Research Article

Synthesis, Characterization, and Anticancer Activity of New Quinazoline Derivatives against MCF-7 Cells

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Two new synthesized and characterized quinazoline Schiff bases 1 and 2 were investigated for anticancer activity against MCF-7 human breast cancer cell line. Compounds 1 and 2 demonstrated a remarkable antiproliferative effect, with an IC50 value of 6.246 × 10−6 mol/L and 5.910 × 10−6 mol/L, respectively, after 72 hours of treatment. Most apoptosis morphological features in treated MCF-7 cells were observed by AO/PI staining. The results of cell cycle analysis indicate that compounds did not induce S and M phase arrest in cell after 24 hours of treatment. Furthermore, MCF-7 cells treated with 1 and 2 subjected to apoptosis death, as exhibited by perturbation of mitochondrial membrane potential and cytochrome c release as well as increase in ROS formation. We also found activation of caspases-3/7, -8, and -9 in compounds 1 and 2. Moreover, inhibition of NF-κB translocation in MCF-7 cells treated by compound 1 significantly exhibited the association of extrinsic apoptosis pathway. Acute toxicity results demonstrated the nontoxic nature of the compounds in mice. Our results showed significant activity towards MCF-7 cells via either intrinsic or extrinsic mitochondrial pathway and are potential candidate for further in vivo and clinical breast cancer studies.

1. Introduction

Quinazoline nucleus is an interesting molecule among the most important classes of an aromatic bicyclic compounds with two nitrogen atoms in their structure. It is consisting of aromatic benzopyrimidine system made up of two fused six member simple aromatic rings benzene and pyrimidine ring [1] Figure I.

Recently, many efforts have been focused by chemists on the modification of quinazoline ring for development of pharmaceutical and clinical compounds [2]. A brief survey about biological importance of quinazoline and their derivatives revealed that a large number of publications began to appear after 1960s. Most of quinazoline derivatives which have been identified consist of wide range of biological and pharmaceutical activities such as anticancer [3], antioxidant [4], antiviral [5], anticonvulsant [6], anti-inflammatory [7], antitubercular [8], anti-HIV [9], analgesic [10], and antimicrobial [11]. In addition, several studies have been conducted to evaluate the pharmacokinetics and toxicity of new quinazoline-based compounds in different animal model to prove the safe nature of their synthesized compounds [12, 13].

Cancer still is a major threat to human beings around the world. Among all diagnosed cancers, breast cancer is the second leading cause of death in women which is diagnosed in nearly 30% of all women in the United States [14, 15]. Normal cell typically dies through apoptosis which regulate cell proliferation and destruction of aberrant cells; however;
in cancer cells, apoptosis is suppressed and required to be triggered which is a key factor in area of anticancer drug development [16]. Among all targets of cancer research, reactive oxygen species (ROS) play an important role in anticancer drug research. Since, generation of excessive ROS will result in perturbation of the mitochondrial membrane potential and release of cytochrome c from mitochondria into the cytosol and consequently activates caspase-9 expression followed by activation of executioner caspases including caspas-3 and -7 which induce execution phase of apoptosis [17]. Furthermore, activation of caspase-8 is closely involved in extrinsic signaling pathway of apoptosis [18] which associated with inhibition of NF-κB translocation [19]. If the activity of this factor is blocked, tumor cells can undergo apoptosis [20].

Although the current anticancer quinazoline-based agents have demonstrated great clinical benefits in cancer treatment [21], we still need to establish better anticancer agents from quinazoline derivatives with minimum adverse side effects [22] that provides much more hope to mankind. We are particularly interested in the present work to develop potential anticancer agents against breast cancer cell line and screen for their possible mechanism either intrinsic or extrinsic mitochondrial pathway. Therefore, we investigated anticancer potential of 3-(5-bromo-2-hydroxybenzylideneamino)-2-(5-bromo-2-hydroxyphenyl)-2,3-dihydroquinazoline-4(1H)-one (1) Figure 3. 2-Aminobenzohydrazide (0.755 g, 5 mmol) reacted with 2-hydroxy-5-bromo benzaldehyde (2.01 g, 10 mmol) according to above-mentioned procedure. Yield: (2.55 g, 98%); mp 236–238 °C; Anal. Calc. for C_{23}H_{13}N_{3}O_{3}Br (517.17): C, 48.77; H, 2.92; N, 8.13. Found: C, 48.63; H, 2.84; N, 8.15. Characteristic IR data (cm\(^{-1}\)): 1661 ν (C=O), 1589 ν (C=N), 1294 ν (C-N), 1158 ν (C-O), 1112 ν (N-N). UV-Vis (dimethylformamide) \(\lambda_{\text{max}}/\text{nm}: 305\) (br), 342 (br) and 365 (br). \(^1\)H-NMR (400 MHz, DMSO-d6/TMS, ppm) \(\square\square\square\) 11.26 (s, H, O Henry), 10.57 (s, H, O Henry), 8.56 (s, H, N=CH), 779 (d, \(J = 777\) Hz, 1H, Ar-H), 765 (d, \(J = 2.36\) Hz, 1H, Ar-H), 756 (d, \(J = 2.23\) Hz, 1H, NH quinazoline ring), 7.42 (dd, \(J_1 = 8.75\) Hz, \(J_2 = 2.42\) Hz, 1H, Ar-H), 7.33 (m, 2H, Ar-H), 7.04 (d, \(J = 2.2\) Hz, 1H, Ar-H), 6.88 (d, \(J = 8.75\) Hz, 2H, Ar-H), 6.83 (d, \(J = 8.16\) Hz, 1H, Ar-H), 6.78 (t, \(J = 7.3\) Hz, 1H, Ar-H), 6.73 (d, \(J = 2.30\) Hz, 1H, CH quinazoline ring), \(^{13}\)C-NMR (100 MHz, DMSO-d6/TMS, ppm) \(\square\square\square\square\) 160.32 (N=O), 156.54 (N=CH), 154.36 and 147.78 (Ar-OH), 146.12 134.45, 133.92, 132.51, 131.27, 128.42, 128.11, 126.64, 120.83, 118.85, 118.11, 117.91, 114.89 and 113.07 (Ar-H), 110.27 and 109.83 (Ar-Br), 66.00 (CH-quinazoline ring).

X-ray quality crystals were grown from DMSO.

2.4. Synthesis of 3-(5-Bromo-2-hydroxy-3-methoxybenzylidenamino)-2-(5-bromo-2-hydroxy-3-methoxyphenyl)-2,3-dihydroquinazoline-4(1H)-one(2) Figure 4. 2-Aminobenzohydrazide (0.755 g, 5 mmol) reacted with 5-bromo-2-hydroxy-3-methoxy benzaldehyde (2.31 g, 10 mmol) according to the procedure described earlier. Yield: (2.80 g, 96%); mp 216–218 °C; Anal. Calc. for C_{33}H_{19}Br_{2}N_{3}O_{5} (576.97): C, 47.86; H, 3.32; N, 7.28. Found: C, 47.81; H, 2.87; N, 7.22. Characteristic IR peeks (cm\(^{-1}\)): 1641 ν (C=O), 1610 (C=O), 1334 ν (N-O), 1270 ν (C-N), 1148 ν (C-O), 1044 ν (N-N). UV-Vis (dimethylformamide) \(\lambda_{\text{max}}/\text{nm}: 305\) (br) and 369 (br). \(^1\)H NMR (400 MHz, DMSO-d6/TMS, ppm) \(\square\square\square\square\square\square\square\) 11.10 (s, H, OH), 9.89 (s, H, OH), 8.59 (s, 1H, N=CH), 779 (d, \(J = 750\) Hz, 1H, Ar-H), 753 (d, \(J = 2.14\) Hz, 1H, NH quinazoline ring), 7.30 (m, 1H, Ar-H), 7.25 (d, \(J = 2.09\) Hz, 1H, Ar-H), 7.16 (d, \(J = 1.95\) Hz, 1H, Ar-H), 7.10(d, \(J = 2.00\) Hz, 1H, Ar-H),
Figure 2: The reaction pathway for the synthesis of compounds (1) and (2).

Figure 3: Compound (1).

Figure 4: Compound (2).

6.80 (m, 3H, Ar-H), 6.68 (d, J = 1.96, 1H, CH quinazoline ring), 3.83 (s, 3H, OCH$_3$), 3.82 (s, 3H, OCH$_3$). $^{13}$C NMR (100 MHz, DMSO-d$_6$/TMS, ppm) 160.08 (N-C=O), 149.133 (Ar-OCH$_3$), 148.93 (Ar-OCH$_3$), 147.50 (-CH=N), 146.50 and 146.04 (Ar-OH), 143.29, 134.47, 128.12, 126.70, 122.70, 120.15, 119.75, 117.88, 116.37, 115.07, 114.88 and 112.90 (Ar-H), 109.88 (2xAr-Br), 65.48 (C-H quinazoline ring), 56.31 and 56.22 (Ar-O-CH$_3$).

2.5. MTT Cytotoxicity Assay. The normal MCF-10 breast cells, normal WRL-68 hepatic cells, and human MCF-7 breast adenocarcinoma cells were purchased by ATCC (Manassas, VA, USA) and maintained in RPMI-1640 (Roswell Park Memorial Institute) medium supplemented with FBS (10%). Cytotoxicity test was performed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Briefly, cells (5 $\times$ 10$^4$ cells/mL) were seeded into 96-well sterile plates (Nunc, Germany). On the next day, cells were treated with different concentrations of (1), (2), and doxorubicin as a positive control. After 24, 48, and 72 hours of incubation, we stained the incubated cells with 20 $\mu$L of MTT (0.121 $\times$ 10$^{-6}$ mol/L) for 3 hours, and then 100 $\mu$L of DMSO was added to dissolve the resulting dark formazan crystals and incubated in dark for 2 hours. Finally, absorbance was measured at 570 nm wavelength using ELISA reader (Hidex, Turku, Finland). The IC$_{50}$ value was calculated and data was reported as the average of three replicates.

2.6. LDH Release Assay. Cytotoxicity effect of the quinazolinone-based compound was also assessed by performing a LDH (lactate dehydrogenase) release assay. In brief, MCF-7 cells were treated with (1) and (2) at different concentrations for 48 hours, the supernatant of the treated cells was transferred into 96-well plates, and then 100 $\mu$L of the LDH reaction solution (PierceTM LDH Cytotoxicity Assay Kit, Thermo Scientific, Pittsburgh, PA) was added. After 30 minutes, the intensity of red colour in the samples presenting the LDH activity was measured at 490 nm using a Tecan Infinite 200 Pro (Tecan, Männedorf, Switzerland) microplate reader.

2.7. Morphological Study. A combination of a cell-permeable DNA-binding dye, that is, acridine orange (AO) with plasma membrane-impermeable, and DNA-binding dye propidium iodide (PI) was used to assess the morphological changes in treated MCF-7 cells. Briefly, MCF-7 cells were plated at a concentration of 1 $\times$ 10$^6$ cell/mL and treated with (1) and (2) at different IC$_{50}$ value for 3 incubation periods (24, 48, and 72 hours) according to Table 2. Moreover, untreated cells also were employed as normal control. Next, plates were incubated in an atmosphere of 5% CO$_2$ at 37 $^\circ$C. The cells were then centrifuged at 300 $\times$ g for 10 minutes and washed twice with cold phosphate buffered saline (PBS). Finally, we stained the cells with equal volume of a mixture of AO/PI (0.107 $\times$ 10$^{-6}$ mol/L) and observed under a UV-fluorescent microscope (Olympus BX51) within 30 minutes before the fluorescence colour
started to fade. Cell morphological assessment was carried out for features such as membrane blebbing, chromatin condensation, and other features of apoptosis.

2.8. Cell Cycle Analysis. Cellomics Cell Cycle kit 1 (Thermo Scientific, Pittsburgh, PA) was used for analyzing the cell cycle distribution induced by the quinazolinone-based compounds. BrdU and phosphohistone H3 dyes are used for simultaneous quantification of nuclear DNA content to distinguish DNA replication in S phase cells and mitosis marker in M phase cells, respectively. Briefly, MCF-7 cells (1 × 10⁶ cells/well) were treated with different concentrations of compounds or DMSO (negative control) for 24 hours. After fixing and staining the cells for BrdU and phosphohistone H3 for 30 minutes as described by the manufacturer’s protocols, we analysed the cells using a Cellomics ArrayScan HCS reader (Thermo Scientific) and quantified the results using a Target Activation Bioapplication module.

2.9. Reactive Oxygen Species (ROS) Assay. The generation of intercellular ROS induced by quinazolinone-based compounds was measured using a Cellomics Oxidative Stress 1 HCS Reagent Kit (Thermo Scientific, Pittsburgh, PA), according to the manufacturer’s protocols. In response to oxidative stress and production of reactive oxygen species (ROS), Dihydroethidium (DHE) dye reagent is converted to fluorescent ethidium and intercalates into DNA. In brief, MCF-7 cells were treated with (1) and (2) at different concentrations for 24 hours. DHE dye was then added to the treated cells and incubated for 30 minutes. Next, cells were fixed and washed with the wash buffer provided with the kit. Finally, the fluorescence intensity reflected the oxidation of the DHE dye to ethidium, which allowed for the measurement of the ROS generation using a fluorescent plate reader at an extension wavelength of 520 nm and an emission wavelength of 620 nm.

2.10. Multiple Cytotoxicity Assay. Cellomics Multiparameter Cytotoxicity 3 Kit was used as previously described by Arbab et al. [23]. This kit enables to analyse crucial apoptotic events, including the loss of cells, changes in cell permeability, cytochrome c release, changes in mitochondrial membrane potential (MMP), morphological features, and nuclear size. Apoptotic events indicated above in the MCF-7 cells after treatment with the quinazolinone-based compounds were simultaneously measured. An ArrayScan high content screening (HCS) system was used to analyse the stained cells in the plates (Cellomics, PA, USA).

2.11. Caspases-3/7, -8, and -9 Activity Assay. Caspase-Glo 3/7, 8, and 9 kit (Promega, Madison, WI) was used to determine the activation of caspases-3/7, -8, and -9 [24]. In brief, the MCF-7 cells were seeded into a white-walled 96-well plate and treated with the different concentrations of (1) and (2) for 24 hours. After adding 100 µL of Glo 3/7, 8, and 9 reagents and incubating for 30 minutes in room temperature, the activity of the caspases in the treated cells was measured as the degree of aminoluciferin-labelled synthetic tetrapeptide cleavage and luciferase enzyme substrate release using a Tecan Infinite 200 Pro microplate reader (Tecan, Männedorf, Switzerland). Moreover, time-dependent manner experiment was also conducted to confirm the expression level of caspases and thus MCF-7 cells were treated with (1) and (2) with concentrations of 8.0 × 10⁻⁶ and 7.6 × 10⁻⁶ mol/L, respectively (Table 2). Then we examined the expression level of caspases-3/7, -8, and -9 in different times.

2.12. NF-κB Translocation Assay. Cellomics NF-κB activation HCS kit (Thermo Scientific, Pittsburgh, PA) was used for measuring the cytosol-to-nucleus translocation of nuclear factor-κB (NF-κB). Briefly, MCF-7 cells were treated with different concentrations of the quinazolinone-based compounds for 3 hours [25]. The treated MCF-7 cells were then stimulated with TNF-α (1 ng/mL) for 30 minutes. Cells were then fixed and stained according to the manufacturer’s protocols. Finally, cells were evaluated using an Array Scan HCS Reader, and we quantified the results using a Cytoplasm to Nucleus Translocation Bio application software.

2.13. Acute Toxicity Study. The acute toxicity study was performed according to the OECD protocol [26]. 12 healthy female mices were obtained from the Animal House, University of Malaya, Kuala Lumpur, Malaysia (Ethic number PMI/27/07/2010/MAA (R)). The acute toxicity study was conducted to confirm the expression level of caspases and thus MCF-7 cells were treated with (1) and (2) with concentrations of 8.0 × 10⁻⁶ and 7.6 × 10⁻⁶ mol/L, respectively (Table 2). Then we examined the expression level of caspases-3/7, -8, and -9 in different times.

2.14. Statistical Analysis. The results are reported as the mean values ± standard deviation (SD) for 3 independent experiments. An analysis of variance (ANOVA) was performed using the Prism statistical software package (Graph Pad Software, USA). P values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. IR Spectral and Electronic Absorptions Studies. IR spectral study of (1) and (2) revealed the presence of characteristic amicid carbonyl absorption bands at 1661 and 1672 cm⁻¹; however, azomethine group absorption bands appeared at 1589 and 1606 cm⁻¹, respectively [27, 28]. In addition, strong absorption bands of ν (N-N) appeared at 1087 and 1072 cm⁻¹. Another absorption bands at 3266 and 3273 cm⁻¹ of ν (N-H) is an evidence for formation of quinazoline ring
[29, 30]. Strong absorption bands of ν (C-O) located at 1271 and 1244 cm⁻¹ for compounds 1 and 2, respectively [31]. Absorption bands centered at 1422 and 1439 cm⁻¹ are attributed to C-N groups [32]. In addition, absorption bands at 571 cm⁻¹ and 577 cm⁻¹ assigned to C-Br group for (1) and (2), respectively [33]. All these main absorption bands confirmed the formation of the compounds. Furthermore, electronic absorption spectrum data of the Schiff bases were recorded in dimethylformamide (DMF) solvent. Three broad bands appeared at 305 nm, 342 nm, and 365 nm for (1). Two bands at 305 nm and 369 nm for (2) were assigned to π → \( \pi^* \) and \( \pi \rightarrow \pi^* \) electronic transitions, respectively, of the azomethine group [26, 34].

3.2. 1H-NMR and \(^{13}\)C NMR Studies. 1H-NMR and \(^{13}\)C-NMR spectra of Schiff bases ligands were recorded in dimethylsulfoxide DMSO-d6 with chemical shifts expressed in ppm using tetramethylosilane TMS as internal standard (see Figures 5, 6, 7, and 8).

1H-NMR spectra displayed two sharp singlet signals at 10.57 ppm and 11.26 ppm for compound (1). 11.10 ppm and 9.89 ppm for compound (2) were assigned to (O-H) groups. A
singlet signal appeared in the region of 8.56 ppm for (1) and 8.49 ppm for compound (2) attributed to proton of H-C=N azomethine group [27, 30].

Two doublet signals appeared in the region of 7.56 ppm with $J = 2.24$ Hz and 6.73 ppm with $J = 2.40$ which assigned to protons of the N-H group and C-H of quinazoline ring [35], respectively. The other ten signals observed in the region between 7.42 and 7.78 ppm attributed to aromatic rings protons. $^1$H-NMR spectra of (1) displayed fifteen signals while (2) displayed seventeen signals; a number of these signals are in good agreement to number of hydrogen atoms. In addition, two sharp signals were assigned to two hydroxyl group and one sharp signal was attributed to azomethine group for compounds. $^{13}$C-NMR spectra confirmed the $^1$H-NMR spectral results. A signal appeared at 160.32 ppm which was attributed to carbon of amide group N-C=O. Two signals at 156.54 ppm and 154.36 ppm assigned to carbons of Ar-OH group were also observed. Two signals at 110.27 and 109.83 ppm were assigned to carbons of Ar-Br while signal appeared at 146.12 attributed to azomethine group -N=CH-, while carbon of quinazoline ring appears at 66.00 ppm. Other signals in the compound belong to carbons of aromatic rings. $^{13}$C-NMR spectrum showed twenty-one resonance signals for (1) and 23 signals for (2), respectively, which were in
3.3. X-Ray Crystallography. The X-ray crystal structure of the compound in Figure 9 consists of two coplanar 2-hydroxy-5-bromophenyl groups bonded to a distorted (envelope) dihydroquinazoline. There is a significant twist between dihydroquinazoline-4(1H)-one and attached 2-hydroxy-5-bromophenyl as a result of torsion angle of the methylene amine linkage. The compound consists of two hydrogen bonding: one is intramolecular N2...H3A with a distance of 1.874 Å and the other is intermolecular O4...H3N: with distance of 2.062 Å between dihydroquinazoline and solvated dimethylsulfoxide (DMSO) (see Table 1).

3.4. MTT Cytotoxicity Assay. MTT cytotoxicity assay was performed to assess the antiproliferation effect of both compounds on MCF-7 cancer cell [36]. The result showed that compounds (1) and (2) significantly inhibited the proliferation of MCF-7 cells; however, they exhibited no suppressive activity against human normal MCF-10 breast cells and normal WRL-68 hepatic cells compared to IC_{50} value of compounds toward MCF-7 cells. In this assay, IC_{50} value of doxorubicin was also recorded as positive control, Table 2.

3.5. LDH Cytotoxicity Assay. The cytotoxicity effect of the quinazolinone-based compounds was also assessed by lactate dehydrogenase (LDH) release test on MCF-7 cells treated with different concentrations of (1) and (2) for 48 hours incubation [25]. Both compounds induced significant cytotoxicity at concentrations of $4 \times 10^{-6}$, $8 \times 10^{-6}$, and $16 \times 10^{-6}$ mol/L and significantly increased the release of LDH in treated cells compared to control cells, Figure 10. The significant levels of

Table 1: X-ray crystallographic data table for compound 1.

| Identification code | Compound 1 |
|---------------------|------------|
| Empirical formula   | C_{23}H_{21}Br_{2}N_{3}O_{4}S |
| Formula weight      | 595.31 |
| Temperature/K       | 100(2) |
| Crystal system      | Monoclinic |
| Space group         | C2/c |
| a/Å                 | 21.906(5) |
| b/Å                 | 9.071(2) |
| c/Å                 | 25.497(6) |
| α/°                 | 90.00 |
| β/°                 | 109.622(5) |
| γ/°                 | 90.00 |
| Volume/Å³           | 4773(2) |
| Z                   | 8 |
| ρ_{calc} mg/mm³     | 1.657 |
| m/mm⁻¹              | 3.520 |
| F(000)              | 2384.0 |
| Crystal size/Å³     | 0.35 × 0.22 × 0.14 |
| Radiation           | MoKα (λ = 0.71073) |
| 2Θ range for data collection | 4.9 to 54° |
| Index ranges        | −27 ≤ h ≤ 18, −11 ≤ k ≤ 11, −32 ≤ l ≤ 32 |
| Reflections collected | 12389 |
| Independent reflections | 5055 [R_{int} = 0.0376, R_{sigma} = 0.0490] |
| Data/restraints/parameters | 5055/0/318 |
| Goodness-of-fit on F² | 1.097 |
| Final R indexes [I ≥ 2σ(I)] | R_{1} = 0.0500, wR_{2} = 0.1154 |
| Final R indexes [all data] | R_{1} = 0.0655, wR_{2} = 0.1212 |
| Largest diff. peak/hole/e Å⁻³ | 1.36/−0.93 |

Crystal data for compound 1 (M = 595.31): monoclinic, space group C2/c (no. 15), a = 21.906(5) Å, b = 9.071(2) Å, c = 25.497(6) Å, β = 109.622(5)°, V = 4773(2) Å³, Z = 8, T = 100(2) K, μ(MoKα) = 3.520 mm⁻¹, D_{calc} = 1.657 g/mm³, 12389 reflections measured (4.9 ≤ 2Θ ≤ 54), 5055 unique (R_{int} = 0.0376, R_{sigma} = 0.0490) which were used in all calculations. The final R_{1} was 0.0500 (>2σ(I)) and wR_{2} was 0.1212 (all data).

Figure 9: Crystal structure of compound (1).

Figure 10: The LDH release assay revealed the significant cytotoxicity of the quinazolinone-based compound on MCF-7 cells at concentrations $4 \times 10^{-6}$, $8 \times 10^{-6}$, and $16 \times 10^{-6}$ mol/L.
3.6. Morphological Observation. AO and PI excite, respectively, green and orange fluorescent under fluorescent microscope when they are intercalated into DNA. Late apoptotic and necrotic cells can take both AO and PI; however, viable and early apoptotic cells can stain only by AO [37]. Morphological changes of (1) and (2) treated MCF-7 cell were examined after 24 and 48 h treatment with IC_{50} values shown in Table 2. Cell morphological assessment showed the effects of compounds to be more prominent in treated MCF-7 cells when compared to untreated cells, Figure 11.

The untreated MCF-7 cells exhibited green healthy intact nuclei. After 24 hours of treatment, chromatin condensation and membrane blebbing (indicated by small protrusions of the membrane) are most significant features of early apoptotic events. These characteristics were more pronounced at 48 hours of treatment, which was associated with the presence of orange colour as a result of the PI-positive band of denaturated DNA. In addition, the presence of secondary necrosis was more prominent, as

LDH release have been observed at a concentration of $8 \times 10^{-6}$ and $16 \times 10^{-6}$ mol/L.

Table 2: The IC_{50} concentration of the quinazolinone-based compounds against MCF-7, MCF-10, and WRL-68 cell lines after 24, 48, and 72 h.

| Compound | Cell line       | Classification      | 24 h          | IC_{50} (µg/mL)     | 48 h          | 72 h          |
|----------|-----------------|---------------------|---------------|---------------------|---------------|---------------|
| (1)      | MCF-7           | Breast cancer cells | $8.06 \times 10^{-6}$ mol/L | $7.59 \times 10^{-6}$ mol/L | $6.24 \times 10^{-6}$ mol/L |
|          | MCF-10          | Normal breast cells | $0.48 \times 10^{-6}$ mol/L | $0.48 \times 10^{-6}$ mol/L | $0.48 \times 10^{-6}$ mol/L |
|          | WRL-68          | Normal hepatic      | $0.77 \times 10^{-6}$ mol/L | $0.77 \times 10^{-6}$ mol/L | $0.77 \times 10^{-6}$ mol/L |
| (2)      | MCF-7           | Breast cancer cells | $7.60 \times 10^{-6}$ mol/L | $6.62 \times 10^{-6}$ mol/L | $5.91 \times 10^{-6}$ mol/L |
|          | MCF-10          | Normal breast cells | $0.48 \times 10^{-6}$ mol/L | $0.48 \times 10^{-6}$ mol/L | $0.48 \times 10^{-6}$ mol/L |
|          | WRL-68          | Normal hepatic      | $0.77 \times 10^{-6}$ mol/L | $0.77 \times 10^{-6}$ mol/L | $0.77 \times 10^{-6}$ mol/L |
| Doxorubicin | MCF-7       | Breast cancer cells | $4.47 \times 10^{-6}$ mol/L | $4.19 \times 10^{-6}$ mol/L | $3.82 \times 10^{-6}$ mol/L |
the prolonged incubation of treated MCF-7 cells might induce secondary necrosis following the late apoptosis. Morphological changes of treated MCF-7 cells indicated the induction of apoptosis by selected quinazolinone-based compounds. The criteria of cell morphology identification are as follows: green intact nucleus, viable cells; dense green areas of chromatin condensation in the nucleus, early apoptosis; dense orange areas of chromatin condensation, late apoptosis; and orange intact nucleus, secondary necrosis [38].

3.7. Cell Cycle Analysis. Cell cycle distribution was also investigated to test if (1) and (2) cause a cell cycle stage-inhibition [25]. The images of cell-stained with BrdU and phosphohistone H3 showed no significant difference between treated cells and untreated cells for both quinazoline compounds (Figure 12(a)). The intensities of (1) and (2) treated and untreated MCF-7 cells indicated that the level of BrdU and phosphohistone H3 intensities were reduced (Figure 12(b)). These results indicate that compounds did not induce S/M phases arrest in MCF-7 after 24 hours of treatment.

Figure 12: Cell cycle analysis. (a) Effect of (1) and (2) on cell cycle arrest. After incubation with DMSO or different concentrations of (1) and (2) for 24 h, MCF-7 cells were stained with BrdU and phosphohistone H3 and subjected to the Cellomics ArrayScan HCS reader for cell cycle analysis. (b) Representative bar charts indicating that treatment of both compounds markedly decreased BrdU and phosphohistone H3 fluorescence intensities in treated MCF-7 cells. Data were expressed as the mean ± SD of fluorescence intensity readings for three independent experiments.
As shown in Figure 14(a), untreated cells reveal maximal probe was applied to assess the function of mitochondria. Cells with 4 intensities indicated that the MMP is reduced in the treated slightly stained after 24 hours. Decline in MMP fluorescent dye uptake, whilst the treated cells with (1) and (2) were very concentrations.

3.8. Measurement of Reactive Oxygen Species (ROS). Upgrading in the level of ROS or/and reduction in the level of antioxidants can trigger mitochondrial-initiated events leading to apoptosis [39, 40]. Furthermore, production of ROS can disrupt the homeostasis in the enzyme system of ROS scavenging antioxidants. Generation of ROS was measured in the treated MCF-7 cells with different concentrations of (1) and (2). Exposure to the quinazolinone-based compounds caused the significant production of ROS in the treated MCF-7 cells, 2-fold higher than normal control, at $4 \times 10^{-6}$, $8 \times 10^{-6}$, and $16 \times 10^{-6}$ mol/L concentrations after 24 hours, Figure 13.

3.9. Mitochondria-Initiated Events Analysis. There are convincing evidences that ROS may contribute to cytochrome c release due to disruption of the mitochondrial membrane potential [41–43]. In the present study, the MMP fluorescent probe was applied to assess the function of mitochondria. As shown in Figure 14(a), untreated cells revealed maximal dye uptake, whilst the treated cells with (1) and (2) were very slightly stained after 24 hours. Decline in MMP fluorescent intensities indicated that the MMP is reduced in the treated cells with $4 \times 10^{-6}$, $8 \times 10^{-6}$, and $16 \times 10^{-6}$ mol/L concentrations. In contrast, a significant elevation in cell membrane permeability was also observed at the same concentration after 24 hours exposure of MCF-7 cells to the quinazolinone-based compounds. Moreover, the release of cytochrome c from mitochondria to cytosol increased significantly compared to the control cells after 24 hours which is closely associated with the activity of ROS, Figure 14(b).

3.10. Caspases-3/7, -8, and -9 Activation Analysis. Induction of apoptosis is precisely mediated by caspases cascade via both intrinsic and extrinsic pathway. Caspases play essential role in apoptosis which mainly included two main groups of initiators and executioners [44]. Caspases-8 and -9 are known as initiator caspases through extrinsic and intrinsic pathway, respectively, and facilitate the activation of executioners, such as caspases-3/7. Therefore, the bioluminescent intensities of respective caspases presenting their activities were measured time-dependently in MCF-7 cells treated with different concentrations of (1) and (2) for 24 hours treatment. As shown in Figure 15, compound (1) induced significant expression of caspases-8-, -9, and -3/7 activities in MCF-7 treated cells at $8.0 \times 10^{-6}$ mol/L in different times. Meanwhile, at $7.6 \times 10^{-6}$ mol/L, (2) induced high expression level of caspases-9 and -3/7 activities for different time; however, caspase-8 did not reveal any considerable activation in comparison to untreated cells. Thus, (1) induced apoptosis in MCF-7 via both extrinsic and intrinsic pathway; however, (2) was able to induce apoptosis only through mitochondria with high expression of caspase-9.

3.11. NF-κB Translocation Analysis. The failure in apoptosis is closely associated with activation of nuclear factor kappa B (NF-κB) that elicits a crucial role in cell proliferation and cytokine gene expression. Tumour necrosis factor-α (TNF-α) can stimulate activation and translocation of NF-κB to nucleus, which mediate gene expression of specific genes via DNA-binding activity. Therefore, we then examine the fact that the role of the quinazolinone-based compounds in the inhibition of activated NF-κB was investigated. The result of images and intensities indicated that (1) compound revealed a significant inhibition on the TNF-α-activated NF-κB; however, (2) compound showed no difference with TNF-α positive control, Figures 16(a) and 16(b). Therefore, the result of this assay is an important evidence to confirm that (1) suppresses proliferation of MCF-7 via both mitochondria and TNF receptor pathways [35].

3.12. Acute Toxicity Analysis. In the acute toxicity test, the animals were treated with compounds 1 and 2 at a dosage of 250 mg/kg and were kept under observation for 14 days. All of the animals survived and did not manifest any sign of toxicity at these dosages. There was no detectable sign of hepatic or renal toxicity in the treated group as compared with the control group in either histological analysis (Figure 17) or blood biochemistry test (Tables 3 and 4).

4. Conclusion

The synthesized quinazolinone Schiff bases (1) and (2) established their structures by elemental analysis, spectroscopic techniques, and X-ray diffraction studies. They have shown anticancer potential against MCF7 breast cancer cells. It was found out that compounds possess the capability of inducing intrinsic and extrinsic apoptosis pathway, which was well regulated by caspase enzymes. Moreover, the active role of mitochondria in the cell death was confirmed by reducing the MMP, release of cytochrome c, and ROS elevation. Our results showed that compounds are promising anticancer agents. However, further research in the area of in vivo studies on the compounds might be vital for the development of new pharmaceuticals drugs.
Figure 14: Effects of the Quinazoline Schiff bases on nuclear morphology, membrane permeability, mitochondrial membrane potential (MMP), and cytochrome c release. (a) Representative images of MCF-7 cells treated with medium alone and at $4 \times 10^{-6}$, $8 \times 10^{-6}$, and $16 \times 10^{-6}$ mol/L concentrations of compounds and stained with Hoechst 33342 for nuclear, cytochrome c, membrane permeability, and MMP dyes. Both compounds induced a noteworthy elevation in membrane permeability and cytochrome c release and a marked reduction in mitochondrial membrane potential (magnification: 200x). (b) Representative bar charts indicating dose-dependent increased cell permeability reduced MMP and increased cytochrome c release in treated MCF-7 cells.
Figure 15: Relative luminescence time-dependent expression of caspases-3/7, -8, and -9 in MCF-7 cells treated with (1) and (2) at concentrations of $8 \times 10^{-6}$ mol/L and $7.6 \times 10^{-6}$ mol/L, respectively, after 24 hours incubation.

Figure 16: NF-κB translocation. (a) Photographs of the intracellular targets of stained MCF-7 cells that were exposed to (1) and (2) at concentrations of $8 \times 10^{-6}$ mol/L and $7.6 \times 10^{-6}$ mol/L, respectively, for 3 hours and then stimulated for 30 minutes with 1 ng/mL TNF-α (NF-κB activation). (b) Representative bar chart showing translocation of TNF-α-induced NF-κB nuclear in MCF-7 cells for different concentrations.

Table 3: Effects of 250 mg/kg of 1 and 2 on liver function test.

| Groups | Total protein (g/L) | Albumin (g/L) | Globulin (g/L) | AP (IU/L) | ALT (IU/L) | AST (IU/L) | GGT (IU/L) |
|--------|---------------------|---------------|----------------|-----------|------------|------------|------------|
| Vehicle | $62 \pm 1.5$        | $12.4 \pm 0.65$ | $51.4 \pm 1.1$ | $85.3 \pm 3.2$ | $58.3 \pm 3.5$ | $251 \pm 8.4$ | $3.5 \pm 0.1$ |
| 1 (250 mg/kg) | $65 \pm 1.3$        | $12.9 \pm 0.59$ | $53.1 \pm 1.8$ | $91.4 \pm 3.4$ | $67.7 \pm 4.6$ | $245 \pm 7.5$ | $3.3 \pm 0.07$ |
| 2 (250 mg/kg) | $60 \pm 1.0$        | $11.8 \pm 0.33$ | $55.6 \pm 1.2$ | $87.2 \pm 2.7$ | $56.1 \pm 4.2$ | $264 \pm 8.3$ | $3.7 \pm 0.09$ |

Values are expressed as the means ± S.E.M. There are no statistically significant differences between the measurements of different groups. Significance was set at $P < 0.05$. 
Figure 17: Histological sections in acute toxicity test (H&E staining, 20x). Histological sections of liver (first row) and kidney (second row). Untreated mice (control group) received 5 mL/kg vehicle (5% Tween 20) ((a) and (d)). Animals treated with 250 mg/kg are ((b) and (e)) and ((c) and (f)) for compounds 1 and 2, respectively. There are no significant differences in structures of liver and kidney between treated and control group.

Table 4: Effects of 250 mg/kg of 1 and 2 on renal function test.

| Groups | Sodium (mmol/L) | Potassium (mmol/L) | Chloride (mmol/L) | CO₂ (mmol/L) | Anion gap (mmol/L) | Urea (mmol/L) |
|--------|-----------------|-------------------|------------------|-------------|-------------------|--------------|
| Vehicle | 145.6 ± 0.91    | 8.7 ± 0.15        | 109.4 ± 0.32     | 20.5 ± 0.34 | 27.0 ± 0.6        | 6.5 ± 0.7    |
| 1 (250 mg/kg) | 151.5 ± 0.58    | 9.5 ± 0.18        | 110.8 ± 0.27     | 22.8 ± 0.68 | 26.6 ± 0.9        | 8.4 ± 0.5    |
| 2 (250 mg/kg) | 153.3 ± 0.76    | 9.4 ± 0.2         | 114.3 ± 0.33     | 19.2 ± 0.21 | 22.7 ± 0.5        | 7.7 ± 0.8    |

Values are expressed as the means ± S.E.M. There are no statistically significant differences between the measurements of different groups. Significance was set at \( P < 0.05 \).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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