, 1983; Denizot and Lang, 1986)

2. Materials and Methods

2.1. Chemical reagents

The reagents RPMI-1640 medium, MTT and DMSO (sigma co., St. Louis, USA), Fetal Bovine serum (GIBCO, UK).

Corresponding Author: Sadia A. Hessein, Department of Chemistry, Faculty of Science (Girl's), Al-Azhar University, Nasr City, 11884, Cairo, Egypt.
E-mail: sadiamohamed.5919@azhar.edu.eg

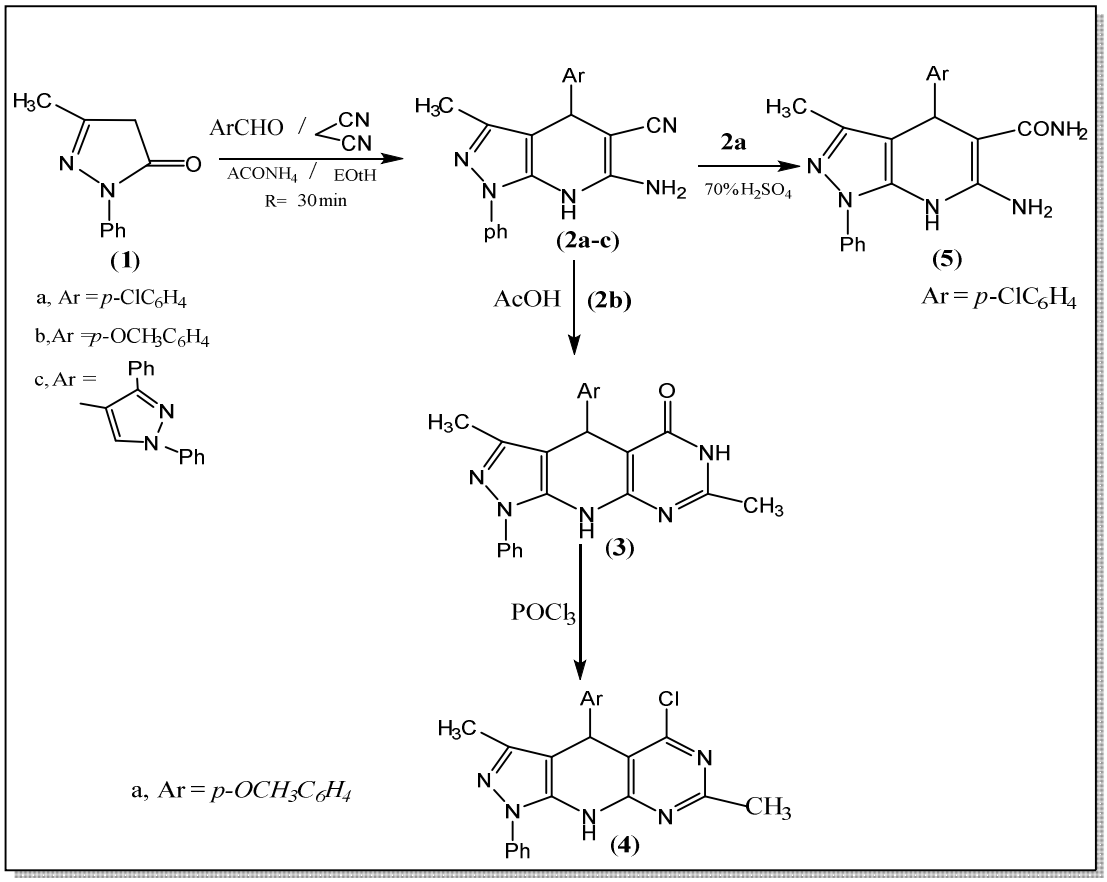
2.2. MTT assay

The cell lines mentioned above were used to determine the inhibitory effects of compounds on cell growth using the MTT assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100µg/ml streptomycin at 37 C in a 5% Co_2 incubator. The cell lines were seeds in a 96-well plate at a density of $1.0x10^4$ cells/well. at 37 C for 48 h under 5% Co_2. After incubation the cells were treated with different concentration of compounds and incubated for 24 h. After 24 h of drug treatment, 20 µl of MTT solution at 5mg/ml was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl is added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at absorbance of 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X100.

3. Results and Discussion

3.1. Chemistry

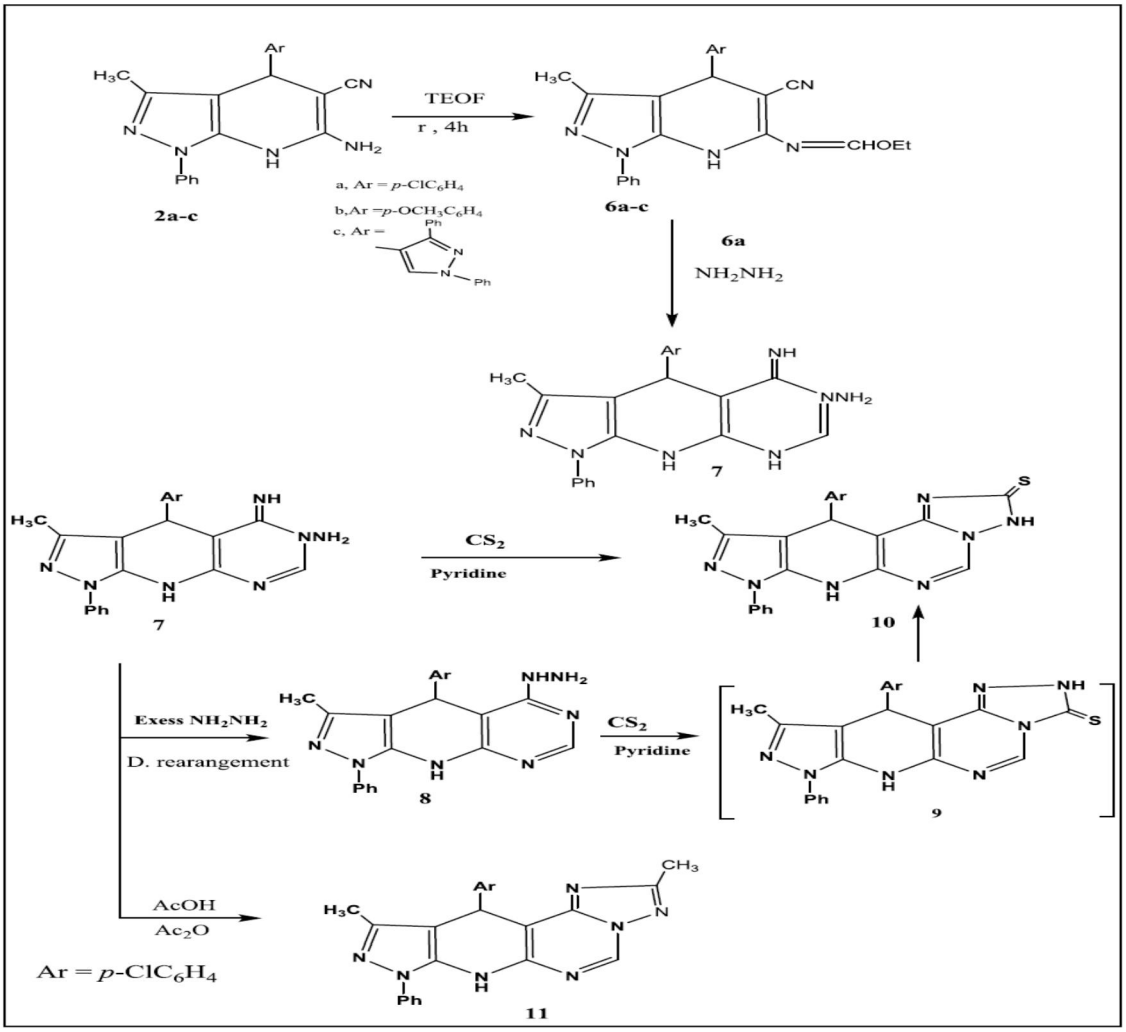
One pot four components reaction of 5-methyl-2-phenyl-2, 4-dihydro-3H-pyrazol-3-one (1) with different aldehydes, malononitrile and ammonium acetate in ethanol afforded the corresponding pyrazolo [3, 4-b]pyridine-5-carbonitrile (2a-c)scheme (1).The novel synthesized 2a-c were deduced through satisfactory elemental analysis and spectral data.The IR spectra of 2a-c showed the characteristic absorption bands in the region of 2185- 2198 cm^{-1} for CN group in addition to the absorption bands in the region of 3206-3481 for NH_2 &NH groups. The ^{1}HNMR spectrum of compound 2b showed three assigned singlet signals at δ 3.88, 4.60 and 6.86 ppm assignable to OCH_3, CH and NH_2 groups while, ^{13}C NMR showed a new signal at δ55.3 attributed to OCH_3 group. Pyrazolopyridine 2a-c was proved to be a convenient precursor for the synthesis of some novel pyrazolopyridine bearing pyrimidine moiety. Thus, refluxing of compound 2b with a mixture acetic acid and conc. Hydrochloric acid (1:1) produced, tricyclic product formulated as 4-(4-methoxyphenyl)-3, 7-dimethyl-1-phenyl-1, 4, 6, 9-tetrahydro-5H-pyrazolo [4', 3':5, 6]pyrido [2, 3-d]pyrimidin-5-one (3) Scheme (1). IR spectrum of 3 showed absorption bands at 1609 and 1663 cm^{-1} corresponding to $\diagdown$C=N$\diagup$ and C=O groups respectively. ^{1}H NMR revealed four singlet signals at δ 1.89, 2.11, 4.15 and 4.52 ppm attributed for CH_3-pyrimidine, CH_3-pyrazole, OCH_3 and CH-pyridine in addition to Ar-H and 2NH groups. ^{13}C NMR exhibited signals at δ 13.4, 25.9 and 173.4 ppm corresponding to CH_3, CH-pyrazolopyridine and CO groups respectively. Thermal reaction of compounds 3 with phosphorus oxychloride produced chloropyrimidine 4 Scheme (1).The structure of the reaction products was confirmed based on elemental analysis and spectral analytical data. IR measurement of 4 showed the disappearance of carbonyl function group. Mass spectrum showed a molecular ion peak at m/z 417.9(28.2%) corresponding to molecular formula $C_{23}H_{20}ClN_5O$ Acid hydrolysis of pyridine-5-carbonitrile 2a with 70% conc. H_2SO_4 produced 6-amino-4-(4-chlorophenyl)-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b]pyridine-5-carboxamide (5) Scheme (1). Elemental analysis and spectral data were in favor of the proposed structure (see experimental).



Scheme 1: Synthetic pathways for compounds of **2a-c, 3- 5.**

In addition, condensation of compounds 2a-c with triethylorthoformate produced, formimidate derivatives 6a-c (Scheme 2). IR spectrum of 6a as an example exhibited the lack of NH_2 band. ^{1}H NMR revealed triplet signal at δ 1.23 for OCH_2CH_3, quartet signal at δ 4.11 attributed to OCH_2CH_3 in addition to a new single signal at δ 8.51 for N=CH group. ^{13}C NMR exhibited signals at δ 15.2 for OCH_2CH_3, 62.06 corresponding to OCH_2CH_3 and a signal attributed to N=CH group at δ157.2 ppm.

We have extended our synthetic program to utilize formimidate 6a as the second starting material to furnish pyrido [2, 3-d] pyrimidine derivative incorporated pyrazole moiety. Thus, treatment of compound 6a with hydrazine hydrate in boiling ethanol afforded a single product formulated as 4-(4-chlorophenyl)-5-imino-3-methyl-1-phenyl-1, 4, 5, 9-tetrahydro-6H-pyrazolo [4', 3':5, 6]pyrido [2, 3-d]pyrimidin-6-amine (7) (Scheme 2). The elemental analysis and spectral data revealed the required structure. The IR spectrum of 7 exhibited absorption bands at 3195, 3301 &3356 cm^{-1} corresponding to NH and NNH_2 groups. ^{1}H NMR spectrum of 7 showed new singlet signals at δ 1.92 ppm, 5.18 and 5.67 corresponding to CH_3, CH-pyrimidine and NH_2 groups, respectively in addition to the rest of the molecule. Refluxing of compound 7 in ethanol with excess of hydrazine hydrate which acts as a base isomerized into 5-hydrazino derivative 8 according to Dimorth rearrangement type. Treatment of compound 8 with carbon disulfide in dry pyridine produced, 11-(4-chlorophenyl)-10-methyl-8-phenyl-8, 11-dihydro-3H-pyrazolo [4', 3':5, 6] pyrido [3, 2-e] [1, 2, 4]triazolo [1, 5-c]pyrimidine-2(7H)-thione (10) via the rearrangement of isomer 9 into 10. Also, triazolo [1, 5-c]pyrimidine 2-thione 10 was achieved by heating of 5-imino pyrimidine 7 with carbon disulfide in dry pyridine to afford a product in all aspects (m.p., mixed m.p. and spectral data) with thione 10 (Scheme 2). Its ^{13}C NMR spectrum exhibited a new signal at δ 190.2 ppm characterized for C=S.Moreover, the corresponding 11-(4-chlorophenyl)-2, 10-dimethyl-8-phenyl-8, 11-dihydro-7H- pyrazolo [4', 3':5, 6]pyrido [3, 2-e] [1, 2, 4]triazolo [1, 5-c]pyrimidine (11) was obtained upon the reaction of compound 7 with a mixture of acetic anhydride/ acetic acid(1:1) under reflux condition.



Scheme 2: Synthetic pathways for compounds **6a-c, 7- 11**

3.2. Biological activity

3.2.1. Cytotoxicity and antitumor evaluation:

All the recently synthesized compounds were evaluated for their in-vitro anticancer effect via the standard MTT method against Hepatocellular carcinoma (HePG-2) and Colorectal adeno carcinoma (Caco-2). The cell line was obtained from ATCC via holding company for biological products and vaccines (VACSERA), Cairo, Egypt. Doxorubicin was used as a standard anticancer drug for comparison. As mentioned in table (1) which indicated that an excellent cytotoxic activity against the two cell lines (HepG2) and (Caco-2) was displayed by formimidate 6c. Compound 5 showed strong cytotoxic activity against (HePG-2) but showed moderate activity against (Caco-2) and 6b exhibited strong cytotoxic activity against two cell lines antitumor. In addition, the moderate activity against two cell lines was demonstrated by compounds 2c, 8 and 11. Further, pyrazolo pyridine 2b and pyrazolo pyridopyrimidinone 3 showed the moderate activity toward (HePG-2) but showed the moderate activity against (Caco-2). While, formimidate 6a exhibited strong cytotoxic activity against (Caco-2) but showed the weak activity against (HepG2). The weak activity against the two cell lines demonstrated by 4 and 7 but 6a exhibited the weak activity against (HePG-2) and moderate activity against (Caco-2). Hence, by comparing the experimental cytotoxicity of the newly synthesized compounds reported in this study to their structures, we may conclude the structure activity relationship's (SAR's).

Table 1: Cytotoxic activity of some compounds against human tumor cells.

No.	Comp.	In vitro Cytotoxicity IC50 (µM)	
		HePG-2	Caco-2
••	**DOX**	4.50±0.2	12.49±1.1
1	**2a**	21.76±1.7	27.23±2.3
2	**2b**	31.01±2.3	56.64±3.0
3	**2c**	23.28±1.9	43.48±2.1
4	**3**	32.49±2.4	67.8±3.8
5	**4**	64.45±3.5	62.23±3.3
6	**5**	9.78±0.8	27.93±2.1
7	**6a**	71.86±4.0	42.83±2.7
8	**6b**	9.29±0.7	18.23±1.4
9	**6c**	5.86±0.3	11.47±1.1
10	**7**	58.76±3.2	75.81±3.8
11	**8**	39.67±2.6	49.27±3.2
12	**10**	31.86±2.4	42.1±2.1
13	**11**	28.79±2.1	35.48±2.5

• **IC50 (µM)**: 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak) and above 100 (non-cytotoxic)
•• **DOX:** Doxorubicin

3.2.2. Structure activity relationship

The presence of the basic skeleton of some heterocyclic rings is necessary for the reasonable range cytotoxic activity against cell lines HePG-2 and Caco-2. Pyrazolo [3, 4-b]pyridin-6-yl) formimidate(6c) showed an excellent cytotoxicity against two cell lines (HepG2) (5.86±0.3)mm and (Caco-2) (11.47±1.1)mm closed to the standard , this is due to the presence of pyrazolo pyridine moiety, ethoxy and methyl groups (donating groups + ve inductive effect). Also, 6b showed strong cytotoxic activity towards the two cell lines to the presence methoxy group (donating groups + ve inductive effect) but less than 6c. Acid hydrolysis of (2a) and converting it into carboxamide 5 causing as in case of 6b increase in cytotoxic activity against the tested cell lines (HepG2) (9.78±0.8)mm and (Caco-2) (27.93±2.1)mm due to the presence of , CH_3, NH_2 groups. The moderate activity was displayed by 2c against the two cell lines (HepG2) (23.28±1.9) mm and (Caco-2) (43.48±2.1) mm to the presence of cyano group and chloride atom (electron withdrawing atoms, -ve inductive effect). Cyclization of compound 6a into hydrazinyl pyrimidine 8 enhance the cytotoxic activity against the tested human cancer cell lines (HepG2) (39.67±2.6)mm and (Caco-2) (49.27±3.2)mm due to the presence of hydrazinyl and methyl groups in addition to fused pyrazolo pyridino pyrimidine moiety. Similarly, the presence of four fused rings system(pyrazolo [4', 3':5, 6]pyrido [3, 2-e] [1, 2, 4]triazolo [1, 5-c]pyrimidine derivatives) as in compounds 10 and 11 increase the activity to moderate higher than in compound 8, (HepG2) (31.86±2.4)mm, (Caco-2) (42.1±2.1)mm for triazole-2-thione 10 and (HepG2) (28.79±2.1)mm, (Caco-2) (35.48±2.5)mm for triazole 11. The weak activity was demonstrated by 6a against (HepG2) (71.86±4.0) mm but showed moderate activity against (Caco-2) (42.83±2.7) mm) due to the presence of cyano group and chloride atom. Also, compound 4 showed weak cytotoxic activity against the two cell lines (HepG2) (64.45±3.5) mm, (Caco-2) (62.23±3.3)mm due to the presence of chloride atom despite the presence of pyrimidine moiety and two methyl groups. Despite the presence of pyrazolopyridino pyrimidine moiety in addition to the presence of lone pair of electron on amino group and methyl groups in compound 7, the chloride atom present in para position of aromatic ring (-ve inductive effect) and imino group reduce the cytotoxicity against two cell lines cell lines HePG-2(58.76±3.2) mm and Caco-2 (75.81±3.8) mm.

4. Experimental

4.1. Chemistry

All melting points are recorded on digital Gallen Kamp MFB-595 instrument and may be uncorrected. The IR spectra (KBr) (cm–1) were measured on a JASCO spectrophoto-meter. 1H NMR spectra were recorded on Bruker spectro-meters (at 500 MHz) and are reported relative to deuterated solvent signals in deuterated dimethyl sulfoxide (DMSO-*d*6). ^{13}C NMR spectra were recorded on Bruker Spectrometers (at 125 MHz) in deuterated dimethyl-sulfoxide (DMSO-d_6). The purity of the synthesized compounds was monitored by TLC. Elemental analyses were carried out by the Micro

analytical Research Center, Faculty of Science, Cairo University. Analytical results for C, H and N were within –/+ 0.4 of the calculated values. The antioxidant screening and minimal inhibitory concentrations of the tested compounds were carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

General procedure for the synthesis of pyrazolo [3, 4-b] pyridine-5-carbonitrile (2a-c)

A mixture of 5-methyl-2-phenyl-2, 4-dihydro-3H-pyrazol-3-one (1) (0.01mol), appropriate aromatic aldehydes namely (*p*-chlorobenzaldehyde, *p*-methoxy benzaldehyde and 1, 3-diphenyl-1H-pyrazole-4-carbaldehyde) (0.01 mol) in each, malononitrile (0.01 mol) and ammonium acetate (2gm) in ethanol (20 mL) was heated under reflux for 30 min. The solid so formed was filtered off and recrystallized from the suitable solvent.

Synthesis of 6-amino-4-(4-chlorophenyl)-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b] pyridine-5-carbonitrile (2a)

Compound 2a

Yield: 75% as orange crystals from ethanol; Mp.: 210–212 °C; IR: (ν /cm $^{-1}$) 1587(*C = N*), 2198(CN) 3251&3481 [NH/NH_2], ^{1}H NMR (500 MHz, DMSO- *d_6)*: δ/ppm = 1.56 (s, 3H, CH_3), 4.41 (s, 1H, CH-pyridine), 5.12 (s, 2H, NH_2, exchangeable by D_2O), 6.32 (d, 2H *J* = 8.8 Hz, Ar-H), 6.8-7.08(m, 4H, Ar-H), 7.11- 7.6 (m, 3H, Ar-H),), 8.38 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-*d_6*) δ/ppm: 13.6 (CH_3), 26.6 (CH-pyridine), 67.35 (C-CN), 112.1(C-CN), 112.91, 113.20(Ar-C), 122.88, 124.49, 133.15, 140.80, 142.73, 144.08, 156.5 (Ar-C + pyrazole + pyridine*)*, 167.1 (C–NH_2*)* : Anal. Calcd. for C_{20} H_{16} N_5Cl (361.83): C, 66.39 ; H, 4.64 ; Cl, 9.80 ; N, 19.36 : Found, 66.23 ; H, 4.22; Cl, 9.61 ; N, 19.13%.

Synthesis of 6-amino-4-(4-methoxyphenyl)-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b] pyridine-5-carbonitrile (2b)

Compound 2b

Yield: 80% as red crystals from acetone; Mp.: 222–224 °C; IR: (ν /cm $^{-1}$) 1588(*C = N*), 2188(CN), 3111, 3220 & 3317 [NH/ NH_2), ^{1}H NMR (500 MHz, DMSO- *d_6)*: δ/ppm = 1.76 (s, 3H, CH_3), 3.88 (s, 3H, OCH_3), 4.60 (s, 1H, CH-pyridine), 6.86 (s, 2H, NH_2, exchangeable by D_2O), 6.89-7.18 (m, 5H Ar-H), 7.29 (d, 2H, *J* =7.11 Hz, Ar-H), 7.97 (d, 2H *J* = 8.13 Hz, Ar-H), 8.38 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-*d_6*) δ/ppm:14.1 (CH_3), 26.3 (CH-pyridine), 55.3 (OCH_3), 68.35 (C-CN), 112.91, 113.20(Ar-C), 117.01(C-CN), 122.88, 124.49, 133.15, 140.80, 142.73, 144.08, 156.5 (Ar-C + pyrazole + pyridine), 170.03 (C–NH_2*)*: Anal. Calcd. for C_{21} H_{19} N_5O (357.42): C, 70.57 ; H, 5.36 ; N, 19.59 : Found, 70.36; H, 5.24; N, 19.21%.

Synthesis of 6-amino-4-(1, 3-diphenyl-1H-pyrazol-4-yl)-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b]pyridine-5-carbonitrile(2c)

Compound 2c

Yield: 80% as red crystals from acetone; Mp.: 176–178 °C; IR: (ν/cm^{-1}) 1589(C=N) ; 2185 (CN), 3206(NH), 3315 & 3382(NH_2); ^{1}HNMR(500 MHz, DMSO- *d_6)*: δ/ppm = 2.1(s, 3H, CH_3), 4.63 (s, 1H, CH-pyridine), 6.12 (br, 2H, NH_2, exchangeable by D_2O), 6.7-6.91(m, 5H, Ar-H), 7.11- 7.6 (m, 5H, Ar-H), 7.93- 8.16 (m, 6H, Ar-H + H-pyrazole),), 10.2 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-*d_6*) δ/ppm:13.4 (CH_3), 25.9 (CH-pyridine), 71.02 (C- CN), 111. 1, 113.24(Ar-C), 114.6(C-CN), 121.18, 126.4, 133.15, 141.02, 142.33, 144.8, 157.8 (Ar-C + pyrazole + pyridine*)*, 172.08 (C–NH_2*)*; MS (EI, 70 eV): *m/z* (%) = 469.1 (M^+) (42.4%), 392 (54.7%), 325 (83%), 257 (72.6%), 191 (74.4%), 178 (65%), 134 (56%), 75 (100%), 57 (69%), 54 (59.4%); Anal. Calcd. for $C_{29}H_{23}$ N_7 (469.55): C, 74.18 ; H, 4.94 ; N, 20.88 : Found: 74.14 ; H, 4.38 ; N, 20.62%.

Synthesis of 4-(4-methoxyphenyl)-3, 7-dimethyl-1-phenyl-1, 4, 6, 9-tetrahydro-5H-pyrazolo [4', 3':5, 6]pyrido [2, 3-d]pyrimidin-5-one (3)

A compound of 2b (0.01mol) in a mixture of (20 mL) acetic acid / conc. HCl (1:1) was heated under reflux for 3h. The solid so formed was filtered off and recrystallized from acetic acid to form **3.** Yield: 66% as yellow crystals ; Mp.: 333–335 °C; IR: (ν/cm^{-1}) 1663(C=O) 3105& 3206(2NH), ^{1}HNMR(500 MHz, DMSO-*d_6*):*δ*/ppm= 1.89(s, 3H, CH_3-pyrimidine), 2.11(s, 3H, CH_3-pyrazole),

4.15(s, 3H, OCH_3), 4.52 (s, 1H, CH-pyridine), 7.13-7.3 (m, 3H, Ar-H), 7.31- 7.68 (m, 6H, Ar-H), 8.02(s, 1H, NH, exchangeable by D_2O), 8.33 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-d_6) δ/ppm:13.4 (CH_3- pyrazole), 25.9 (CH_3-pyrimidine), 35.2 (CH-pyridine), 55.1(OCH_3), 111.1, 113.24, 114.6, 121.18, 126.4, 133.15, 141.02, 142.33, 144.8, (Ar-C + pyrazole), 157.8(2C, NH-C-N + N=C-NH), 173.4 (CO) ; MS (EI, 70 eV): *m/z* (%) = 399 (M^+) (42.4%), 392 (54.7%), 325 (83%), 257 (72.6%), 191 (74.4%), 178 (65%), 134 (56%), 75 (100%), 57 (69%), 54 (59.4%); Anal. Calcd. for $C_{23}H_{21}N_5O_2$ (399.45): C, 69.16 ; H, 5.30 ; N, 17.53 : Found: 69.14 ; H, 5.18 ; N, 17.22 %.

Synthesis of 5-chloro-4-(4-methoxyphenyl)-3, 7-dimethyl-1-phenyl-4, 9-dihydro-1H-pyrazolo [4', 3':5, 6]pyrido [2, 3-d]pyrimidine (4)

A mixture of 3 (0.01mol) and phosphorus oxychloride (20 mL) was heated under reflux for 6h then the reaction mixture was poured into ice- water. The solid so formed was filtered off and recrystallized from acetone to form 4.Yield: 58% as deep red crystals; Mp.: 312–315 °C; IR: (ν/cm^{-1}) 1601(C=N), 3124 (NH) ;^{1}HNMR(500 MHz, DMSO- d_6): δ/ppm = 1.26(s, 3H, CH_3- pyrimidine), 2.3(s, 3H, CH_3- pyrazole), 3.79(s, 3H, OCH_3), 4.63 (s, 1H, CH-pyridine), 6.7-6.91(m, 4H, Ar-H), 7.93- 8.16 (m, 5H, Ar-H),), 10.2 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-d_6) δ/ppm:13.7(CH_3-pyrazole), 24.1 (CH_3-pyrimidine), 36.22 (CH-pyridine), 55.02(OCH_3), 109.1, 112.06, 117.18, 122.6, 126.15, 141.22, 142.7, 144.9, 156.6 (Ar-C + pyrazole + pyridine), 161.02 (2C, N=C – NH+ N-C-Cl) ; MS (EI, 70 eV): *m/z* (%) = 419.1 (M^{+2}) (37.4%), 382 (73.7%), 315 (11.1%), 273 (26%), 221 (62.4%), 177 (15%), 134 (17%), 77 (100%), 57 (21%), 54 (31.4%); Anal. Calcd. for $C_{23}H_{20}ClN_5O$ (417.9): C, 66.11 ; H, 4.82 ; Cl, 8.48 ; N, 16.76 : Found: 66.06 ; H, 4.66 ; Cl, 8.26 ; N, 16.32%.

6-amino-4-(4-chlorophenyl)-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b] pyridine-5-carboxamide(5)

Compound 2a (0.01mol) was added portion wise to (20 mL) 70% conc. Sulfuric acid, with stirring for 1 hour. The reaction mixture was poured into ice water, neutralized, filtered, dried and recrystallized from P.E 80-100°C to form 5.Yield: 58% as yellow crystals; Mp.: 112–114 °C; IR: (ν/cm^{-1})1665(CO) 3216 & 3381 [NH /2NH_2] ;^{1}HNMR(500 MHz, DMSO- d_6): δ/ppm = 2.12(s, 3H, CH_3-pyrazole), 4.27 (s, 1H, CH-pyridine), 4.61(s, 2H, NH_2, exchangeable by D_2O), 6.21((s, 2H, $CONH_2$, exchangeable by D_2O) 7.02-7.7(m, 4H, Ar-H), 7.82- 8.08 (m, 5H, Ar-H), 9.17 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-d_6) δ/ppm:13.21 (CH_3), 36.6 (CH-pyridine), 78.11(C-$CONH_2$) 109.1, 112.06, 117.18, 122.6, 126.15, 141.22, 142.7, 144.9, 156.6 (Ar-C + pyrazole + pyridine), 162.02 (C-NH_2) 169.2 ($CONH_2$) ; MS (EI, 70 eV): *m/z* (%) = 379. (M^+) (48.4%), 369 (22.7%), 292 (44 %), 275 (18.3%), 192 (100%), 134 (17%), 77 (90%), 54 (45.4%); Anal. Calcd. for $C_{20}H_{18}ClN_5O$ (379.85): C, 63.24 ; H, 4.78; Cl, 9.33 ; N, 18.44 : Found: 63.16 ; H, 4.42 ; Cl, 9.22 ; N, 18.06 %.

General procedure for the synthesis of pyrazolo [3, 4-b] pyridin-6-yl) formimidate (6a-c)

A mixture of 2a-c (0.01 mol) in each and triethylorthoformate (20 mL) was heated under reflux for 4 h. The solid so formed was filtered off and recrystallized from the proper solvent.

Ethyl N-(4-(4-chlorophenyl)-5-cyano-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b] pyridin-6-yl) formimidate (6a)

Compound 6a

Yield: 58% as red needles from ethanol; Mp.: 165–167 °C; IR: (ν/cm^{-1}) 1599(C=N) ; 2222 (CN), 3129(NH) ; ^{1}HNMR(500 MHz, DMSO- d_6): δ/ppm = 1.23(t, 3H, OCH_2CH_3), 2.17(s, 3H, CH_3), 4.11(q, 2H, OCH_2CH_3), 4.62 (s, 1H, CH-pyridine), 6.52-7.07(m, 4H, Ar-H), 7.33- 7.9 (m, 5H, Ar-H), 8.51(s, 1H, N=CH), 10.81 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-d_6) δ/ppm: 14.4 (CH_3), 15.2 (OCH_2CH_3), 27.9 (CH-pyridine), 62.06 (OCH_2CH_3), 83.02 (C-CN), 112.2, 114.6 (Ar-H), 118.1(C-CN), 120.18, 124.1, 132.17, 141.6, 142.13, 144.8, 149.7 (Ar-C + pyrazole), 157.8(N=CH), 167.2(NH-C-N=CH) ; Anal. Calcd. for $C_{23}H_{20}ClN_5O$ (417.9): C, 66.11 ; H, 4.82 ; Cl, 8.48 ; N, 16.76 : Found: 66.04 ; H, 4.36 ; Cl, 8.26 ; N, 16.66 %.

Ethyl N-(5-cyano-4-(4-methoxyphenyl)-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b]pyridin-6-yl)formimidate (6b)

Compound 6b

Yield: 88% as yellow sheets from P. E. 80-100 ; Mp.: 134–136 °C; IR: (ν/cm^{-1}) 1610(C=N) ; 2215 (CN), 3111(NH) ; ^{1}HNMR(500 MHz, DMSO- *d_6)*: δ/ppm = 1.55(t, 3H, OCH_2CH_3), 2.3(s, 3H, CH_3), 3.72 (s, 3H, OCH_3), 4.21(q, 2H, OCH_2CH_3), 4.77 (s, 1H, CH-pyridine), 6.41 (d, 2H J = 8.6 Hz, Ar-H), 6.8-7.12(m, 4H, Ar-H), 7.61-7.93 (m, 4H, Ar-H), 8.62(s, 1H, N=CH), 9.06 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-*d_6*) δ/ppm:14.1 (CH_3), 15.6 (OCH_2CH_3), 26.18 (CH-pyridine), 56.6 (OCH_3), 63.2 (OCH_2CH_3), 79.6 (C-CN), 111.6, 113.2, 116.1 (Ar-C), 118.8(C-CN), 120.18, 124.1, 132.17, 141.6, 142.13, 144.8, 149.7 (Ar-C+ pyrazole), 158.1(N=CH), 163.2(NH-C-N=CH) ; Anal. Calcd. for $C_{24}H_{23}N_5O_2$ (413.48): C, 69.72 ; H, 5.61; N, 16.94 : Found: 69.48 ; H, 5.33 ; N, 16.7 %.

Ethyl N-(5-cyano-4-(1, 3-diphenyl-1H-pyrazol-4-yl)-3-methyl-1-phenyl-4, 7-dihydro-1H-pyrazolo [3, 4-b]pyridin-6-yl)formimidate (6c)

Compound 6c

Yield: 67% as yellow crystals from ethanol; Mp.: 161–163 °C; IR: (ν/cm^{-1}) 1605(C=N) ; 2212 (CN), 3332(NH) ; ^{1}HNMR(500 MHz, DMSO- *d_6)*: δ/ppm = 1.21(t, 3H, OCH_2CH_3), 2.02(s, 3H, CH_3), 3.86(q, 2H, OCH_2CH_3), 4.61 (s, 1H, CH-pyridine), 6.62 (d, 2H, J = 8.2 Hz, Ar-H) 6.8-7.41(m, 5H, Ar-H), 7.61-7.89 (m, 6H, Ar-H), 7.92 (d, 2H, J = 7.18 Hz, Ar-H), 8.01(s, 1H, pyrazole), 8.55(s, 1H, N=CH), 10.06 (s, 1H, NH, exchangeable by D_2O); ^{13}CNMR (125 MHz, DMSO-*d_6*) δ/ppm: 13.1 (CH_3), 15.09 (OCH_2CH_3), 26.1 (CH-pyridine), 62.7 (OCH_2CH_3), 78.3 (C-CN), 112.6, 114.6, 116.1 (Ar-C), 117.6(C-CN), 123.17, 124.3, 128.11, 132.2, 141.7, 143.6, 144.8, 148.1, 149.3, 149.7 (Ar-C + pyrazole), 156.2(N=CH), 165.6(NH-C-N=CH) ; Anal. Calcd. for $C_{32}H_{27}N_7O$ (525.62): C, 73.12 ; H, 5.18; N, 18.65 : Found: 73.01 ; H, 5.03 ; N, 18.44 %.

Synthesis of 4-(4-chlorophenyl)-5-imino-3-methyl-1-phenyl-1, 4, 5, 9-tetrahydro-6H-pyrazolo [4', 3':5, 6]pyrido [2, 3-d]pyrimidin-6-amine (7)

A mixture of 6a (0.01 mol) and hydrazine hydrate (3 mL) in ethanol (20mL) was stirred 1 h. at room temperature. The solid that formed was filtered, washed by ethanol, dried and recrystallized from acetone. Yield: 66% as yellow crystals; Mp.: 220–122 °C; IR: (ν/cm^{-1}) 1597(C=N), 3195, 3301 &3356 [3NH/NNH_2] ; ^{1}HNMR(500 MHz, DMSO- *d_6)*: δ/ppm = 1.92(s, 3H, CH_3), 5.18 (s, 1H, CH-pyridine), 5.67(s, 2H, NNH_2 exchangeable by D_2O) 6.66(d, 2H, J = 7.22 Hz, Ar-H), 7.3 -7.53(m, 5H, Ar-H), 7.73 (d, 2H, J=8.11, Ar-H), 8.1(s, 1H, CH-pyrimidine), 8.87 (s, 1H, NH, exchangeable by D_2O), 9.03 (s, 2H, 2NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-*d_6*) δ/ppm: 13.2(CH_3) , 29.9 (CH-pyridine), 88.01 (C-C=NH), 112.2, 114.6, 118.1, 120.18, 124.1, 132.17, 141.6, 142.13, 144.8, 149.7 (Ar-C + pyrazole + CH-pyrimidine), 157.4(HN-C-N, NH=C) ; Anal. Calcd. for $C_{21}H_{18}ClN_7$ (403.87): C, 62.45; H, 4.49 ; Cl, 8.78 ; N, 24.28 : Found: 62.11 ; H, 4.12 ; Cl, 8.7 ; N, 24.03 %.

4-(4-chlorophenyl)-5-hydrazinyl-3-methyl-1-phenyl-4,9-dihydro-1H-pyrazolo [4',3':5,6]pyrido [2, 3-d]pyrimidine (8)

A mixture of 7 (0.01 mol) and a few drops of hydrazine hydrate in ethanol (20mL) was heated under reflux 2h. The solid that formed was filtered, washed by ethanol, dried and recrystallized from ethanol to give 8. Yield: 75% as yellow crystals; Mp.: 126–128 °C; IR: (ν/cm^{-1}) 1607(C=N), 3201 & 3223 [$NHNH_2$/NH] ; ^{1}HNMR(500 MHz, DMSO- *d_6)*: δ/ppm = 1.62(s, 3H, CH_3), 4.72 (s, 1H, CH-pyridine), 5.26(s, 3H, $NHNH_2$ exchangeable by D_2O), 6.77(d, 2H, J = 8.02Hz, Ar-H), 7.11 -7.63(m, 4H, Ar-H), 7.73-7.88 (m, 3H, Ar-H), 7.91(s, 1H, CH-pyrimidine), 9.27 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-*d_6*) δ/ppm:13.11(CH_3) , 40.9 (CH-pyridine), 89.01 (C-C-$NHNH_2$), 112.4, 113.2 (2C, Ar-C), 117.21(C-$NHNH_2$), 119.1, 120.4, 123.7, 127.2, 133.3, 140.6, 142.2, 144.8, 149.7 (Ar-C + pyrazole), 156.3(CH-pyrimidine), 172.4(HN-C-N-CH) ; Anal. Calcd. for $C_{21}H_{18}ClN_7$ (403.87): C, 62.45 ; H, 4.49 ; Cl, 8.78 ; N, 24.28 : Found: 62.11 ; H, 4.13 ; Cl, 8.7 ; N, 24.1 %.

11-(4-chlorophenyl)-10-methyl-8-phenyl-8, 11-dihydro-3H-pyrazolo [4', 3':5, 6]pyrido [3, 2-e] [1, 2, 4]triazolo [1, 5-c]pyrimidine-2(7H)-thione (10)

To a solution of 7 or 8 (0.01 mol) for each and carbon disulfide (10 mL) in pyridine(20mL) was refluxed 5 h. then the reaction mixture was cooled, poured in cold water and 1mL of conc. hydrochloric acid was added. The solid that formed was filtered, dried and recrystallized from ethanol in each case to afford 73% yield of the title product which identical to that prepared from compound **8,** as deep red crystals; Mp.: 190–192 °C; IR: (ν/cm^{-1}) 1519(C=S), 1611(C=N) ; 3324 (br, 2NH) ; ^{1}HNMR(500 MHz, DMSO-d_6): δ/ppm = 2.1(s, 3H, CH_3), 4.71 (s, 1H, CH-pyridine) 6.88(d, 2H, J = 7.22 Hz, Ar-H), 7.2 - 7.44(m, 5H, Ar-H), 7.72(d, 2H, J=8.11, Ar-H), 8.3(s, 1H, CH-pyrimidine), 8.87 (s, 1H, NH, exchangeable by D_2O), 9.9 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-d_6) δ/ppm: 13.2(CH_3) , 32.1 (CH-pyridine), 91.11 (C-C=N-CS), 112.2, 114.6, 118.1, 120.18, 124.1, 132.17, 141.6, 142.13, 144.8, 149.7 (Ar-C + pyrazole + CH-pyrimidine), 151.6(C=N-C=S)), 156.1(HN-C-N=C), 190.2 (CS); MS (EI, 70 eV): *m/z* (%) = 446. (M $^{+1}$) (22.4%), 398 (36.1%), 321 (11.7 %), 230 (91.3%), 190 (17%), 134 (17%), 74 (100%), 53 (45.4%); Anal. Calcd. for $C_{22}H_{16}Cl\ N_7S$ (445.93): C, 59.26 ; H, 3.62 ; Cl, 7.95 ; N, 21.99 ; S, 7.19 : Found: C, 59.17 ; H, 3.6 ; Cl, 7.62 ; N, 21.71 ; S, 7.07.

11-(4-chlorophenyl)-2, 10-dimethyl-8-phenyl-8, 11-dihydro-7H-pyrazolo [4', 3':5, 6]pyrido [3, 2-e] [1, 2, 4] triazolo [1, 5-c]pyrimidine (11)

A solution of **7** (0.01 mol) in a mixture of glacial acetic acid / acetic anhydride (20mL) (1:1) was heated under reflux 5 h. Then the reaction mixture poured into water, filtered, dried and recrystallized from ethanol. Yield: 80% as orange crystals; Mp.: 211–213 °C; IR: (ν/cm^{-1}) 1607(C=N) ; 3105 (NH) ; ^{1}HNMR(500 MHz, DMSO- d_6): δ/ppm = 1.95(s, 3H, CH_3), 2.4(s, 3H, N=C-CH_3), 5.61 (s, 1H, CH-pyridine), 7.31 -7.44(m, 5H, Ar-H), 7.53-7.8 (m, 5H, Ar-H+ CH-pyrimidine), 9.57 (s, 1H, NH, exchangeable by D_2O); ^{13}C NMR (125 MHz, DMSO-d_6) δ/ppm: 13.2 (CH_3-pyrazole), 13.91(CH_3-triazole) , 40.1 (CH-pyridine), 88.01 (C-C=N-C-), 112.2, 114.6, 118.1, 120.18, 124.1, 132.17, 141.6, 142.13, 144.8, 149.7, (Ar-C + pyrazole + CH-pyrimidine), 151.6 (C- C=N-C-), 177.4(HN-C-N=CH) ; Anal. Calcd. for $C_{23}H_{18}Cl\ N_7$ (427.9): C, 64.56 ; H, 4.24 ; Cl, 8.28 ; N, 22.91 : Found: 64.26 ; H, 4.11 ; Cl, 8.02 ; N, 22.6 %.

4. Conclusion

In the present work all the novel heterocyclic compounds incorporated, pyrazolo [3,4-b] pyridine moieties exhibited from very strong to moderate cytotoxic activity against two cell lines. The screening results revealed that the synthesized compounds 6c, 6b and 5 displayed promising inhibitory activity against the growth of the tested human cancer cell lines.

References

Abd El-All, A.S., A.S. Hassan, S.A. Osman, H.A.A. Yosef, W.H. Abdel-Hady, M.A. El-Hashash, S.R. Atta-Allah, M.M. Ali, and A.A. El Rashedy, 2016. Acta. Pol. Pharm., 73:79.

Abdellatif, K.R.A. and R.B. Bakr, 2018. New Advances in Synthesis and Clinical Aspects of Pyrazolo[3,4-d]Pyrimidine Scaffolds. Bioorg. Chem., Bioorg. Chem., 78, 341–357.

Ahsan, M.J., 2013. Anticonvulsant activity and neuroprotection assay of 3-substituted-N-aryl-6,7-dimethoxy-3a,4-dihydro-3 H-indeno [1,2-c] pyrazole-2-carboxamide analogues. Arab J. Chem., 3:644–650.

Ali, T.E.S, 2009. Synthesis of some novel pyrazolo[3,4-b]pyridine and pyrazolo[3,4-d]pyrimidine derivatives bearing 5,6-diphenyl-1,2,4-triazine moiety as potential antimicrobial agents. Eur. J Med Chem. 44:4385-4392.

Denizot, F. and R. Lang, 1986. . Rapid colorimetric assay for cell growth and survival modification to the tetrazoliumn dye procedure giving improved sensitivity and reliability. J. Immunol. Methods. , 89, 271-277.

El-Naggar, M., A.S. Hassan, H.M. Awad, and M.F. Mady, 2018. Design, Synthesis and Antitumor Evaluation of Novel Pyrazolopyrimidines and Pyrazoloquinazolines. Molecules .23:1249-1268.

Esvan, Y.J., F. Giraud, E. Pereira, V. Suchaud, L. Nauton, V. Théry, L.G. Dezhenkova, D.N. Kaluzhny, V.N. Mazov, A.A. Shtil, F. Anizon, and P. Moreau, 2016. Synthesis and biological activity of pyrazole analogues of the staurosporine aglycon K252c . Bio. Med. Chem., 24, (14) ,c , 3116-3124.

Hassan, A.S., G.O. Moustafa and H.M. Awad, 2017. Synthesis and in vitro anticancer activity of pyrazolo[1,5-a]pyrimidines and pyrazolo[3,4-d][1,2,3]triazines. Synth. Commun., 47(21):1963.

Hassan, A.S., H.M. Awad, A.A. Magd-El-Din, and T.S. Hafez, 2018. Synthesis and in vitro antitumor evaluation of novel Schiff bases. Med Chem. Res, 27(3):915-927.

Hassan, G.S., D.E. Abdel Rahman, E.A. Abdelmajeed, R.H. Refaey, M. Salem, Al. and Y.M. Nissan, 2019. New pyrazole derivatives: Synthesis, anti-inflammatory activity, cycloxygenase inhibition assay and evaluation of mPGES. Eur. J. Med. Chem., 171, 332-342.

Hessein, S.A. and H.A. Sorror, 2014. Synthesis of some novel benzofuran, pyrazole, pyrimidine, pyridine, pyridopyrimidinedihione , benzopyrone and thiophene derivatives of antioxidant activity .Egypt. J. Biomed. Sci., 45, 97-115.

Mohi-El-Deen, E.M., E.A. Abd El-Meguid, Sh. Hasabelnaby, E.A. Karam, and E.S. Nossier, 2019. Synthesis, Docking Studies, and In Vitro Evaluation of Some Novel Thienopyridines and Fused Thienopyridine–Quinolines as Antibacterial Agents and DNA Gyrase Inhibitors. Molecules, 24(20):3650-3669.

Mosmann, T., 1983. . Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods.65, 55-63.

Naguib, B.H. and H.B. El-Nassan, 2016. Synthesis of new thieno[2,3-b]pyri-. dine derivatives as pim-1 inhibitors. J. Enzyme Inhib. Med. Chem. 31:1718-25.

Naim, M.J., O. Alam, N. Fawaz, J. Alam Md., and P. Alam, 2016. Current status of pyrazole and its biological activities. J. Pharm BioAllied Sci., 8(1): 2-17.

Salem, M.S. and M.A.M. Ali, 2016. Novel Pyrazolo[3,4-b]pyridine Derivatives: Synthesis, Characterization, Antimicrobial and Antiproliferative Profile . Bio. Pharm Bull. ;39(4):473-483.

Zhao, B., Y. Li, P. Xu, Y. Dai, C. Luo, Y. Sun, J. Ai, M. Geng, and W. Duan, 2016. Discovery of Substituted 1H-Pyrazolo[3,4-b]pyridine Derivatives as Potent and Selective FGFR Kinase Inhibitors . ACS Med. Chem. Lett. 7(6):629- 634.