Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(9):4-12

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Ultrasonic assisted synthesis, anticancer and antioxidant activity of some novel pyrazolo[3,4-*b*]pyridine derivatives

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ABSTRACT

*3-Amino-4,6-dimethyl-1H-pyrazolo[3,4-b]pyridine (**2**) has been used as versatile precursor to prepare several heterocyclic compounds. Compound **2** reacted with N-(aryl)-2-chloro-acetamides **3a-d**, 4-subsituted benzaldehyde **6a**, **b** and indoline-2,3-dione derivatives **8a-e** to yield **4a-d**, **7a**, **b** and **9a-e**, respectively. The newly synthesized compounds are characterizing by IR, mass and ^{1}H NMR analyses. Ultrasonic irradiations gave a lower reaction time to give products **4a-d**, **7a-b** and **9a-e** in higher yields than those obtained by the previous conventional method where their yields increased from 70-79% to 87-93%. All the newly synthesized compounds were screened to evaluate their anticancer and antioxidant activity. The obtained results showed that compounds **4b** and **4c** showed a significant activities against the liver cancer cells (HepG-2) with inhibition percentages of 51% and 68.3%, and with IC_{50} = 120.8 and 73.3 µM, respectively. The antitumor activity of **4b** and **4c** against HCT116 showed inhibition percentages of 90% and 89.7% with IC_{50} = 55.6 and 55.7 µM, respectively. Furthermore, the synthesized compounds were examined in vitro for their antioxidant activities against DPPH radicals. The results obtained revealed that all of the tested compounds showed dose dependent DPPH inhibition activities, which were reflected by their IC_{50}. Among the newly synthesized compound **4c** showed the highest antioxidant activity with IC_{50}= 72.0 µM.*

Keywords: Pyrazolopyridine, *N*-phenylacetamide, Isatin, Ultrasonic, Antioxidant, Anticancer

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INTRODUCTION

Fused pyridines have been attracted considerable attention of researchers due to their great usefulness in synthetic chemistry and due to a very wide spectrum of their biological activities. Pyrazolopyridines especially pyrazolo[3,4-*b*]pryridines which are isosters of bioactive indoles or indazoles [1, 2], they represent important building blocks in both natural and synthetic bioactive compounds [3]. Pyrazolo[3,4-*b*]pyridines have been shown antibacterial [4, 5] and antiviral effects [6]. Various pyrazolo[3,4-*b*]pryridines have been found to exhibit pharmacological properties such as anti-inflammatory [7] and anxiolytic activity [8] along with xanthine oxidase inhibitors, cholesterol formation inhibitor and anti-alzheimer [9]. Moreover, pyrazolo[3,4-*b*]pyridines also exhibit promising biological activities including dopamine D3-receptor antagonist, dopamine D4 antagonist [10, 11] and adenosine A1-receptor antagonist [12, 13].

Pyrazolo[3,4-*b*]pyridine skeleton have proven to be interesting classes of heterocycles due to diverse biological properties including antioxidant and antitumor activities [14-17].

Ultrasound irradiation has been increasingly used in organic synthesis in the last three decades. Ultrasonic-assisted organic synthesis (UAOS) is a powerful and green approach which is becoming popular for accelerating organic compound synthesis [18, 20]. Comparing with traditional methods, this method is more conveniently and easily controlled. A large number of organic reactions have been carried out in higher yield, shorter reaction times and milder conditions under ultrasound irradiation [21, 22].

In this study, we are aim to synthesis novel functionalized compounds starting from 3-amino-4,6-dimethylpyrazolo[3,4-*b*]pyridine (**2**) by refluxing and under ultrasonic irradiation as an approach for green chemistry to evaluate their anticancer and antioxidant activity.

EXPERIMENTAL SECTION

2.1. Chemistry

2.1.1. General

All melting points were uncorrected and measured using an Electro-thermal IA 9100 apparatus (Shimadzu, Japan). Microanalytical data were performed by Vario El-Mentar apparatus (Shimadzu, Japan), National Research Centre, Cairo, Egypt. IR spectra were recorded (as potassium bromide pellets) using KBr disc technique on a Perkin-Elmer 1650 Spectrophotometer, National Research Centre-Cairo, Egypt. NMR experiments were determined on a JEOL-Ex-300 MHz in deuterated dimethylsulphoxide (DMSO – d_6) and chemical shifts were expressed as parts per million; ppm (δ values) against TMS as an internal reference (Faculty of Science, Cairo University, Cairo, Egypt). Mass spectra were recorded on Shimadzu GCMS-QP-1000EX mass spectrometer at 70 eV (Cairo University, Cairo, Egypt). Reactions carried out under ultrasonic irradiation were performed by Fischer Sonicator (with frequency of 25 kHz and nominal power 600 W).

2.1.2. Synthesis of 4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-amine (2).

This compound was prepared according to the reported method [23] by the reaction of 2-chloro-4,6-dimethylnicotinonitrile (**1**) with hydrazine hydrate in *n*-butanol gave 79% yield; m.p. 280-282 °C; yield 80%; IR ν 1620 (C=N), 3300 (NH), 3470 (NH_2) cm^{-1}. ^{1}H NMR (DSMO-d_6) δ 2.42 (s, 3H, CH_3), 2.57 (s, 3H, CH_3-C=N), 5.05 (s, 2H, D_2O exchangeable, NH_2), 6.59 (s, 1H, pyridine H), 11.70 (s, 1H, D_2O exchangeable, NH); MS ($C_8H_{10}N_4$) *m/z* = 162 (M^+).

2.1.3. Synthesis of *N*-(aryl)-2-chloro-acetamide 3a-d.

These compounds were prepared according to the reported method [24] by the reaction four equivalent of chloroacetyl chloride was added drop wise over one hour to the aqueous amine solution in THF (30 ml). Then the solution was left to stir overnight. The desired product was isolated as precipitate after pouring reaction mixture to an ice-cold water. The precipitate was filtered, washed with cold water and dried. Recrystalised using 95% ethanol 42-45% yield.

2.1.4. 2-(3-amino-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl)-*N*-arylacetamides 4a-d.

A solution of the appropriate amides **3a-d** (2 mmol) in isopropanol (20 mL), K_2CO_3 (0.69 g, 5 mmol) was added and reflux for 30 min. For this solution, 4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-amine (**2**) (0.324 g, 2 mmol) was added and the reflux was continued for 12h, then left to cool and evaporate the solvent. The resulted precipitate washed with distilled water, filtered, dried and then recrystallized from EtOH/DMF to afford compounds **4a-d**, respectively.

2.1.4.1. 2-(3-Amino-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl)-*N*-phenylacetamide (**4a**).
Yield (76%); m.p. 230-232 °C; IR ν 3411-3195 (NH+NH_2), 1661 (C=O) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.45 (s, 3H, CH_3), 2.58 (s, 3H, CH_3), 4.92 (s, 2H, CH_2), 5.25 (s, 2H, D_2O exchangeable, NH_2), 6.66 (s, 1H, pyridine H), 7.03-7.58 (m, 5H, ArH), 10.18 (s, 1H, D_2O exchangeable, -CONH); MS ($C_{16}H_{17}N_5O$) *m/z* = 295 (M^+).

2.1.4.2. 2-(3-Amino-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl)-*N*-(3-bromophenyl) acetamide (**4b**).
Yield (73%); m.p. 185-187 °C; IR ν 3406-3188 (NH+NH_2), 1660 (C=O) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.41 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 4.93 (s, 2H, CH_2), 5.29 (s, 2H, D_2O exchangeable, NH_2), 6.58 (s, 1H, pyridine H), 7.02-8.02 (m, 4H, ArH), 10.42 (s, 1H, D_2O exchangeable, -CONH); MS ($C_{16}H_{16}BrN_5O$) *m/z* = 374 (M^+).

2.1.4.3. 2-(3-Amino-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl)-*N*-(3-chlorophenyl) acetamide (**4c**).
Yield (71%); m.p. 150-152 °C; IR ν 3387-3186 (NH+NH_2), 1671 (C=O) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.42 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 4.94 (s, 2H, CH_2), 5.05 (s, 2H, D_2O exchangeable, NH_2), 6.58 (s, 1H, pyridine H), 7.05-7.89 (m, 5H, ArH), 10.10 (s, 1H, D_2O exchangeable, -CONH); MS ($C_{16}H_{16}ClN_5O$) *m/z* = 329 (M^+).

2.1.4.4. 2-(3-Amino-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl)-*N*-(4-tolyl)acetamide (**4d**).
Yield (73%); m.p. 215-217 °C; IR ν 3411-3194 (NH+NH_2), 1656 (C=O) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.25 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 4.90 (s, 2H, CH_2), 5.28 (s, 2H, D_2O exchangeable, NH_2), 6.65 (s, 1H, pyridine H), 7.10 (d, *J* = 7.5 Hz, 2H, ArH), 7.43 (d, *J* = 7.5 Hz, 2H, ArH), 10.13 (s, 1H, D_2O exchangeable, -CONH); MS ($C_{17}H_{19}N_5O$) *m/z* = 309 (M^+).

2.1.5. Synthesis of compounds 6a, b.
The starting materials, 4-piperidinobenzaldehyde (**6a**) and 4-morpholinobenzaldehyde (**6b**) were prepared by nucleophilic substitution of 4-fluorobenzaldehyde (1 equiv) with cyclic secondary amines (1.5 equiv) in dimethylsulfoxide or DMF and in the presence of potassium carbonate (1.6 equiv). The reaction mixture was heated at 100°C for 20 hours. Then the mixture was cooled down to room temperature and diluted with ether, and then extracted with water. The combined organic layer was washed with brine, dried over MgSO4, and concentrated in vacuo. The resulting crude product was purified by column chromatography on silica gel with 10% EtOAc:Hexane [25].

2.1.6. Synthesis of compounds 7a, b.
The appropriate aldehyde 4-(piperidin-1-yl)benzaldehyde (**6a**) or 4-morpholinobenzaldehyde (**6b**) (1 mmol) was added to a stirred solution of **2** (0.162 g, 1 mmol) in refluxing in absolute ethanol (20 mL)), and glacial acetic acid (0.5 mL). The reaction mixture was refluxed for 4 h. The obtained product was filtered, washed with ethanol, dried and crystallized from EtOH/DMF to afford compounds **7a** and **7b**, respectively.

2.1.6.1. *N*-(4,6-Dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1-(4-(piperidin-1-yl) phenyl) methanimine (**7a**).
Yield (76%); m.p. 220-222 °C; IR ν 3429 (NH), 1594 (C=N) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 1.57-1.88 (m, 6H, piperidine H), 2.48 (s, 3H, CH_3), 2.63 (s, 3H, CH_3), 3.37-3.31 (m, 4H, piperidine H), 7.05-7.80 (m, 5H, 4 ArH + pyridine H), 8.87 (s, 1H, -CH=N-), 12.92 (s, 1H, D_2O exchangeable, NH); MS ($C_{20}H_{23}N_5$) *m/z* = 333 (M^+).

2.1.6.2. *N*-(4,6-Dimethyl-1H-pyrazolo[3,4-*b*]pyridin-3-yl)-1-(4-morpholinophenyl) methan imine (**7b**).
Yield (70%); m.p. 295-297 °C; IR ν 3431 (NH), 1592 (C=N) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.45 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 3.28-3.03 (m, 4H, morpholine H), 3.74-3.77 (m, 4H, morpholine H), 6.85 (s, 1H, pyridine H) 7.04 (d, *J* = 9.0 Hz, 2H, ArH), 7.85 (d, *J* = 9.0 Hz, 2H, ArH), 8.94 (s, 1H, -CH=N-), 12.95 (s, 1H, D_2O exchangeable, NH); MS ($C_{19}H_{21}N_5O$) *m/z* = 335 (M^+%).

2.1.7. Synthesis of compounds 9a-e
The appropriate indoline-2,3-dione derivatives **8a-e** (1 mmol) was added to a suspension of 4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-amine (**2**) (0.162 g, mmol) (1 mmol) in absolute ethanol (10 mL), and glacial acetic acid (0.5 mL) was added to the mixture. The reaction mixture was refluxed for 2 h. The precipitate formed was collected by filtration, washed with ethanol, dried and crystallized from EtOH/DMF to afford compounds **9a-e**, respectively.

2.1.7.1. 3-((4,6-Dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)imino)indolin-2-one (**9a**)
Yield (79%); m.p. 280-282 °C; IR ν 3433, 3160 (2NH), 1735 (C=O), 1605 (C=N) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.56 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 6.91-6.94 (m, 2H, ArH), 6.97 (s, 1H, pyridine H), 7.41-7.46 (m, 1H, ArH), 8.21-8.24 (m, 1H, ArH), 10.92 (s, 1H, D_2O exchangeable, isatin NH), 13.65 (s, 1H, D_2O exchangeable, NH); MS ($C_{16}H_{13}N_5O$) *m/z* = 291 (M^+).

2.1.7.2. 5-Chloro-3-((4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)imino)indolin-2-one (**9b**)
Yield (75%); m.p. >300 °C; IR ν 3423, 3111 (2NH), 1729 (C=O), 1618 (C=N) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.57 (s, 3H, CH_3), 2.72 (s, 3H, CH_3), 6.94-6.97 (m, 1H, ArH), 7.03 (s, 1H, pyridine H), 7.45-7.53 (m, 1H, ArH), 8.74-8.75 (m, 1H, ArH), 11.04 (s, 1H, D_2O exchangeable, isatin NH), 13.94 (s, 1H, D_2O exchangeable, NH); MS ($C_{16}H_{12}ClN_5O$) *m/z* = 325 (M^+).

2.1.7.3. 5-Bromo-3-((4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)imino)indolin-2-one (**9c**)
Yield (74%); m.p. >300 °C; IR ν 3525, 3108 (2NH), 1726 (C=O), 1614 (C=N) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.57 (s, 3H, CH_3), 2.72 (s, 3H, CH_3), 6.90-6.92 (m, 1H, ArH), 7.03 (s, 1H, pyridine H), 7.62-7.65 (m, 1H, ArH), 8.87 (s, 1H, ArH), 11.05 (s, 1H, D_2O exchangeable, isatin NH), 13.93 (s, 1H, D_2O exchangeable, NH); MS ($C_{16}H_{12}BrN_5O$) *m/z* = 370 (M^+).

2.1.7.4. 3-((4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)imino)-5-fluoroindolin-2-one (**9d**)
Yield (77%); m.p. >300 °C; IR ν 3448, 3203 (2NH), 1727 (C=O), 1619 (C=N) cm^{-1}; ^{1}H NMR (DSMO-d_6) δ 2.49 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 6.91-6.95 (m, 1H, ArH), 7.02 (s, 1H, pyridine H), 7.33-7.34 (m, 1H, ArH), 8.50-

8.54 (m, 1H, ArH), 10.93 (s, 1H, D_2O exchangeable, isatin NH), 13.89 (s, 1H, D_2O exchangeable, NH); MS ($C_{16}H_{12}FN_5O$) *m/z* = 309 (M^+).

2.1.7.5. 3-((4,6-Dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)imino)-5-nitroindolin-2-one (**9e**)
Yield (79%); m.p. >300 °C; IR ν 3398, 3194 (2NH), 1737 (C=O), 1620 (C=N) cm^{-1}; ^1H NMR (DSMO-d_6) δ 2.44 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 6.98-8.33 (m, 4H, ArH + pyridine), 11.38 (s, 1H, D_2O exchangeable, isatin NH), 11.71 (s, 1H, D_2O exchangeable, NH); MS ($C_{16}H_{12}N_6O_3$) *m/z* = 336 (M^+).

2.1.8. Ultrasonic irradiations synthesis of compounds **4a-d**, **7a**, **b** and **9a-e**.
The procedure was similar to that described in conventional methods except that the mixture was capped in closed Erlenmeyer flask and subjected to ultrasonic irradiations (25 kHz and nominal power 600 W) at room temperature for the appropriate time until completion of the reaction (monitored by TLC). The resulting solid was collected by filtration and purified by crystallization from the appropriate solvent.

2.2. Biological activity

2.2.1. In-vitro anticancer activity

Cell culture of Hep-G2 (human liver carcinoma cell line) and HCT 116 (human colorectal carcinoma) were purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 and in DMEM medium respectively. Both media were supplemented with 10% heat-inactivated FBS, 100U/ml penicillin and 100U/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

2.2.2. MTT cytotoxicity assay

The cytotoxicity activity against Hep-G2 and HCT 116 human cell lines was estimated using the 3-[4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells [26, 27]. Cells were dispensed in a 96 well sterile microplate (5 x 10^4 cells/well), and incubated at 37^{o}C with 100 µM/ml of each tested compound or Doxorubicin® (positive control) for 48 h in a serum free medium prior to the MTT assay. After incubation, media were carefully removed, 40 µL of MTT (5 mg/mL) were added to each well and then incubated for an additional 4 h. The purple formazan dye crystals were solubilized by the addition of 200 µL of acidified isopropanol. The absorbance was measured at 570 nm using a microplate ELISA reader (Biorad, USA). The relative cell viability was expressed as the mean percentage of viable cells compared to the untreated control cells.

2.2.3. *In-vitro* antioxidant activity

1,1-Diphenyl-2-picryl hydrazyl (DPPH) was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and methanol were of HPLC grade and all other reagents and chemicals were of analytical reagent grade. Antioxidant activity of each compound and standards (ascorbic acid and rutin) was assessed based on the radical scavenging effect of stable DPPH free radical [28]. 10 µl of each tested compound or standard (from 0.0 to 100 µM) was added to 90 µl of a 100 µM methanolic solution of DPPH in a 96-well microtitre plate (Sigma-Aldrich Co., St. Louis, MO, US). After incubation in dark at 37°C for 30 min, the decrease in absorbance of each solution was measured at 520 nm using an ELISA micro plate reader (Model 550, Bio-Rad Laboratories Inc., California, USA). Absorbance of blank sample containing the same amount of DMSO and DPPH solution was also prepared and measured. All experiments were carried out in triplicate. The scavenging potential was compared with a solvent control (0% radical scavenging) and the standard compounds. Radical scavenging activity was calculated by the following formula:

% Reduction of absorbance = [(AB - AA) / AB] x 100, where: AB – absorbance of blank sample and AA – absorbance of tested compound (t = 30 min). The concentration of each compound required to scavenge 50% of DPPH (IC_{50}) was determined as well [29, 30].

Statistical analysis

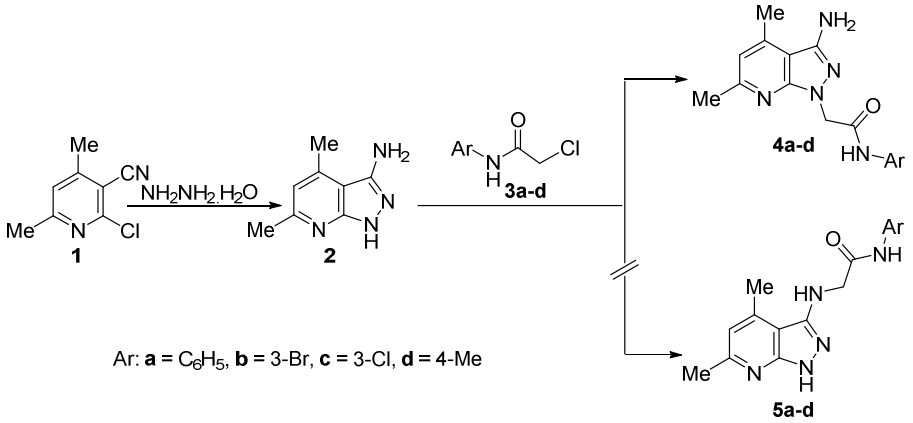
All experiments were conducted in triplicate (n = 3). All the values were represented as mean ± SD. Significant differences between the means of parameters as well as IC_{50}s were determined by probit analysis using SPSS software program (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

3.1. Chemistry

The reaction of 2-chloro-4,6-dimethyl pyridine-3-carbonitrile (**1**) with hydrazine hydrate afforded the 3-amino-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine (**2**) [23]. Furthermore, the amino group and cyclic NH function of pyrazole ring of compound **2** is a favorable units to react with electrophiles usually result in the formation of N-C bond.

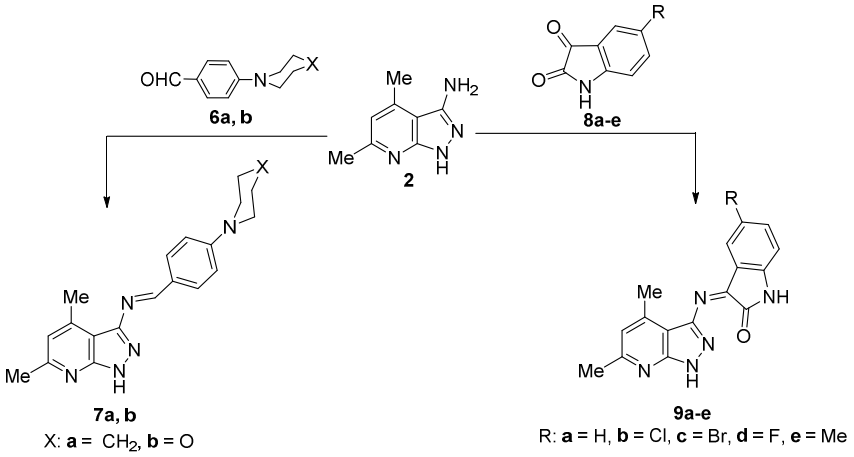
Therefore, the reactivity of compound **2** towards 2-chloro-*N*-arylacetamides **3a-d** [24] as carbon electrophiles was utilized. The cyclic NH function of pyrazole ring of compound **2** is more accessible than amino group to react with electrophiles **3a-d** due to its acidity and subsequently its anion stability with potassium carbonate. Thus, treatment of compound **2** with **3a-d** in a heated isopropanol, in the presence potassium carbonate, afforded the corresponding 2-(3-amino-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-1-yl)-*N*-arylacetamides **4a-d**, respectively (Scheme 1) not the possible products **5a-d**.



Scheme 1. Synthesis of compounds 4a-d

The IR spectrum of compounds **4a-d** showed an overlapped NH+NH_2 absorption bands in the region at 3411-3186 cm^{-1} in addition to absorption band due to stretching of C=O group at 1671-1656 cm^{-1} region. ^{1}H NMR spectra of **4a-d** showed two D_2O exchangeable singlet signals at δ 10.42-10.10 and δ 5.29-5.05 region due to NH and NH_2 groups, respectively. They showed also a singlet signal at δ 4.94-4.90 region which represent the CH_2 protons in addition to the singlet signals of two methyl groups at δ 2.58-2.40 and the signal of pyridine proton at δ 6.66-6.58. Mass analysis of **4a-d** showed a peak, in each case, corresponding to their molecular ion peak.

Furthermore, the reaction of **2** with 4-(piperidin-1-yl)benzaldehyde (**6a**) or 4-morpholinobenzaldehyde (**6b**) [25] in refluxing absolute ethanol and in the presence of catalytic amount of glacial acetic acid afforded hydrazones **7a**, **b**, respectively. The IR spectra of compounds **7a, b** showed N-H and C=N bands in the region 3431-3429 cm^{-1} and 1594-1592 cm^{-1}, respectively. The ^{1}H NMR spectrum of compounds **7a, b** showed singlet signal within the δ 12.95-12.92 region corresponding to NH proton (D_2O exchangeable) and δ 8.94-8.87 region corresponding to CH proton. Compound **7a** showed two multiplet signals at δ 1.57-1.88 and δ 3.37-3.31 corresponding to 6H and 4H of piperidine protons, respectively, while compound **7b** showed two multiplet signals at δ 3.28-3.03 and δ 3.74-3.77 corresponding to two sets of four morpholine protons. Mass spectra of **7a** showed M^+ at *m/z* 333while **7b** showed M^+ at *m/z* 335.



Scheme 2. Synthesis of compounds 7a, b and 9a-e

Finally, indoline-2,3-dione derivatives **8a-e** were reacted with 4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-3-amine (**2**) in absolute ethanol in the presence of acetic acid to yield the corresponding Schiff bases **9a-e**. The IR spectra of **9a-e** exhibited the characteristic absorption band of 2NH groups in the region *δ* 3448-3108 cm^{-1} in addition to absorption bands of C=O group at *δ* 1737-1726 cm^{-1}. The characteristic singlet signals of D_2O-exchangeable NH protons appeared in the region δ 11.38-10.92 and 13.89-11.71 for isatin and pyrazole NH, respectively.

The reactions described in Schemes 1 and 2 were also carried out under ultrasonic irradiations (at room temperature) to give products **4a-d**, **7a-b** and **9a-e**. The results are reported in (Table 1). Compounds **4a-d**, **7a-b** and **9a-e** were found to be consistent in all respects with the ones produced by the conventional method. It is worthy to note that the reactions carried out by ultrasonic irradiations were done in lower time compared with conventional method. Also, the synthesized products were formed in pure form directly and the yields were increased from 70-79% to 87-93%.

Table 1. Comparison between reaction times and yields for conventional and ultrasonic irradiation methods

Comp.	Conventional method		Ultrasonic method	
	Yield (%)	Reflux (h)	Yield (%)	Room tempt. (h)
4a	76	12	91	2.5
4b	73	12	92	2.5
4c	71	12	89	2.5
4d	73	12	91	2.5
7a	76	4	92	3.5
7b	70	4	87	3.5
9a	79	2	93	3.5
9b	75	2	90	3.5
9c	74	2	89	3.5
9d	77	2	90	3.5
9e	79	2	93	3.5

3.2. Biological Activity

3.2.1. Anti-tumor activity

The synthesized compounds were examined *in vitro* for their anti-tumor activities against Hep-G2 human liver cancer cell line and HCT 116 human cell line using MTT assay. The percentage of the intact cells was measured and compared to the control. The activities of the derivatives against carcinoma cells were compared with the cytotoxicity of Doxorubicin® (Table 2 and Figure 1).

Table 2. Anticancer activity of the synthesized compounds against Hep-G2 human liver cancer cell line and HCT 116 human colorectal carcinoma cell line

Comp.	IC_{50} (µM)	
	Hep-G2	HCT 116
2	>1000 ± 15.7	261.2 ± 8.3
4a	NA	>1000 ± 17.2
4b	120.9 ± 6.2	55.6 ± 2.6
4c	73.3 ± 3.7	55.7 ± 2.8
4d	181.6 ± 8.1	469.1 ± 7.9
7a	416.8 ± 11.1	>1000 ± 11.9
7b	508.0 ± 5.2	NA
9a	173.8 ± 8.3	347.8 ± 8.4
9b	858.5 ± 9.4	942.4 ± 11.8
9c	NA	NA
9d	>1000 ± 17.2	>1000 ± 16.3
9e	138.2 ± 7.9	193.5 ± 6.3

NA = No Activity

The obtained results showed that only two compounds; **4b** and **4c** showed high cytotoxic activities against the liver cancer cells (HepG-2) with inhibition percentages of 51% and 68.3%, respectively, at concentration of 100 ppm. The IC_{50} for both compounds were found to be 120.9 and 73.3 µM, respectively. The other compounds did not show significant activities. The results of the antitumor activity against HCT116 showed high anticancer activities only for both compounds **4b** and **4c** with inhibition percentages of 90% and 89.7%, respectively, at concentration of 100 ppm. The IC_{50} for both compounds were found to be 55.6 and 55.7 µM, respectively.

The results of the anticancer activity revealed that, the induction of electron withdrawing groups as bromo- and chloro-groups (**4b** and **4c**, respectively) in meta-position, leads to enhance the anti-cancer activity compared to *para*-position (**4d**). In addition, the substitution at 2-position of compound 2 reveals more significant anticancer activity (**4a-d**) compared to the substitution at 3-position (**9a-d**).

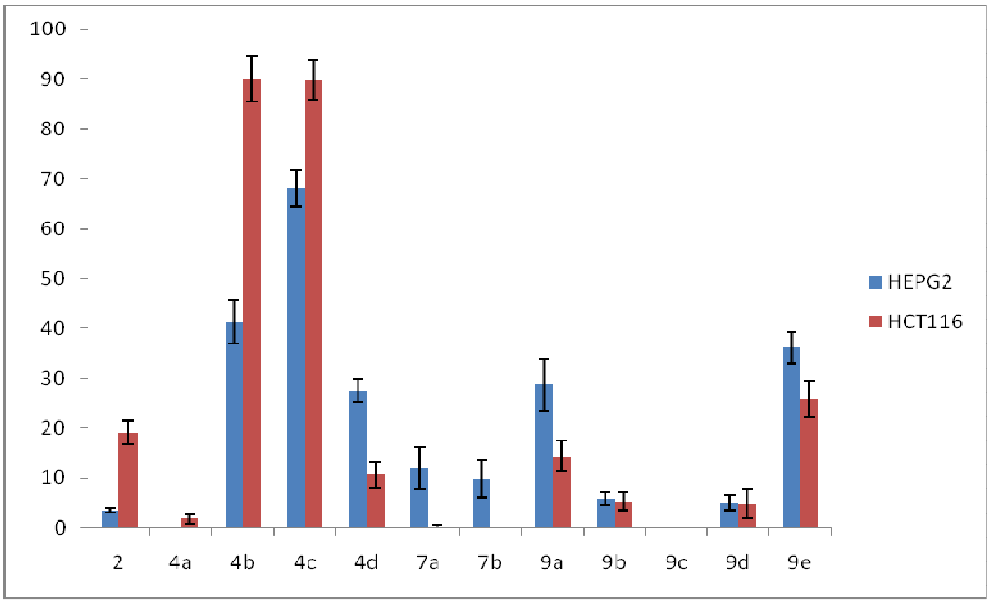


Figure 1. Anticancer activity of the synthesized compounds using MTT assay against HEPG2 and HCT116 cancer cell lines

3.2.2. In vitro antioxidant activity

The synthesized compounds were examined *in vitro* for their antioxidant activities against DPPH radicals (Table 3, Figure 2). The results obtained revealed that, all of the tested compounds showed dose dependent DPPH inhibition activities, which were reflected by their IC_{50} values as summarized in Table 2. The activities of the compounds appeared in the following order: **rutin** > **Vit C** > **2** > **4c** > **4a** > **4d** > **7a** > **4b** > **7b** > **9c** > **9d** > **9e** > **9b** > **9a**.

Table 3. Antioxidant activity of the synthesized compounds using DPPH assay

Comp.	IC_{50} (µM)
2	69.0 ± 3.2
4a	103.0 ± 5.1
4b	115.7 ± 6.0
4c	72.0 ± 2.7
4d	111.0 ± 5.2
7a	114.0 ± 1.9
7b	117.0 ± 3.8
9a	160.0 ± 2.6
9b	155.5 ± 6.2
9c	128.0 ± 3.9
9d	136.5 ± 2.6
9e	155.0 ± 3.8
Rutin	27.3 ± 2.1
Vit C	48.7 ± 1.9

Comparing the activity of the twelve compounds to the standard antioxidants and well known potent DPPH inhibitors (rutin and vit C); It is clear that the best scavenging properties was gained by compound **2** (IC_{50}; 69.0 µM**)** followed by compound **4c** (IC_{50}; 72.0 µM**)**. However, none of these compounds showed as high activity as the standards.

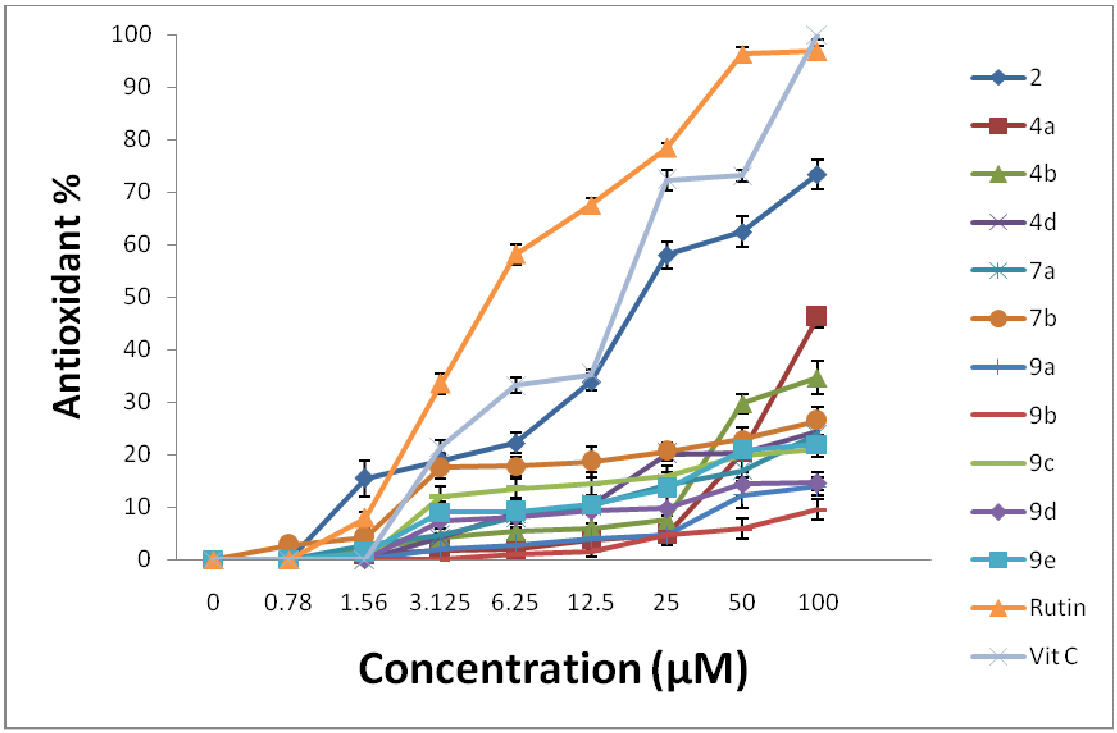


Figure 2. antioxidant activity of the synthesized compounds using DPPH assay

CONCLUSION

The objective of this work was established by testing a powerful and green approach Ultrasonic-assisted organic synthesis (UAOS) by the synthesis of some novel pyrazolo[3,4-*b*]pyridines **4a-d**, **7a**, **b** and **9a-e**, respectively. Compounds **4b** and **4c** showed a significant activities against the liver cancer cells (HepG-2) with inhibition percentages of 51% and 68.3%, and with IC_{50} = 120.8 and 73.3 µM, respectively. The antitumor activity of **4b** and **4c** against HCT116 showed inhibition percentages of 90% and 89.7% with IC_{50} = 55.6 and 55.7 µM, respectively. Compound **4c** showed the highest antioxidant activity with IC_{50}= 72.0 µM.

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