Synthesis of some novel 2-(3-cyano-6-(thiophen-2-yl)-4,4′-bipyridin-2-yloxy) acetohydrazide derivatives: assessment of their cytotoxic activity

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Abstract
A new series of pyrazole, bipyridine, N-amide derivatives and Schiff bases was synthesized using compound 2-(3-cyano-6-(thiophen-2-yl)-4,4′-bipyridin-2-yloxy) acetohydrazide (3) as a starting material. The compounds structures were confirmed depending on the spectroscopic methods and elemental analysis. Also, the compounds were evaluated as anticancer agents by the compounds screened towards adenocarcinoma breast cancer cell line (MCF-7). The compounds showed a promising cytotoxic effect against human breast cancer cells. Compound 7c showed the most effective activity compared to other compounds with (IC50 = 0.6 ± 0.01 μg mL−1) in comparison with the reference drug doxorubicin (IC50 = 1.6 ± 0.02 μg mL−1). While compound 3 is closely active with doxorubicin. Also compounds 2, 4, 6, 7a, 7b and 7d showed noticeable cytotoxic effect. Early and late apoptotic cells were detected using Acridine orange/Ethidium bromide staining technique. The results of biologically screening of the tested compounds give an idea about the importance in the compounds acting against breast cancer and may lead to the discovery of a potent anticancer agent.

Keywords: Pyridine, Schiff bases, Breast cancer, Apoptotic cells, Thiophene

Introduction
Cancer disease is one of the most widely spread diseases nowadays especially breast cancer. Breast cancer comes in various forms either histological or clinical because it is a heterogeneous disease. Its treatment is done through chemotherapy and/or hormone therapy. Heterocyclic compounds that incorporating pyridine moiety appear miscellaneous pharmacological properties such as anticancer [1], antimicrobial [2, 3], anticonvulsant [4], antiviral [5], anti-HIV [6], antifungal and, antibacterial activities [7]. Also the antitumor activity of pyridine ring enhanced by introducing different substituents such as hydrazide bearing either thiazole, thiophene, benzothiophene, triazole or pyrazole, and cyanoacetohydrazide [8]. Studying Structure-activity relationship (SAR) of the compounds is due to the well-reported anticancer activity of these rings. Compounds containing a pyridine group that includes a cyano group have excellent antitumor activity as reported in the previous publications [9–15]. Based on the reported biological activity of these heterocyclic moieties [16, 17], Schiff bases [18–20], triazoles [21, 22], quinolones and spiro compounds [23, 24] as anticancer agents [25] and continuing of my research on the chemistry of the biologically active compounds [25–30]. Herein, I designed new biologically active compounds using 2-(6′-(4-chlorophenyl)-3′-cyano-3,4′-bipyrindin-2′-yloxy) acetohydrazide(3) as a building block.

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and studying their antitumor activity against breast cancer cell line.

**Results discussion**

**Chemistry**

In this research, a one-pot manner was used for the synthesis of compound 2-oxo-4-(pyridin-4-yl)-6-(thiophen-2-yl)-1,2-dihydropyridine-3-carbonitrile (1) where all the reaction components, 2-acetylthiophene, 4-pyridine carboxaldehyde, ammonium acetate, and ethyl cyanoacetate were added in the presence of ceric ammonium nitrate (CAN) and then refluxed in ethanol. The resulting compound 1 then alkylated with ethyl bromoacetate in ethanol and in the presence of a catalytic amount of potassium carbonate to give the alkylated derivative ethyl 2-(3-cyano-6-(thiophen-2-yl)-4,4′-bipyridin-2-yloxy) acetate (2). The structure of compound 2 was confirmed depending on the spectral data. For example, in the 1H NMR spectrum, the characteristic signals of the ethoxy group appeared at 1.18 ppm for (CH₃) and at 4.15 ppm for (OCH₂) and the signal for (NH) group at 8.79 ppm was disappeared. Hydrazinolysis of compound 2 gave the acid hydrazide 3. In the acid hydrazide 1H NMR spectrum the signals of the ethoxy groups at 4.15 and 1.18 ppm were disappeared and new signals appeared at 8.75 and 12.48 for the (NH-NH₂) group. All other signals appeared at their expected position as illustrated in the experimental section. 2-(3-Cyano-6-(thiophen-2-yl)-4,4′-bipyridin-2-yloxy)acetohydrazide (3), is used as a starting matter for the synthesis of all target compounds in this work (Scheme 1).

Compound 3 was cyclized into different heterocyclic moieties. Cyclization of 3 with ethyl acetoacetate and/or acetylacetone gave the corresponding 2-(2-(3-methyl-5-oxo-4,5-dihydropyrazol-1-yl)-2-oxo ethoxy)-6- (thiophen-2-yl)-4,4′-bipyridine-3-carbonitrile (4) and/or 2-(2-(3,5-dimethyl-1H-pyrazol-1-yl)-2-oxoethoxy)-6-(thiophen-2-yl)-4,4′-bipyridine-3-carbonitrile (5), respectively. The compounds’ structures were confirmed based on their spectroscopic data and their elemental analysis wherein both compounds, the characteristic signals of (NH-NH₂) group disappeared. In compound 4 new signals appeared at 1.84 ppm for (CH₃) group and at 2.88 for (CH₂) in pyrazole ring. While in compound 5 new signals...
at 1.81, 2.01 for (2CH₃) have appeared. Also in the $^{13}$C NMR spectra of compound 4 a new signal for the new carbonyl group in pyrazolone ring have appeared. All the appeared signals are in accordance with the expected values. Cyclization of compound 3 with ethyl cyanoacetate or diethyl malonate gave the corresponding 2-[(2-(5-
dioxopyrazolidin-1-yl)-2-oxoethoxy)-6-(thiophen-2-yl)-
4,4′-bipyridin-3-carbonitrile (6) (Scheme 2). In the $^1$H NMR spectrum of compound 6 a characteristic signal of (CH₂) at 2.51 ppm in pyrazolidine ring have appeared.

A new series of expectedly biologically active N-amide derivatives and Schiff bases was synthesized. Schiff bases 7a–d were obtained through condensation of compound 3 with different aldehydes namely 3-pyridine carboxaldehyde, 3,4- Dihydroxy benzaldehyde, anisaldehyde and vanillin, in acetic acid. In all Schiff bases the signal characteristic to the (NH₂) group was disappeared and the signal of (NH) group at 12.48 ppm was shifted to new positions at 12.48, 9.99, 12.49 and 8.84 ppm. In compounds 7a, 7b, 7c, and 7d respectively. All the characteristic signals of the arylidine groups were appeared at their expected positions as shown in the experimental part, Compound 7c structure was confirmed based on the spectroscopic data in (Fig. 1) The reaction of 3 with p-toluenesulfonyl chloride in absolute ethanol afforded the corresponding 2-(3-cyano-6(thiophen-2-yl)-4,4″-
bipyridin-2-yloxy)N-(tosylmethylene)aceto hydrazide (8) (Scheme 3). Compound 8 structure was confirmed based on the spectroscopic data and the elemental analysis.

**In vitro anticancer screening**

The in vitro cytotoxic activities of compounds 1, 2, 3, 5, 6, 7a–d and 8 were determined using SRB assay towards breast cancer cell line (MCF-7) over concentration range of 0.01 to 1000 μg. The tested compounds exhibited a variable cytotoxicity profile against the tested human breast cancer cells. (Table 1 and Fig. 2). doxorubicin is a reference drug in this study The IC₅₀ is the compounds concentrations reduce the cell viability to 50%. The data in Table 1 and Fig. 2 indicate the cytotoxicity profile of the newly synthesized compounds against breast cancer cells. The results showed considerable cytotoxicity against cancer cell, most of the compounds showed highly cell killing significant on MCF-7 cells; some of them were revealed a strong activity, others were found to be on par near the reference drug toxicity (IC₅₀ = 1.3 μg mL⁻¹).

The Schiff base 7c (IC₅₀ = 0.6 μg mL⁻¹) is the most potent compound in this evaluation and it showed higher

![Scheme 2](image-url)  
**Scheme 2** Synthesis of pyrazole derivatives (4-6). Reagents and conditions: (i) ethyl acetoacetate/AcOH, reflux; (ii) diethyl malonate/AcOH or ethyl cyanoacetate/AcOH, reflux; (iii) acetylacetone/AcOH reflux.
Fig. 1 Analysis for the compound 7C. a Infrared spectrum, b nuclear magnetic resonance spectrum, c mass spectrum.
activity than doxorubicin itself; then the sulphonamide derivative 3 (IC50 = 1.3 μg mL−1) have activity nearly to the reference drug. Compounds 1, 3, 6, 7a, 7b, and 7d had a highly toxic effect against breast cancer cell lines with IC50's ranging from 1.3 to 4.7 μg mL−1 compared to doxorubicin, and the compound 2 has a moderate cytotoxic effect with IC50 = 8.2 μg mL−1. While compound 5 has a weak activity with IC50 = 28.7 μg mL−1 compared with other compounds and compared to doxorubicin.

After staining cells using double stains AO/EtBr, cells appeared in the form of four colors as follows: living cells (normal green nuclei), early-programmed cell death (apoptotic) (bright green nucleus with segmented chromatin), late-programmed cell death (apoptotic) (orange nucleus with chromatin condensation or fragmentation) and necrotic cells (Kernel of uniformly colored orange cells).

The uniformly stained green cells with normal, round and intact nuclei that indicates the healthy cell control. Whereas, the highly cell killing with late apoptotic observed by treatment with compound 1 and some necrotic cell also observed with the compound itself; on the other hand there are no necrotic cells with compounds 2 and 3 compared to compound 1, and the derivative acetohydrazide 3 have high rate of late apoptotic compared to compounds 1 and 2 (Figs. 3 and 4).

The compound 5 killing the cells with early apoptotic way was more pronounced compared to compounds 3 and 6. Compound 6 has a necrotic cells after treatment compared to compounds 3 and 5. Also, compound 3 have cells with late apoptotic more than compounds 5 and 6 (Figs. 5 and 6).

Compound 7d has a highly late apoptotic effect on cancer cells compared to 3, 7a, 7b, 7d and compound 7c then 3 have early apoptotic more than 7a, 7b, and 7d is lower (Figs. 7 and 8).

Whereas, compound 8 has early apoptotic killing effect and cell necrotic against cancer cells more than compound 3, while compound 3 has a more cell late apoptotic effect than compound 8 (Figs. 9 and 10).

The biological activity of the tested compounds were indicated the promising cell killing effect of the 4,4′ bipyridine moiety in the compounds acting towards breast tumor cells.

**Table 1** The IC50 (μg mL−1) of the compounds 1, 2, 3, 5, 6, 7a–d, 8 against breast cancer (MCF-7) cell line

| Compounds | MCF-7 IC50 (µg) |
|-----------|----------------|
| Dox.      | 1.6±0.02       |
| 1         | 4±0.18         |
| 2         | 8.2±0.7        |
| 3         | 13±0.04        |
| 5         | 28.7±1.4       |
| 6         | 4.7±0.3        |
| 7a        | 3.5±0.2        |
| 7b        | 4.4±0.2        |
| 7c        | 0.6±0.01       |
| 7d        | 2.7±0.04       |
| 8         | 3±0.2          |

**Conclusions**

In this paper used compound 2-(3-cyano-6-(thiophen-2-yl)-4,4′-bipyridin-2-yl)acetohydrazide 3 to synthesis a novel substituted pyrazole, bipyridine, N-amide derivatives and Schiff bases. The anticancer activity of the compounds was assessed against breast cancer cell line (MCF-7). The data obtained for the tested compounds shows the possible importance of these compounds to act as anticancer agents where compound 7c showed better activity than the standard drug itself. While other compounds such as compound 3 is equiepotent with the standard drug. Compounds 2, 4, 6, 7a, 7b and 7d showed obvious activities but less than the reference.
Fig. 2 The dose response curves of the cytotoxicity of different compounds towards MCF-7 tumor cell line. Cells were exposed to plant extract with different concentrations for 72 h. Cell viability was determined by SRB stain.

Fig. 3 Nuclear morphological conersions of MCF-7 cells, after treated using chemical compounds 1, 2, and 3 compared with reference drug doxorubicin. Compounds stimulate different nuclear changes such as condensation and fragmentation of chromatin, nuclei condensation, as demonstrated by acridine orange/Ethidium bromide staining at 200×. Yellow arrows indicate live cell, pink arrows indicate early apoptotic Red arrows indicate necrotic and blue arrows indicate late apoptotic cells.
Materials and methods

Chemistry

Melting points were measured on a Gallenkamp apparatus, and are uncorrected. The desired time for completing the reaction was monitored by TLC. The IR spectra were recorded using (KBR) plates on a Shimadzu 470 IR spectrometer. The 1H and 13C NMR spectra were measured on a Bruker 400DRX-Avance NMR spectrometer at 400 MHz and chemical shifts (δ) are in ppm relative to TMS (tetramethylsilane). Mass spectra were measured on GC/MS with electron impact ionization by to (70 eV).

Elemental analyses were performed on Perkin-Elmer 2400 series II CHN elemental analyser.

**Synthesis of 2-oxo-4-(pyridin-4-yl)-6-(thiophen-2-yl)-1,2-dihydro pyridine-3-carbonitrile (1)**

4-Pyridine carboxaldehyde (0.01 mol), 2-acetyl thiophene (0.01 mol), ethyl cyanoacetate (0.01 mol), ammonium acetate (0.15 mol) and 5 mol% of CAN in ethanol (25 mL) in a 50 mL round-bottom flask were refluxed for 2 h. After completion of the reaction, the solid product obtained was collected, filtered, washed several times by water dried and then crystallized from ethanol to give compound 1 as yellow crystals in yield 89%, m.p. 205 °C. IR (KBr): 3093 (NH), 2218 (CN), 1673 (C=O) cm⁻¹. 1H NMR (DMSO-d6) δ: 7.24–8.67 (m, 8H, Ar–H, thiophene and pyridine rings), 8.79 (s, 1H, NH) ppm. 13C NMR (DMSO-d6) δ: 163.53 (C=O), 162.81, 150.71 (2C), 143.52 (2CH), 142.89, 142.32 (2C), 131.68, 129.59, 129.01 (3CH), 122.50 (2CH), 121.9 (C), 116.14 (CN), 113.38 (CH) ppm. MS: m/z (%): 279 (M⁺, 20), 224 (100). Anal. Calc. (%) for C₁₅H₉N₃OS: C, 64.50; H, 3.25; N, 15.04; S, 11.48. Found: C, 64.55; H, 3.18; N, 15.11; S, 11.45.

**Synthesis of ethyl 2-(3-cyano-6-(thiophen-2-yl)-4,4'-bipyridin-2-yloxy) acetate (2)**

A mixture of compound 1 (0.01 mol), ethyl bromoacetate (0.01 mol), and anhydrous potassium carbonate (0.15 mol) in acetone was refluxed for 2 h. After
completion of the reaction the mixture was poured onto the ice, the product separated was collected by filtration, dried, and crystallized from ethanol to give 2 as pale yellow needles in yield 75%, m.p. 159–160 °C. IR (KBr): 2224 (CN), 1753 (C=O), 1600 (C=N) cm⁻¹. ¹H NMR (DMSO-d₆) δ: 1.18 (t, 3H, J=6.8, CH₃), 4.15 (q, 2H, J=6.8, OCH₂), 5.06 (s, 2H, CH₂), 7.24-8.82 (m, 8H, Ar=H, thiophene and pyridine rings) ppm. ¹³C NMR (DMSO-d₆) δ: 163.26(C=O), 153.48 (C), 150.71 (C), 143.38 (2CH), 142.38 (C), 132.59, 129.88, 129.64 (3CH), 123.51 (2CH), 114.95 (CN), 113.14 (CH), 91.57 (2C), 64.25 (OCH₂), 61.29 (CH₂), 14.65 (CH₃) ppm. MS: m/z (%): 365 (M⁺, 100). Anal. Calc. (%) for C₁₉H₁₅N₃O₃S. C, 62.45; H, 4.14; N, 11.50; S, 8.77. Found: C, 62.48; H, 4.19; N, 11.45; S, 8.68.

**Synthesis of 2-(3-cyano-6-(thiophen-2-yl)-4,4′-bipyridin-2-yloxy) aceto hydrazide (3)**

A mixture of hydrazine hydrate (99%, 0.04 mol), and compound 2 (0.01 mol), was refluxed in 20 mL absolute ethanol for 5 h. The reaction mixture was poured on an ice-water. The product formed was filtered of, washed with water, and dried, and crystallized from ethanol to give 3 as yellow crystals in yield 65%, m.p 226 °C. IR (KBr): 3402.43, 3334.92 (NH₂), 3267 (NH), 2212 (CN), 1741 (C=O), 1620 (C=N) cm⁻¹. ¹H NMR (DMSO-d₆) δ: 4.67
(s, 2H, CH₂), 7.18–8.58 (m, 8H, Ar–H, thiophene and pyridine rings), 8.75 (d, 2H, NH₂), 12.48 (s, 1H, NH) ppm. \( ^{13}\)C NMR (DMSO-d₆) δ: 160.82 (C=O), 153.20, 153.16 (2C), 150.53 (2CH), 143.38 (2C), 143.10 (C), 131.25, 129.45, 129.19 (3CH) 123.47 (2CH), 116.33 (CN), 111.44 (CH), 101.86 (CH), 85.76 (C), 56.50 (OCH₂) ppm. MS (m/z, %): 351 (M⁺, 20), 101 (100). Anal. Calc. (%) for C₁₇H₁₃N₅O₂S. C, 58.11; H, 3.73; N, 19.93; S, 9.12. Found: C, 58.17; H, 3.68; N, 19.96; S, 9.18.

**General procedure for the synthesis of compounds 4-6**

An equimolar amount of ethyl acetoacetate, acetylacetone and/or ethyl cyanoacetate (or diethyl malonate) and a mixture of compound 3 (0.01 mol) was refluxed in 15 mL acetic acid for 5 h. The produced product after cooling was filtered off, washed with water, dried, and crystallized with acetic acid to give compounds 4, 5, and 6 respectively.

2-(2-((3-methyl-5-oxo-4,5-dihydropyrazol-1-yl)-2-oxo)thoxy)-6-(thiophen-2-yl)-4,4′-bipyridine-3 carbonitrile

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**Fig. 8** Rate of apoptotic MCF-7 tumor cells after 48 h treatment with chemical compounds (mean ± SD of three independent experiments in three repeats each) compared to cell control

**Fig. 9** Morphological and nuclear changes of MCF-7 tumor cells after treatment by chemical compounds 3, and 8 compared with reference drug doxorubicin. Compounds stimulate different nuclear changes such as chromatin fragmented and condensation, nuclei condensation, as demonstrated by acidine orange/Ethidium bromide staining at 200×. Yellow arrows indicate live cell, pink arrows indicate early apoptotic Red arrows indicate necrotic and blue arrows indicate late apoptotic cells.
Pale yellow crystals in yield 71%, m.p. 202–204 °C. IR (KBr): 2347.37 (CN), 1670.35 (C=O), 1637.56 (C=O), 1620 (C=N) cm⁻¹. ¹H NMR (DMSO-d6) δ: 1.84 (s, 3H, CH₃), 2.88 (s, 2H, CH₂), 4.67 (s, 2H, CH₂), 7.21–8.79 (m, 8H, Ar–H, thiophene and pyridine rings) ppm. ¹³C NMR (DMSO) δ: 175.18 (C=O), 170.05 (2C=O), 161.99 (C=O), 159.51 (C=O), 158.02 (C=O), 149.79 (2CH), 144.50 (CH), 144.77 (C), 143.10 (CH), 129.45, 128.97, 127.44 (3CH), 124.10 (CH), 111.44 (CN), 101.86 (CH), 58.16 (OCH₂), 42.59 (CH₂), 22.88 (CH₃) ppm. MS: m/z (%): 417.03 [M⁺, 19], 293 (100). Anal.Calc. (%) for C₁₉H₁₃N₅O₃S. C, 60.42; H, 3.62; N, 16.78; S, 7.68. Found C, 60.47; H, 3.68; N, 16.75; S, 7.65.

3-(3,5-Dimethyl-1H-pyrazol-1-yl)-2-oxoethoxy)-6-(thiophen-2-yl)-4,4'-bipyridine-3-carbonitrile (5) Pale yellow crystals in yield 50%, m.p. 197–198 °C. IR (KBr): 3265 (NH), 2347.37 (CN), 1670.35 (C=O), 1637.56 (C=O), 1620 (C=N) cm⁻¹. ¹H NMR (DMSO-d6) δ: 1.81 (s, 3H, CH₃), 2.01 (s, 3H, CH₃), 4.66 (s, 2H, CH₂), 6.21–8.75 (m, 9H, CH pyrazole, pyridine and thiophene rings) ppm. ¹³C NMR (DMSO) δ: 197.41 (C=O), 174.87 (C=O), 161.99 (C=N) cm⁻¹. ¹H NMR (DMSO-d6) δ: 4.66 (s, 2H, CH₂), 8.70–7.18 (m, 13H, Ar–H, thiophene, pyridine and CH alphatic), 9.99 (s, 1H, NH), 12.49 (br. s, 1H, OH), 13.71 (br. s, 1H, OH) ppm. ¹³C NMR (DMSO) δ: 151.56 (C), 150.38 (C), 149.67 (C), 147.73 (CH), 144.95 (CH), 144.77 (C), 143.10 (CH), 129.46, 128.97, 127.45 (3CH), 124.10 (3CH), 111.44 (CN), 101.87 (CH), 59.08 (OCH₂) ppm. MS: m/z (%): 440 [M⁺, 20], 374 (100). Anal.Calc. (%) for C₂₁H₁₆N₅O₄S.C, 62.76; H, 3.66; N, 19.08; S, 7.28. Found C, 62.76; H, 3.70; N, 19.11; S, 7.32.

General procedure for synthesis of Schiff bases 7a-d
A mixture of compound 3 (0.01 mol) and the appropriate aromatic aldehyde (3-pyridincarboxaldehyde, 3, 4-dihydroxy benzaldehyde, anisaldehyde and vanillin (0.01 mol) in the presence of a catalytic amount of piperedine, in absolute ethanol (10 mL) was refluxed for 2 h. After cooling, the formed precipitate was filtered off, dried, and crystallized from acetic acid to afford the corresponding Schiff base 7a-d.
CH3), 8.88–7.03 (m, 13H, Ar–H, thiophene, pyridine and CH aliphatic), 12.49 (s, 1H, NH–H) ppm. 13C NMR (DMSO): δ = 162.74 (C=O), 153.63 (C=N), 153.17 (C), 152.23 (C), 151.56 (C), 149.58 (2CH), 144.22 (CH), 143.27 (C), 143.10 (CH), 128.97, 128.06, 127.44 (3CH), 123.37 (2CH), 111.44 (CN), 107.84 (CH), 101.87 (CH), 55.91 (OCH2), 47.13 (OCH3) ppm. MS: m/z (%): 469 [M + 27], 462 (100). Anal. Calc. (%) for C25H19N5O4S: C, 63.95; H, 4.08; N, 14.92; S, 3H, CH3), 4.08 (s, 2H, CH2), 8.26–7.12 (m, 13H, Ar–H, thiophene, pyridine and CH aliphatic), 8.84 (s, 1H, NH), 12.58 (br. s, 1H, OH) ppm. 13C NMR (DMSO): δ = 153.60 (C=O), 153.17 (C), 152.23 (C), 145.93 (C), 145.54 (CH), 144.41 (C), 143.09 (C), 141.89 (C), 141.86 (C), 140.34 (C), 139.24 (C), 139.13 (C), 138.13 (C), 137.88, 137.47 (3CH), 125.76 (2CH), 112.49 (CN), 115.80 (CH), 113.53 (CH), 111.44 (CN), 106.31 (CH), 101.87 (CH), 55.81 (OCH3), 44.04 (OCH2) ppm. MS: m/z (%): 485 [M + 27], 482 (100). Anal. Calc. (%) for C25H19N5O4S2: C, 58.07; H, 3.67; N, 13.55; S, 6.83. Found C, 63.91; H, 4.11; N, 14.96; S, 6.87.

(E)-2-[3-(cyano-6-((thiophen-2-yl)-4,4′-bipyrindin-2-yl)oxy)-N′-4-hydroxy-3 methoxy benzylidene) acetohydrazide (7d)

Pale Yellow crystals in yield 80%, m.p.240–241 °C. IR (KBr): 3402.43 (OH), 3339 (NH), 2222 (CN), 1654.92 (C=O), 1547.09, 1513.68 (C=C), 128.97, 128.06, 127.44 (3CH), 123.37 (2CH), 111.44 (CN), 107.84 (CH), 101.87 (CH), 55.91 (OCH2), 47.13 (OCH3) ppm. MS: m/z (%): 469 [M + 27], 462 (100). Anal. Calc. (%) for C25H19N5O4S2: C, 58.01; H, 3.70; N, 13.53; S, 12.39. Found C, 58.07; H, 3.67; N, 13.55; S, 12.34.

Synthesis of (E)-2-[3-(cyano-6-(thiophen-2-yl)-4,4′-bipyrindin-2-yl)oxy)-N′-(4-hydroxy-3 methoxy benzylidene) acetohydrazide (8)

A mixture of p-toluene sulfonyl chloride (1 mmol) and compound 3 (1 mmol) in 10 mL of absolute ethanol was refluxed for 3 h. The formed precipitate was filtered, washed with water, dried, and recrystallized from dioxane to get buff crystals in yield 60%, m.p.279 – 280 °C. IR (KBr): 3350 (NH), 2200 (CN), 1700 (C=O), 1645.92 (C=O), 1516 (C=O), 1416 (C), 1398 (C), 1298, 1279, 1277 (3CH), 123.38 (2CH), 115.80 (CH), 113.53 (CH), 111.44 (CN), 106.31 (CH), 101.86 (CH), 55.81 (OCH3), 44.04 (OCH2) ppm. MS: m/z (%): 485 [M + 14], 306.76 (100). Anal. Calc. (%) for C25H19N5O4S2: C, 61.88; H, 3.89; N, 14.45; S, 6.56.

Acridine orange/ethidium bromide staining for detection of early and late apoptotic cells

DNA binding dyes Acridine orange (AO) and Ethidium bromide (EtBr), were used for the morphological detection of viable, apoptotic and necrotic cells. AO is taken up by both non-viable and viable cells that emit green fluorescence when intercalated into DNA. EtBr is taken up by non-viable cells whereas; it is excluded by viable cells and emits red fluorescence by intercalation into DNA. Cells were seeded on cover slide inside six well plates. Cells were incubated in CO2 incubator with 37 °C temperature and 5% CO2 for 24 h then treated with IC50% concentration of the chemical compounds and incubated for 48 h. Cells were washed with cold PBS 1× for three times. Cells were stained with a mixture Acridine Orange 100 µg/ml/Ethidium Bromide (AO/EB) 100 µg/ml in PBS 1x with 10% FBS on each well and then incubated for 5 min in RT. The cover slides with cultured stained cells were transfer immediately to new slides and the cells were ready to be visualized by the blue filter of the fluorescence microscope [34, 35].

In vitro cytotoxic activity

Cell culture

Breast carcinoma (MCF-7) human cell line was obtained from the American type culture collection (ATCC). Cells were maintained in RPMI-1640 supplemented with (100 µg/mL); penicillin (100 units/mL) and heat-inactivated fetal bovine serum (10% v/v) in a humidified, 5% (v/v) CO2 atmosphere at 37° [31, 32].

Cytotoxicity assay

The cytotoxicity of the chemical compounds was evaluated against (MCF-7) human tumor cell using Sulforhodamine B assay (SRB) in King Khalid University, biology department. 80% confluency growing cells were trypsinized and cultured in a 96 well tissue culture plate for 24 h before treatment with the chemical compounds. Cells were exposed to the six different concentrations of each compound (0.01, 0.1, 1, 10, and 1000 µg/ml); untreated cells (control) were added. The cells were incubated with the concentrations for 72 h and subsequently fixed with TCA (10% w/v) for 1 h at 4 °C. After several washings, cells were stained by 0.4% (w/v) SRB solution for 10 min in dark place. Excess stain was washed with 1% (v/v) glacial acetic acid. After drying overnight, the SRB-stained cells were dissolved with Tris–HCl and the color intensity was measured in microplate reader at 540 nm. The relation between viability percentage of each tumor cell line and compounds concentrations was analyzed to get the IC50 (dose of the drug which reduces survival to 50%) using Sigma Plot 12.0 software [33].

Abbreviations

MCF-7: Breast cancer cell line; SAR: Structure–activity relationship; CAN: Ceric ammonium nitrate; 1H NMR: Nuclear magnetic resonance spectroscopy; SRB: Sulforhodamine B; IC50: The half maximal inhibitory concentration (IC50); AO/EtBr: Acridine Orange Ethidium Bromide; TLC: Thin-layer chromatography; The IR spectra: Infrared spectroscopy; KBr: Potassium bromide; TMS: Tetramethylsilane; GC/MS: Gas chromatography–mass spectrometry; 13C NMR: Carbon-13
Nuclear Magnetic Resonance Spectroscopy; DMSO: Dimethyl Sulfoxide; ATCC: American type culture collection; SRB: Sulphorhodamine B assay; AO: Acridine orange; EtBr: Ethidium bromide; PBS: Fetal bovine serum; PBS: Phosphate-Buffered Saline.

Acknowledgements
The author thank Dr. Heba A. E. Elhady Mohamed for her helpful discussion and review of this work.

Authors’ contributions
All work was performed by the sole author. The author read and approved the final manuscript.

Funding
This research received no external funding.

Availability of data and materials
All the data supporting findings are contained within the manuscript.

Competing interests
No competing of interests.

Received: 28 March 2020   Accepted: 20 May 2020

Published online: 02 June 2020

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