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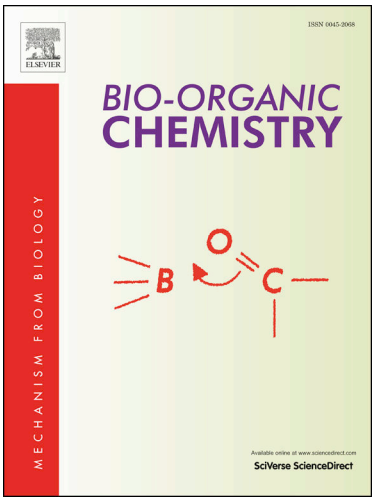

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Novel Indole Schiff Base β-Diiminato Compound as an Anti-Cancer Agent Against Triple-Negative Breast Cancer: Invitro Anticancer Activity Evaluation and In Vivo Acute Toxicity Study

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Abstract

Breast cancer is the most prevalent cancer among women globally, with triple-negative breast cancer (TNBC) associated with poor prognosis and low five-year survival rates. Schiff base compounds, known for their extensive pharmacological activities, have garnered significant attention in cancer drug research. This study aimed to evaluate the anticancer potential of a novel β-diiminato compound and elucidate its mechanism of action. The compound's effect on cell viability was assessed using MTT assays in breast cancer cell lines including MCF-7 and MDA-MB-231. Cytotoxic effects were further analyzed using trypan blue exclusion and lactate dehydrogenase (LDH) release assays. In order to assess the mechanism of inhibitory activity and mode of cell death induced by this compound, flow cytometry of cell cycle distribution and apoptosis analysis were carried out. Apoptosis incidence was initially assessed through cell and nuclear morphological changes (Hoechst 33342/Propidium iodide (PI) staining) and further confirmed by Annexin V/PI staining and flow cytometry analysis. In addition, the effect of this compound on the disruption of mitochondrial membrane potential (MMP) and generation of the reactive oxygen species (ROS) was determined using the JC-1 indicator and DCFDA dye, respectively. The results demonstrated that the 24h treatment with β-diiminato compound significantly suppressed the viability of MDA-MB-231 and MCF-7 cancer cells in a dose-dependent manner with the IC_{50} value of 2.41±0.29 and 3.51±0.14, respectively. The cytotoxic effect of the compound was further confirmed with a dose-dependent increase in the number of dead cells and enhanced LDH level in the culture medium. This compound exerted its anti-proliferative effect by G2/M phase cell growth arrest in MDA-MB-231 breast cancer cells and induced apoptosis-mediated cell death, which involved characteristic changes in cell and nuclear morphology, phosphatidylserine externalization, mitochondrial membrane depolarization, and increased ROS level. Neither hepatotoxicity nor nephrotoxicity was detected in the biochemical and histopathological analysis confirming the safety characterization of this compound usage. Therefore, the results significantly confirmed the potential anticancer activity of a novel β-diiminato compound, as evidenced by the induction of cell cycle arrest and apoptosis, which might

be driven by the ROS‐mediated mitochondrial death pathway. This compound can be a promising candidate for future anticancer drug design and TNBC treatment, and further preclinical and clinical studies are warranted.

Keywords: Breast cancer, β-diiminato compound, apoptosis, anticancer, intrinsic pathway, cell cycle.

1. Introduction

Cancer, recognized as a complex and heterogeneous disease, stands as the second leading cause of death worldwide. By 2020, the incidence of cancer is projected to escalate to 19.3 million new cases annually. Among the 100 cancer types, breast cancer is the most frequently diagnosed, accounting for 12% of all cancer cases globally [1-3]. Triple-negative breast cancer (TNBC) diagnosed by the lack of expression of hormone receptors including estrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor (HER2) is amongst the most lethal breast cancer subgroup which is characterized by a highly aggressive and metastatic phenotype. Consequently, TNBC patients often face a poor prognosis, as indicated by a low five-year survival rate [4-6].

The regulation of normal cell growth involves a delicate balance between cell cycle progression and programmed cell death (apoptosis) [7]. The cell cycle comprises of four different sequential phases including G1, S, G2 (together known as interphase), and M, which are associated with cell preparation for DNA replication, DNA synthesis, cell preparation for cell division, and mitosis, respectively. The normal cell has several checkpoints regulated by key modulators throughout the different phases. Defect in the function of these regulators and overriding such checkpoints leads to uncontrolled proliferation, as detected in many cancers, including breast carcinomas [8, 9]. Besides cell cycle, apoptosis is the hallmark of cancer as well. The apoptosis mechanism is complex and can be initiated by the activation of the extrinsic (receptor-mediated) or intrinsic (mitochondrial-mediated) pathway. Both apoptotic pathways lead to irreversible alterations of cellular macromolecules such as lipids, proteins, or DNA causing cell death. Deficiencies at any point along these pathways can cause malignant transformation of the affected cells, tumor metastasis, and resistance to anticancer drugs [10-12]. Therefore, cell cycle arrest and apoptosis induction have been regarded as effective strategies for eliminating cancer cells in breast cancer treatment as observed in chemotherapy drugs such as cisplatin [13, 14]. However, because of the side effects, resistance to the chemotherapy drugs, and low overall survival rate especially in patients with TNBC cancer, there has been enormous effort focused on the discovery and development of more effective novel therapeutic agents in breast cancer treatment [15, 16].

Schiff bases, a versatile class of compounds in medical chemistry, have exhibited significant biological activities including antibacterial [17], antifungal [18], antioxidant [19], anti-inflammatory [20], antimalarial [21], and antiviral activity [22]. In recent years, they have garnered significant interest as promising agents in the area of drug discovery in cancer. Efforts have been focused on designing and developing novel Schiff bases with enhanced anticancer efficacy and reduced side effects [23-28]. This study aims to evaluate the inhibitory effects, anticancer activity, and cytotoxic mechanisms of the novel indole, Schiff-based β-diiminato compound against the human metastatic breast cancer cell line, MDA-MB-231. Given the highly aggressive nature and frequent chemotherapeutic resistance of these cells, they serve as a valuable model for TNBC research.

2. Methodology

2.1 Synthesis and preparation of novel β-diiminato compound

The novel indole, Schiff based β-diiminato compound, LH_3, was synthesized as previously described [28]. Briefly, it was synthesized by reacting 2-(diformylmethylidene)-3,3-

dimethylindole (0.645 g, 3 mmol) with o-aminophenol (0.654 g, 6 mmol) in ethanol (50 mL) with 0.5 mL of 37% hydrochloric acid under reflux for 5 hours. After partial solvent evaporation and standing at room temperature, β-diiminato ligand crystals formed over several days. An efficient method used refluxing toluene, precipitating it in 0.5 hours, yielding 84% pure product. Its structure was also further confirmed using NMR and IR spectroscopy, verifying the formation of the desired β-diiminato ligand [28]. The compound was obtained from the Chemistry Department of the Faculty of Science of the University of Malaya and further dissolved in dimethyl formamide (DMF) to generate the stock solution of 40 mg/mL. It was further diluted with media to get 100 µg/mL working stock solution for experiments. The maximum concentration of DMF even at the highest concentration of the drugs was less than 0.1% v/v.

2.2 Cell Culture

The human MDA-MB-231 and MCF-7 breast cancer, and WRL-68 normal hepatic cells were cultured in Dulbecco's Modified Eagle Medium (DMEM); non-tumorigenic 184B5 breast cells were grown in mammary epithelial basal medium (MEBM) along with the additives from MEGM kit (Lonza, USA). All cell lines were obtained from the American Type Culture Collection (ATCC, USA) and media were supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. The cells were cultured as the monolayer in tissue culture flasks (Corning, USA), incubated at 37°C in a 5% CO_2 humidified atmosphere, and sub-cultured when they reached approximately 70% confluence.

2.3 MTT colorimetric assay for cell viability

The effect of the β-diiminato compound on cell viability and proliferation of MDA-MB-231, MCF-7, 184B5 and WRL-68 cell lines was assessed using yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) dye. Briefly, cells were seeded in 96-well plates at a density of 7000 cells/well (MDA-MB-231, MCF-7,and WRL-6) or 10,000 cells/well (184B5) and incubated overnight at 37°C in 5% CO_2. On the following day, the cells were treated with different concentrations of the compounds (0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 µg/mL) and incubated for 24h [23, 29] . In addition, untreated control cells and blank wells containing medium were also included in each plate. After incubation time, 50 µL of MTT solution (2 mg/mL in phosphate-buffered saline) was added to each well, the plates were covered with aluminum foil, and incubated for further 2h. Then 100 µL of dimethylsulfoxide (DMSO) was added to the empty wells to dissolve the produced formazan crystal. Plates with cover were then shaken for 15 min at room temperature. The absorbance was measured at 570 nm using a Tecan infinite M1000Pro microplate reader (Tecan, Männedorf, Switzerland). The cell viability was expressed as the percentage of absorbance in the treated cells compared to that in the control cells. The assay was performed in 3 independent studies and the concentration that inhibited 50% of cell viability (IC_{50} value) was determined by non-linear regression analysis using GraphPad Prism™ 5 software. Based on the obtained IC_{50} results, a range of concentrations, including both higher and lower values, was included in the following experiments to investigate the dose-dependent effects of the β-diiminato compound.

2.4 Trypan blue exclusion assay

To investigate the potential of the β-diiminato compound to induce cell death, trypan blue staining was performed. MDA-MB-231 cells were seeded in 6-well tissue culture plates at a density of 5×10^5 cell/well and incubated overnight at 37°C. On the next day, the medium was removed and replaced with fresh culture medium containing 1.25, 2.5 and 5 µg/mL of the compound or without treatment (negative control) and incubated for 24h. After the incubation time, the floating and attached cells were collected and an equal volume of suspended cells in the medium were mixed with 0.4% trypan blue solution (Sigma-Aldrich) at 1:1 ratio. Then viable and non-viable cells as the unstained and blue stained cells, respectively, were counted on a hematocytometer via an inverted microscope. Cell viability was expressed as the percentage of viable cells compared with the control.

2.5 Lactate dehydrogenase (LDH) cytotoxicity assay

LDH as a stable cytoplasmic enzyme is used to determine cell death by measuring lactate dehydrogenase activity released from cells with damaged membranes in the culture medium [30, 31]. To further investigate the cytotoxic effect of β-diiminato compound on MDA-MB-231 breast cancer cells, the LDH cytotoxicity assay was performed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, USA). Briefly, MDA-MB-231 cells were seeded (10^4 cells/well) in a black 96-well plate and incubated overnight at 37°C. On the next day, the media was removed and the fresh medium containing 1.25, 2.5 and 5 µg/mL of the compound were added and the plate was incubated for 18h. Untreated, positive, and blank cell-free control were also included in the plate. After the incubation time, the plate was equilibrated to room temperature, and the CytoTox-ONE™ reagent was added to each well. After shaking for 30 s, the plate was further incubated at 37°C for 15 min and the fluorescent intensity of red color, representing the LDH activity, was measured using a Tecan infinite M1000Pro microplate reader (Tecan, Männedorf, Switzerland) with an excitation and emission wavelengths of 560 and 590 nm, respectively.

2.6 Cell cycle analysis

The effect of β-diiminato compound on cell cycle distribution was assessed by flow cytometry analysis of DNA content after propidium iodide (PI) staining. Briefly, MDA-MB-231 breast cancer cells (10^6) were seeded in T-25 flask and incubated overnight. On the next day, the cells were treated with 0, 2.5 and 5 µg/mL of the compound and incubated for 24h at 37°C in an incubator supplied with 5% CO_2. Then, the floating and attached cells were collected, washed with phosphate buffer saline (PBS) and fixed with 70% ethanol overnight at -20°C. Before the analysis, the fixed cells were washed with cold PBS, resuspended in PI/RNase staining buffer (BD Bioscience) and incubated for 30 min in the dark at room temperature. The distribution of cells in the cell cycle was measured using FACScan flow cytometer (Becton Dickinson, USA) within 1h.

2.7 Assessment of cell morphological changes

2.7.1 Observation of cells by phase contrast microscope

MDA-MB-231 breast cancer cells were seeded at the concentration of 10^5 in a 24-well plate and incubated overnight at 37°C in an incubator supplied with 5% CO_2. Then, the fresh

medium was replaced and the cells were treated with 0, 1.25, 2.5, and 5 μg/mL of the β-diiminato compound and incubated for 24h at 37°C. After the incubation time, cells were visualized under phase-contrast microscopy to evaluate the morphological changes at 100x magnification.

2.7.2 Hoechst 33342/PI staining

Double-fluorescence staining using Hoechst 33342 (HO33342) and PI as a cell membrane permeable and impermeable dye, respectively, is a staining method to evaluate apoptotic cells according to the nuclear morphology. For this purpose, MDA-MB-231 breast cancer cells (**(**10^5) were seeded into a 24-well plate and incubated overnight at 37°C in an incubator supplied with 5% CO_2. On the next day, the cells were treated with different concentrations of the β-diiminato compound including 0, 2.5, and 5 μg/mL, and incubated for 24h as before. After being washed with PBS, the cells were stained with HO33342 (10 μg/mL) and PI (2.5 μg/mL) solution in the dark. Then, live and apoptotic cells were detected by DAPI (for HO33342 staining) and TRITC (for PI staining) filters of the fluorescence-inverted microscope at 200x magnification.

2.8 Annexin-V-FITC/PI assay

In order to further evaluate the mode of cell death and assess the early and late apoptosis induced by the compound, Annexin-V-FITC staining assay was done using the Apoptosis Detection Kit (eBioscience, USA) according to the manufacturer's protocol. Briefly, MDA-MB-231 breast cancer cells at the density of 5×10^5 cells/well were seeded in a 6-well plate and incubated overnight at 37°C in an incubator supplied with 5% CO_2. On the following day, the fresh medium was replaced and the cells were treated with 0, 2.5 and 5 μg/mL concentrations of the β-diiminato compound for 24h. After the incubation time, the floating and attached cells were harvested and washed with PBS. The cell suspension in binding buffer was then stained with Annexin-V-FITC at room temperature for 10 min in the dark and then the pellet was resuspended in binding buffer and stained with PI. The samples were kept in ice and the fluorescence intensity of the control and treated samples were analyzed using the FACScan flow cytometer (Becton Dickinson, USA) system.

2.9 Mitochondrial membrane potential (MMP) analysis

Disruption of MMP is one of the early intracellular events that occurs following induction of apoptosis [32]. JC-1 dye (Molecular probes) is a fluorescent probe that is used for monitoring the changes in the potential of the mitochondrial membrane. Briefly, MDA-MB-231(10^5 cell/well) cells were seeded in a 24-well plate and incubated overnight at 37°C in an incubator supplied with 5% CO_2. Then, the breast cancer cells were treated with 0, 1.25, 2.5, and 5 μg/mL concentrations of the β-diiminato compound for 24h. After that, a warm medium containing the JC-1 dye (2 μM final concentration) was added to each well and further incubated at 37°C for 20 min in the dark. After being washed with PBS, the MMP depletion was observed under a fluorescence microscope (Nikon, ECLIPSE TI-S) through the FITC (green) and TRITC (red) filters at 100x magnification.

2.10 Detection of reactive oxygen species (ROS) generation

ROS was detected using Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, USA) according to the manufacturer's protocol with some slight modifications. 2, 7-dichlorofluorescin diacetate (DCFDA) as a cell-permeable dye enters the cell and reacts with ROS which results in the formation of highly fluorescent compound dichlorofluorescein (DCF). Briefly, MDA-MB-231 cells at the density of 4×10^4 cell/well were seeded in a 96-well black plate and incubated overnight. Then, the medium was removed from each well, and cells were stained with 25 μM DCFDA followed by incubation for 45 min at 37°C in an incubator supplied with 5% CO_2. After being washed with buffer, the cells were treated with 0, 1.25, 2.5, and 5 μg/mL concentrations of the β-diiminato compound and further incubated for 4h at 37°C. Cell-free control was also included as the control. Then, the fluorescence intensity was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a fluorescence microplate reader (Tecan infinite M1000Pro microplate reader, Männedorf, Switzerland). The ROS fold changes were determined compared to the control after blank (background) subtraction.

2.11 Acute toxicity evaluation

In order to evaluate the safety of the compound usage, the acute toxicity study was conducted according to the Organization for Economic Co-operation and Development protocol (OECD) [33]. Since the female rats are slightly more sensitive, female Sprague-Dawley rats (6–8 weeks old) were randomly divided into 3 groups. All experimental procedures were approved by the ethics committee of the Faculty of Medicine, University of Malaya, Malaysia (Ethics Number: 2016-171006/BMS/R/MAA). After overnight fasting with access to the water, the animals were fed orally with a single dosage of 0 (control), 150, and 300 mg/kg of the β-diiminato compound by oral gavage. Before dose administration, the body weight of each animal was determined and the dose was calculated according to the body weight. For the control group, 10% Tween 20 was administered. Feeding was started 3-4 h after dosing and all animals were observed at 30 min, 2, 4, 8, 24 and 48h up to two weeks after administration to monitor any clinical or toxicological symptoms. Animals were sacrificed on the 15^{th} day by an overdose of xylazine and ketamine anesthesia. Blood samples were then collected for serum biochemical tests [34, 35]. In addition, kidney and liver histological analysis was performed using hematoxylin and eosin (H&E) staining.

2.12 Statistical analysis

The experimental data are expressed as a mean ± standard deviation (SD). A statistical comparison was performed using SPSS software v.22 to compare the effect between untreated and treated cells. The results were considered statistically significant compared with the control group if $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

3. Results

3.1 β-diiminato compound significantly reduced breast cancer cell viability

The effect of this compound on the viability of MDA-MB-231, MCF-7 breast cancer cells, and non-tumorigenic (184B5, WRL-68) cells after 24h treatment was determined using MTT assay. This assay is used to assess cellular metabolism and hence viability. In metabolically active cells, MTT is reduced by the mitochondrial succinate dehydrogenase enzymes and results in the formation of purple formazan crystals, which is directly proportional to the number of viable cells in the culture [36]. As shown in Figure 1, after 24h treatment, the β-diiminato compound showed growth inhibition properties and significantly reduced the MDA-MB-231 and MCF-7 cell viability in a dose-dependent manner with the IC_{50} value of 2.41±0.29 and 3.51±0.14 µg/mL, respectively. In addition, the IC_{50} value of cisplatin as a chemotherapeutic drug, on MDA-MB-231 cells was higher compared to compound, at 6.56±1.17 µg/mL. However, this compound did not show a remarkable effect on human non-tumorigenic 184B5 breast cells and WRL-68 hepatic cells compared to its IC_{50} value for cancer cells.

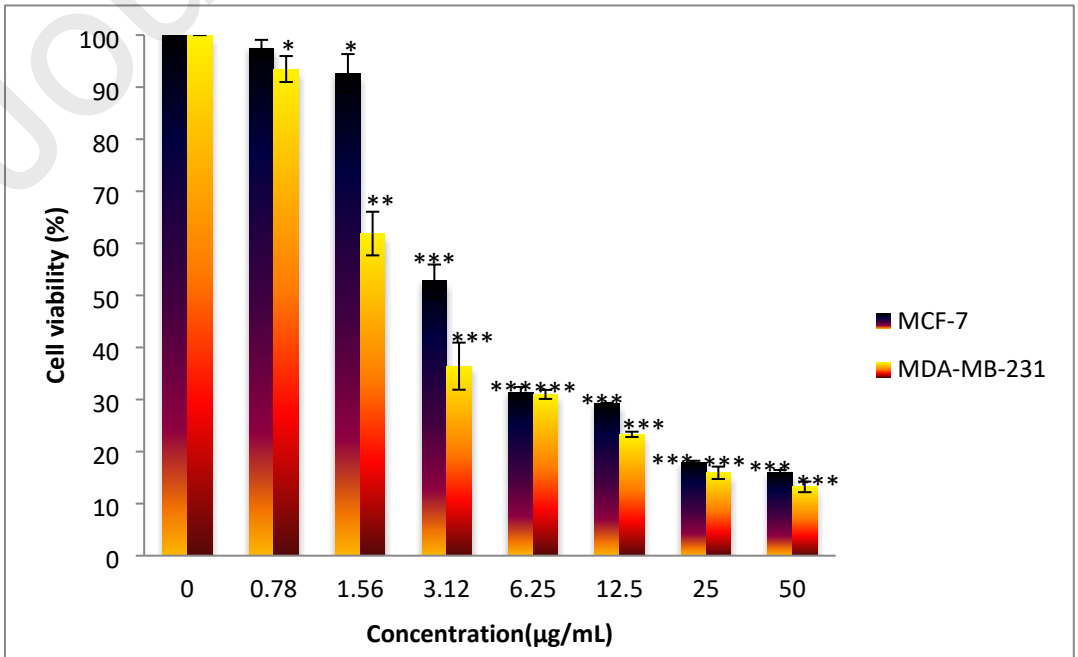

Figure 1. Colorimetric MTT cell viability assay. Human breast MCF-7 and MDA-MB-231 cancer cells were treated with different concentrations of β-diiminato compound including 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 µg/mL for 24h. The result showed a dose-response reduction in cell viability in all cancer cell lines as compared to the control group (0 µg/mL). Here, the viability of untreated cells (control) was considered 100% and the data were presented as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001 were considered statistically significant compared with the control group.

3.2 Trypan blue cytotoxicity assay

In order to determine the cytotoxic effect of the β-diiminato compound, trypan blue staining assay was performed. This assay is based on the principle that the live cells with the intact cell membrane exclude the dye and remain unstained, while the dead are stained with the dye. The number of dead and live cells was determined after 24h exposure to different concentrations of this compound. As shown in Figure 2, the β-diiminato compound inhibited the growth and induced the death in MDA-MB-231 breast cancer cells in a dose-dependent manner.

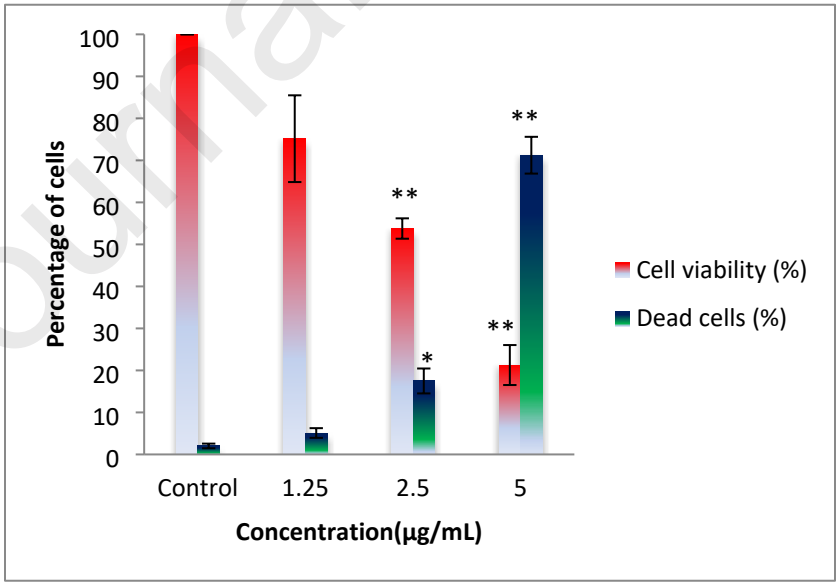

Figure 2. Trypan blue cytotoxicity assay. MDA-MB-231 breast cancer cells were treated with and without the indicated concentrations of the β-diiminato compound for 24h. The results showed decrease in cell viability and an increase in dead cells percentage as compared to the control group (0 µg/mL). Here, the viability of untreated cells was considered 100% and the data were expressed as the mean ± SD (n=3). *P < 0.05 and **P < 0.01 were considered statistically significant compared with the control group.

3.3 LDH-release cytotoxicity assay

Measuring leakage of components such as lactate dehydrogenase enzyme from the cytoplasm into the surrounding culture medium is a biomarker for cell death [30]. Therefore, the cytotoxic effect of the β-diiminato compound was further confirmed through LDH release after 18h treatment with a different concentration on MDA-MB-231 cells. As shown in Figure 3, this Schiff-based compound caused an increase in the LDH release level in the culture medium compared to control in a dose-dependent manner representing loss of membrane integrity either due to apoptosis or necrosis.

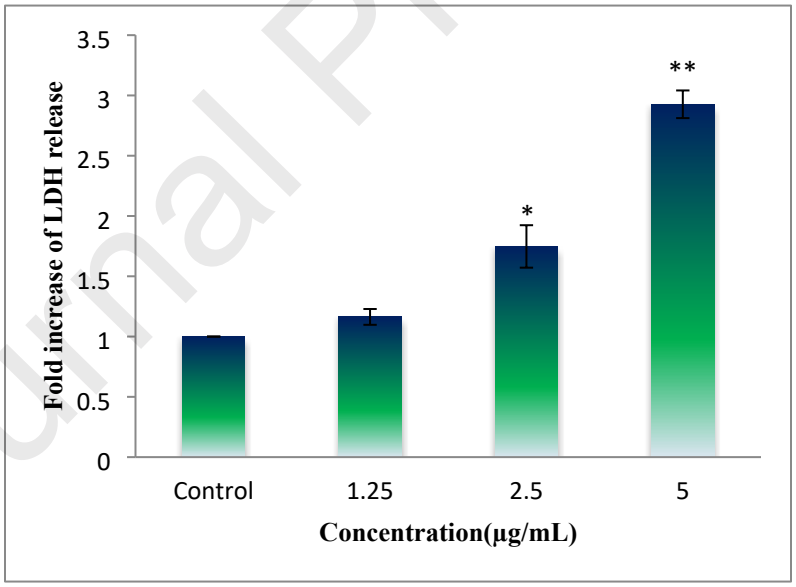

Figure 3. Lactate dehydrogenase (LDH) release assay. MDA-MB-231 breast cancer cells were treated with and without the indicated concentration of the β-diiminato compound for 24h. Increased LDH release in culture medium in treated cells revealed significant cytotoxicity of the compound on breast cancer cells at 2.5 and 5 µg/mL concentrations. Here, the control (untreated) group is referred as 1. The data were expressed as the mean ± SD (n=3). *P < 0.05 and **P < 0.01 were considered statistically significant compared with the control group.

3.4 β-diiminato compound induces G2/M phase arrest in MDA-MB-231 breast cancer cells

The effect of the β-diiminato compound on cell cycle distribution was investigated to obtain insights into the mechanism of its antiproliferative activity by flow cytometry analysis. The results showed that 24h treatment with this compound led to a significant accumulation of cells in G2/M phase that was accompanied by a decrease in the percentage of cells in G0/G1 and S phases in a dose-dependent manner. As can be seen in Figure 4, compared to the control (11.97%), the percentage of cells in the G2/M phase increased to 17.30% and 20.14% at 2.5 and 5 µg/mL, respectively. In addition, the β-diiminato compound induced a significant time-dependent increase in the sub-G1 phase containing only fractional DNA content representing apoptotic cell death. Therefore, the results indicated that the β-diiminato compound inhibited cell proliferation through cell cycle arrest at the G2/M phase and might induce a cytotoxic effect through apoptotic cell death.

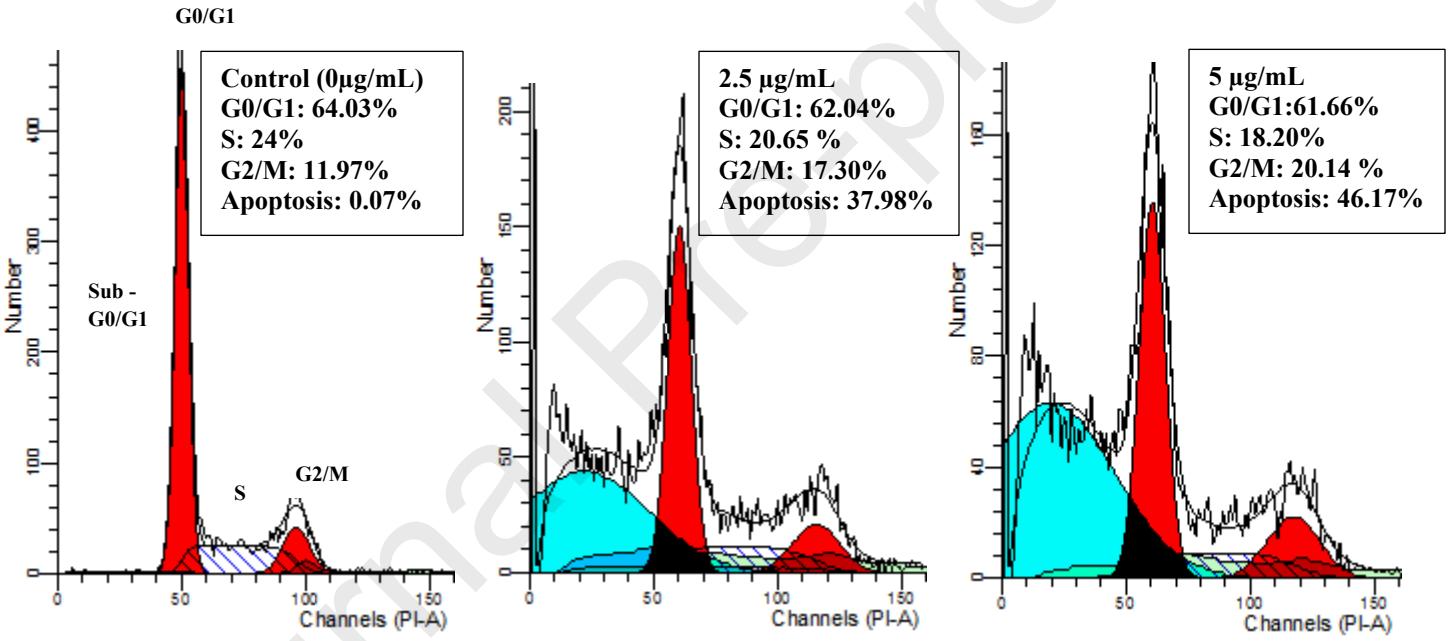

Figure 5. **Flowcytometry analysis of β-diiminato compound-induced cell cycle arrest.** MDA-MB-231 cells were treated with various concentrations of the β-diiminato compound for 24h and the cell cycle distribution was analysis using PI staining and flow cytometry. This compound increased the cell population in G2/M phase and decrease in other phases in dose-dependent manner representing G2/M cell growth arrest. Enhanced sub-G1 accumulation also represented the apoptotic cells containing only fractional DNA content.

3.5 Apoptotic Morphological changes induced by compound

3.5.1 Morphological analysis of cells by microscopy

The effect of the β-diiminato compound on the morphology of MDA-MB-231 cells was determined after 24h treatment by phase contrast microscopy. As shown in Figure 6, the treated cells displayed morphological changes including cell shrinkage, rounding up, and detachment from substratum and membrane blebbing, which are associated with cell death through apoptosis in a dose-dependent manner. However, untreated cells were evenly distributed on the substratum showing a normal adherent state.

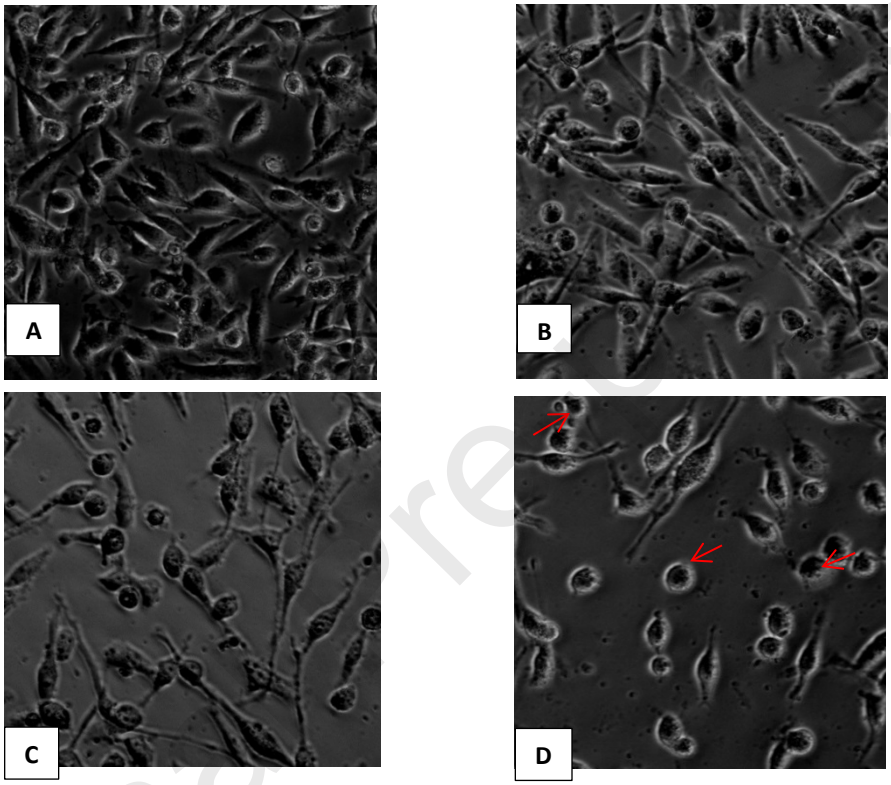

Figure 5. Growth inhibitory effect and morphological changes in β-diiminato compound-treated MDA-MB 231 cells. MDA-MB-231 breast cancer cells were treated with different concentrations of the compound for 24h and the cells were visualized under phase-contrast microscopy (×100) to investigate the morphological changes. Reduction in cell population and induction of apoptotic morphological changes such as shrinkage, rounding of the cell shape, and membrane blebbing (pointed with arrows) were observed in treated cells in a dose-dependent manner as compared to the control. Here, A represents the control (untreated) group. B, C, and D stand for the 1.25, 2.5 and 5 µg/mL concentration of the compound, respectively.

3.5.2 Morphological analysis of nucleus by HO33342/PI staining

Since during apoptosis, the typical alteration in nuclear morphology may happen, morphological features can be considered as indicators of programmed cell death activation [37]. Apoptotic nuclear morphological changes can be investigated using fluorescent dyes. HO33342, a blue-fluorescence dye, is a membrane-permeable dye, which stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells. However, PI, a red-fluorescence dye is only permeable to dead cells, which can only stain the cells in situations where there is a loss of plasma membrane integrity. Consequently, HO33342/PI double staining makes it possible to distinguish normal, early, and late apoptosis using fluorescence microscopy. The morphological observation in the nuclei of MDA-MB-231 cells after 24h treatment with the β-diiminato compound displayed significant morphological alterations compared to untreated control in a dose-dependent manner. As shown in Figure 6, the MDA-MB-231 treated cells showed morphological characteristics of apoptosis including nuclear shrinking, chromatin condensation, and fragmentation. Although untreated cells showed blue healthy intact nuclei, the treated cells displayed bright blue and red/pink fluorescent representing the early and late apoptosis events, respectively. Thereby, the results indicated that the β-diiminato compound might trigger apoptosis morphological changes in human MDA-MB-231 cells.

Merged (HO33342 and PI) PI

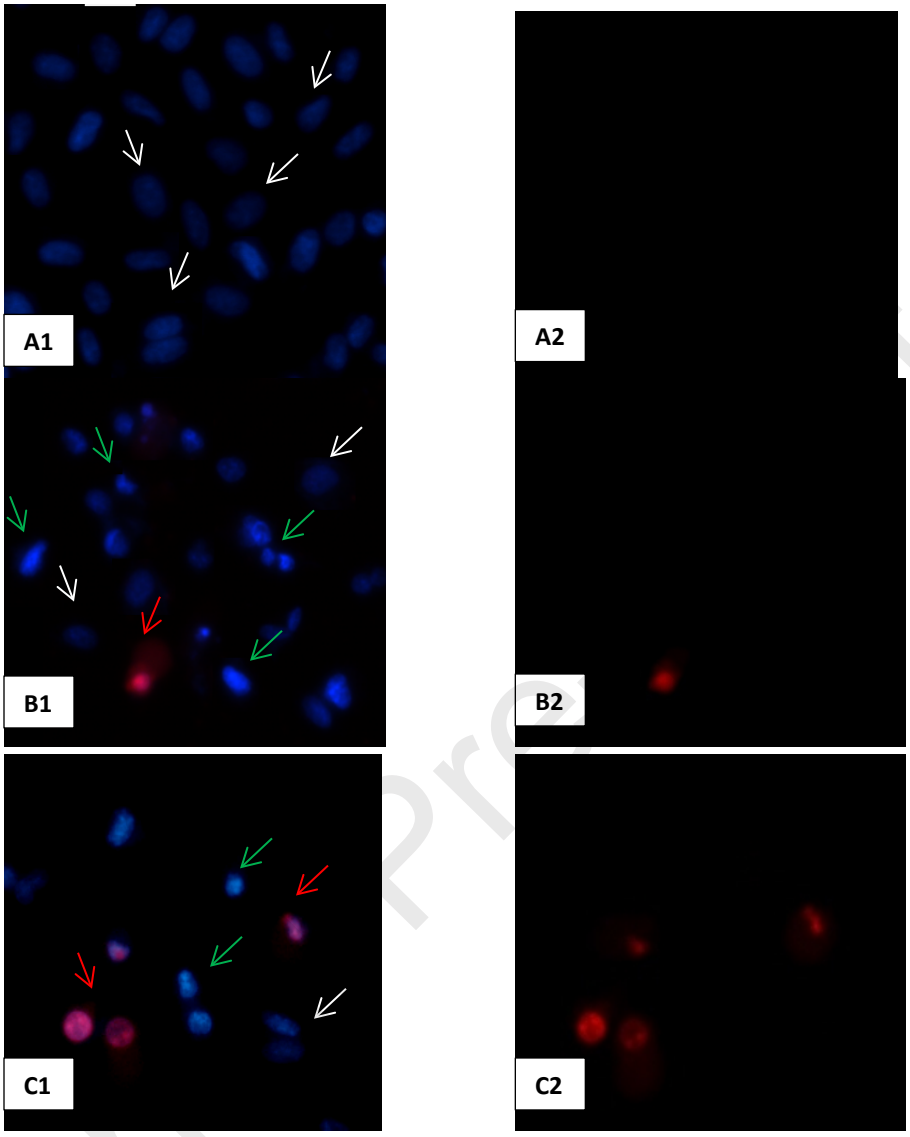

Figure 6. Nuclear morphological changes in compound-treated MDA-MB 231 cells. MDA-MB-231 breast cancer cells were treated with different concentrations of the compound for 24h, stained with HO33342/PI and visualized under a fluorescent microscope (×200). Untreated (control) cells exhibit healthy blue nuclei while the treated cells showed some apoptotic features including nuclear shrinkage, chromatin condensation, and fragmentation. Early and late apoptotic events were identified through bright blue and red/pink fluorescence of condensed chromatin. Here, A, B, and C refer to the untreated (0 µg/mL), 2.5 and 5 µg/mL concentration of the compound, respectively. White, green, and red arrows show normal, early, and late apoptotic nuclei, respectively.

3.6 The β-diiminato compound induced early and late Apoptosis in MDA-MB-231 Cells

Since both cytotoxicity and cell morphological changes showed that the compound may induce cell death in MDA-MB-231 cells, quantification and further confirmation of apoptotic cell death were determined by Annexin V/PI staining after 24h treatment with the β-diiminato compound. As it is shown in Figure 7, early apoptotic events (Annexin-V-FITC+, PI-) of the cells increased from 0.7% in untreated cells to 25% and 47.2%, at higher concentrations including 2.5 µg/mL and 5 µg/mL, respectively. Furthermore, late apoptotic events (Annexin-V-FITC+, PI+) of the cells also enhanced from 0.5% in control cells to 13.3%, 37.4%, treated cells at 2.5 µg/mL and 5 µg/mL respectively. Therefore, the flow cytometry analysis confirmed the potential of the β-diiminato compound to induce apoptotic cell death in a dose-dependent manner in MDA-MB-231 breast cancer cells.

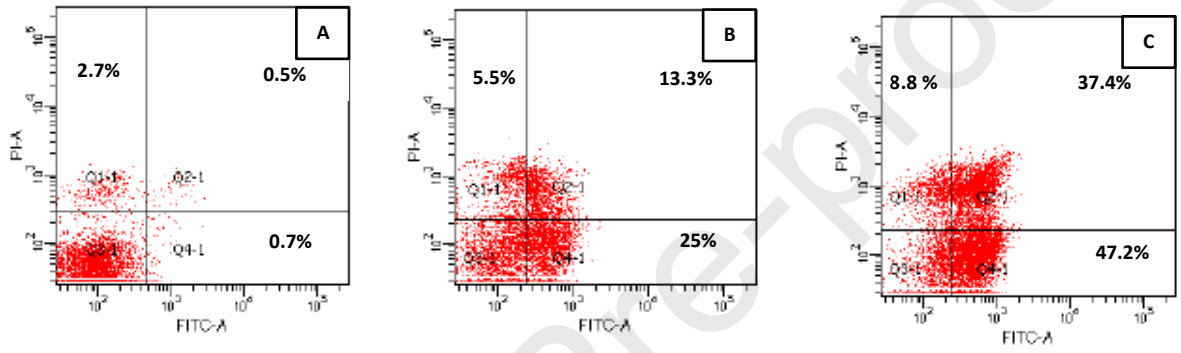

Figure7. Flowcytometry analysis of compound-induced apoptosis. MDA-MB-231 cells were treated with different concentrations of the compound for 24h and the apoptosis was analyzed using Annexin V-FITC/PI staining. Untreated cells were used as the control. Here, Q3-1, Q4-1, Q2-1, and Q1-1 refer to live cells, early apoptosis, late apoptosis, and necrosis, respectively. Flow cytometry analysis revealed the potential of this compound to induce early and late apoptotic events in a dose-dependent manner compared to control. Here, A, B, and C stand for the untreated (0 µg/mL), 2.5 and 5 µg/mL concentration of the compound, respectively.

3.7 The β-diiminato compound disrupted MMP in treated MDA-MB-231 cells

Mitochondria play a significant role in apoptosis regulation and mitochondrial membrane potential, as an important parameter of mitochondrial function is applied as an indicator

of cell health. Therefore, the membrane-permeable JC-1 dye is used for mitochondrial compartment monitoring in apoptosis studies. JC-1 presents potential-dependent accumulation in mitochondria and forms J-aggregates showing a red fluorescent in the mitochondrial matrix of healthy cells. However, in apoptotic cells, JC-1 remains in monomeric form displaying green fluorescent [38]. As shown in Figure 8, compared with the control group, the 24h treatment with the β-diiminato compound caused mitochondrial disruption and dose-dependent reduction of MMP in MDA-MB-231 cells. Consequently, this compound induced mitochondria-mediated apoptosis.

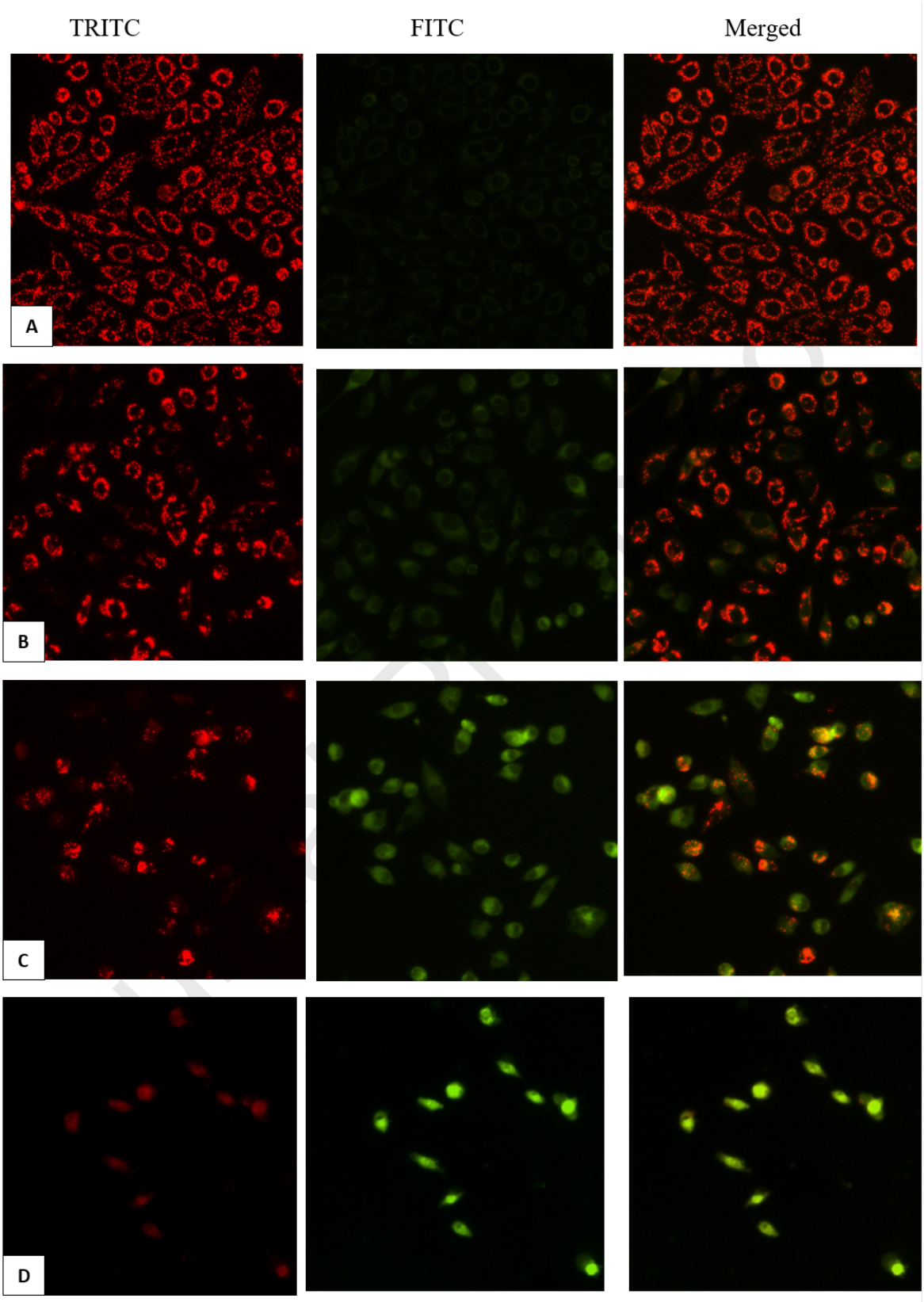
TRITC
FITC
Merged
A
B
C
D

Figure 8. Disruptive effect of the β-diiminato compound on mitochondrial membrane potential (MMP). MDA-MB-231 cells were treated with different concentrations of the β-diiminato compound for 24h. MMP alteration was determined using JC-1 indicator displaying concentration-dependent accumulation in mitochondria under a fluorescent microscope (100×). Since the mitochondrial depolarization is identified by a decrease in the red/green fluorescence of JC-1 dye, the results revealed the potential of this compound to disrupt MMP in dose-dependent manner compared to the control. Here, A represents the control (untreated) group. B, C, and D represent the 1.25, 2.5, and 5 µg/mL concentrations of the compounds, respectively.

3.8 The β-diiminato compound induced oxidative stress generation in treated MDA-MB-231 cells

Since the overproduction of reactive oxygen species can lead to mitochondrial dysfunction and cell death [39, 40], the effect of the β-diiminato compound on the ROS generation was measured using non-fluorescent DCFDA which can be oxidized by intracellular ROS-producing fluorescent DCF. As shown in Figure 9, after 24h treatment with this compound, intracellular ROS levels increased significantly in treated cells in a dose-dependent manner compared to control cells. Therefore, enhanced ROS level is associated with collapse in mitochondrial membrane integrity leading to activation of apoptosis intrinsic pathway.

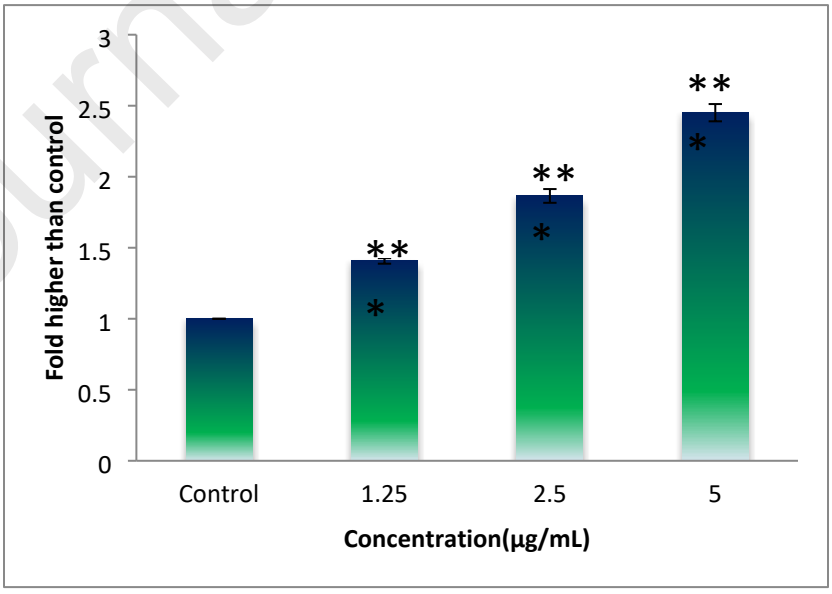

Figure 9. Increased reactive oxygen species (ROS) level induced by the β-diiminato compound. MDA-MB-231 cells were treated with different concentrations of this compound for 4h. The level of ROS was significantly increased in β-diiminato compound-treated cells compared to the untreated (control) cells. Here, the control group is referred as 1. The data were expressed as

the mean ± SD (n=3). ***P < 0.001 was considered statistically significant compared to the control group.

3.9 The β-diiminato compound induced no significant toxicity in *in-vivo* experiment

Acute toxicity is the initial step for the safety evaluation of the compound. Sprague-Dawley rats were treated once either with 10% Tween 20 or β-diiminato compound at dosages of 150 mg/kg and 300 mg/kg. After 14 days of the experiment, no mortality was observed and all animals were alive during the treatment period. In addition, neither physical abnormalities nor behavior changes were observed. Furthermore, biochemical and histopathological analysis of the kidney and liver shown in (Tables 1 and 2) and (Figure 10) indicated that there were no hepatotoxic or nephrotoxic effects in the treated rats as compared to the control group and revealed the safety usage of this compound in the *in-vivo* experiments.

Groups	**Sodium (mmo/L)**	**Potassium (mmo/L)**	**Chloride (mmo/L)**	**CO_2 (mmo/L)**	**Anion gap (mmo/L)**	**Urea (mmo/L)**	**Creatinine (mmo/L)**
Vehicle control	141.3±1.52	4.73±0.20	100.6±1.52	26.3±0.57	18.6±1.52	5.46±0.51	30.3±0.57
150mg/kg	140.3±1.52	4.66±0.20	99.6±1.15	28±1.73	17±2	6.06±0.30	29.3±2.51
300mg/kg	141±1	4.7±0.14	102±1.41	26.5±2.12	20.5±2.12	6.23±0.55	30.66±1.52

Table 1. Effect of the β-diiminato compound on kidney biochemical parameters in rats. The results were expressed as the (mean±SD). There were no statistically significant differences between treated groups compared to control group. Values were considered significant at P < 0.05.

Groups	**Albumin (g/L)**	**Total bilirubin (µmol/L)**	**Alkaline phosphate (IU/L)**	**Alanine Aminotransferase (IU/L)**	**Glutamyl transferase (IU/L)**
Vehicle control	37.33±1.15	2.00±0.00	155.66±40.46	41.00±1.41	6.00±0.00
150 mg/kg	35.66±1.52	2.00±0.00	156±27.22	37±1.52	6.00±0.00

300 mg/kg	36.33±10.57	2.00±0.00	145±18.90	534.58	6.00±0.00

Table 2. Effects of the β-diiminato compound on the liver biochemical parameters in rats. The results were expressed as the mean±SD. There were no statistically significant differences between treated groups compared to control group. Values were considered significant at $P < 0.05$.

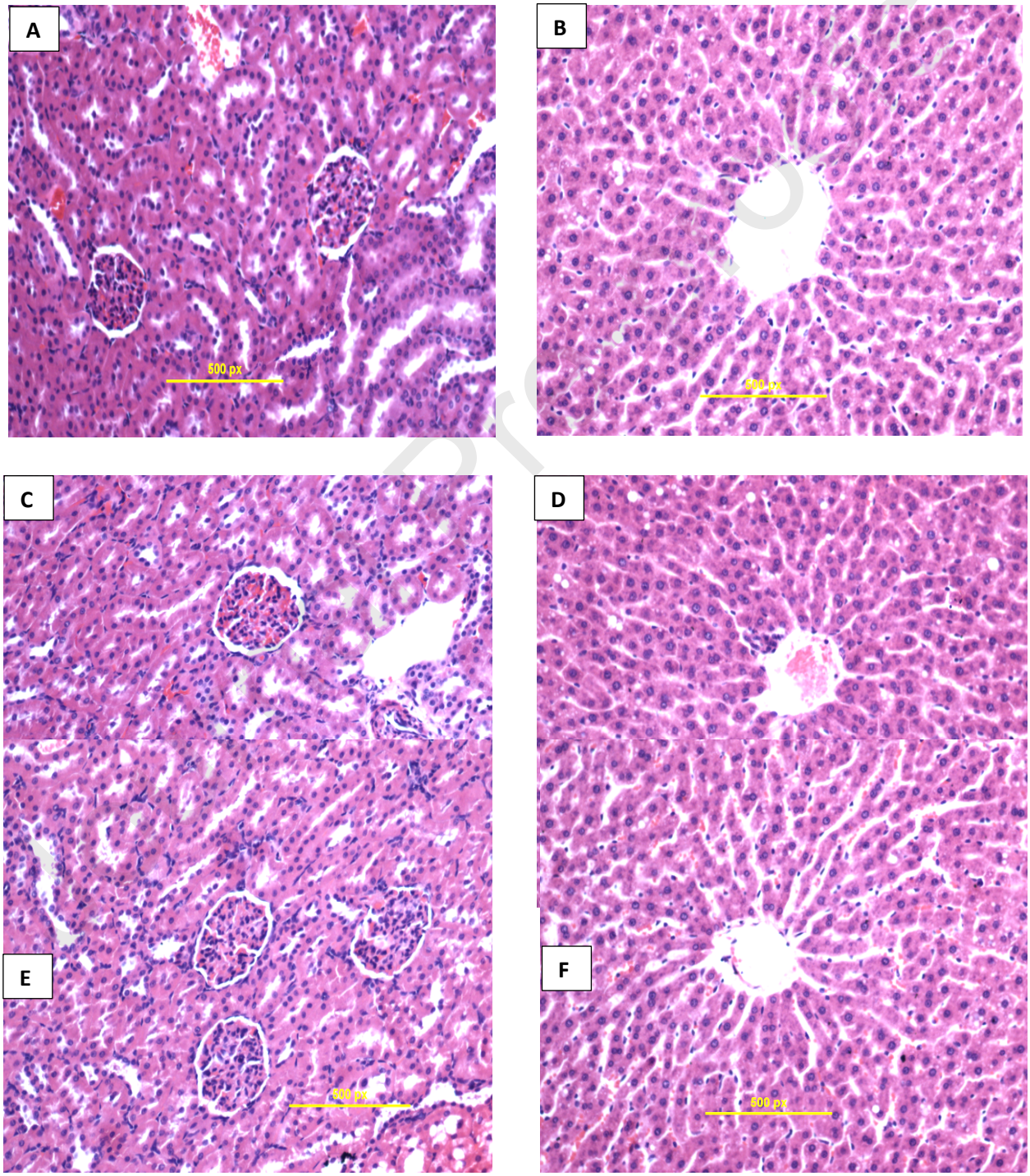

Figure 10. kidney (left) and liver (right) histological investigation of the acute toxicity experiment. Untreated rats as a control group (A and B) received 5 mL/kg of the vehicle (10% Tween20). The rates were treated with 150 mg/kg (C and D) and 300 mg/kg (E and F) of the β-diiminato compound. No significant differences were observed between the treated and vehicle control group (Hematoxylin and Eosin staining, 20X).

4. Discussion

Despite the availability of chemotherapeutic regimens, patients with breast cancer undergoing chemotherapy continue to face significant challenges such as drug resistance, adverse side effects, and other pharmacological limitations [41]. Triple-negative breast cancer (TNBC) relapses more frequently and aggressively than hormone receptor-positive subtypes of breast cancer. TNBC patients have distant metastases happening mostly in the brain, lung, bone, and liver and are associated with poor prognosis, as defined by low five-year survival [5, 6, 42]. Therefore, there is still a crucial need to develop novel and more effective anti-cancer agents, especially for TNBC treatment. Biologically active molecules, Schiff bases, are known to show a variety of pharmacological activities due to characteristic C=N functionality and have been extensively focused on their enhanced activities compared to non-Schiff bases in drug discovery studies. Therefore, the development of a new chemotherapeutic Schiff bases has obtained widespread attention in cancer treatment [43-48].

In the present study, a novel indole Schiff-based compound, β-diiminato ligand, was assessed for its inhibitory and cytotoxic effect on breast cancer cells with a particular focus on TNBC. In addition, its mechanism of cytotoxic action was also investigated. Our study demonstrates that β-diiminato compound exhibits an IC_{50} value of 2.5 µg/mL (6 µM) after 24h of treatment, indicating more potent anticancer activity against TNBC compared to the chemotherapeutic drug cisplatin in inhibiting MDA-MB-231 cell growth. This value is considerably lower compared to other Schiff base derivatives, highlighting its potent anticancer activity. For instance, platinum (II) complexes derived from acylpyrazolone-based Schiff base ligands have been reported with IC_{50} values ranging from 24 to 65 µM after 48 hours against TNBC cells [49]. Similarly, the H2L Schiff base ligand and its Ni(II) complex have shown IC_{50} values of 77.5 µM and 15.87 µM, respectively, in MDA-MB-231 cells after 24h [50]. Additionally, another study investigated chiral Schiff base ligands and their metal complexes, including Pd(II), Fe(II), Ni(II), and Cu(II), finding that after 48h, the ligands themselves had IC_{50} values higher than 200 µM, indicating no significant cytotoxicity. However, some metal complexes exhibited increased antiproliferative effects with IC_{50} values ranging from 9.9 to 99.8 µM, demonstrating their enhanced potency compared to the free ligands in TNBC [51]. These studies underscore the potency of β-diiminato ligand in comparison to the existing Schiff base derivatives and highlight its potential application in cancer treatment.

Additionally, the non-tumorigenic 184B5 breast cells and WRL-68 hepatic cells have been included to evaluate the compound's safety profile. The 184B5 cells allow us to assess the compound's effect on normal breast tissue, while WRL-68 helps evaluate potential liver toxicity,

given the liver's role in drug metabolism. Notably, the β-diiminato compound did not exhibit significant inhibitory effects on either the 184B5 breast cells or WRL-68 hepatic cells compared to its IC_{50} values observed in breast cancer cells. This suggests that the compound may have a selective anticancer activity, with minimal impact on non-cancerous cells, which is an important aspect of its potential safety profile. Furthermore, the single-dose acute oral toxicity test revealed no signs of toxicity or mortality. Both biochemical and histopathological analyses showed no evidence of hepatotoxicity or nephrotoxicity, confirming the safety profile of the compound. These findings are consistent with other studies demonstrating the safety of Schiff base compounds in preclinical models [52-54]. -In addition, loss of membrane integrity in trypan blue positive cells and enhanced LDH level in culture medium confirmed the cytotoxic effect of β-diiminato compound in treated MDA-MB-231 cells, possibly mediated via activation of an apoptosis or necrosis pathway, prompting further investigation.

A balance between cell cycle progression and apoptosis regulates normal cell growth. Therefore, disruption of this balance due to uncontrolled cell cycle arrest or resistance to apoptosis leading to cancer incidence is the major focus of drug discovery in cancer therapy [11, 14]. Flow cytometry analysis of the DNA content of treated cells revealed that β-diiminato compound arrested cell cycle progression in MDA-MB-231 cells by increasing the percentage of cells in the G_2/M phase in a dose-dependent manner compared to the control group. The observed G2/M phase arrest can lead to apoptosis by disrupting cell cycle regulation and activating apoptotic pathways through multiple mechanisms, as supported by studies [55-57]. Prolonged cell cycle arrest at the G2/M checkpoint and subsequent DNA damage can trigger the modification of p53 and other pro-apoptotic factors. This activation leads to the engagement of caspases, particularly caspase-3 and caspase-7, which are crucial in the execution phase of apoptosis. This cascade ensures that cells with irreparable DNA damage do not proceed through mitosis, thereby preventing the propagation of damaged DNA and promoting cell death through apoptosis [55-57]. Furthermore, treated cells also exhibited significant sub-G1 peaks, which are indicative of apoptosis. During apoptosis, endonucleolytic cleavage of genomic DNA results in the formation of small DNA fragments, ~180 bp, which correspond to the sub-G1 phase on flow cytometry histograms [58-60].

The deregulation of apoptotic signaling pathways is central to almost all cancer types. Therefore, apoptosis induction has been recognized as a novel strategy for the identification of anticancer drug. Further experiments were performed to determine the mode of cell death induced by the β-diiminato compound. Microscopic analysis and HO33342/PI staining showed distinctive morphological changes associated with typical apoptosis features including cell shrinkage, detachment, membrane blebbing, and chromatin condensation and fragmentation. Externalization of phosphatidylserine (PS) as the biochemical characteristic of apoptosis [61, 62] was also detected using Annexin V, recombinant PS-binding protein, and PI as a marker for early and late apoptosis, respectively. FACS analysis demonstrated an increase in the early and late apoptotic events in treated MDA-MB-231 cells in a dose-dependent manner compared to control cells confirming the cell death induction through apoptosis [63, 64].

Mitochondria as the intracellular organelles, which are involved in many processes essential for cell survival, play a significant role in apoptosis regulation [65, 66]. Mitochondrial membrane potential is an important parameter of mitochondrial function and apoptosis mediated by the mitochondrial pathway is often associated with the loss of MMP [32]. JC-1 staining revealed that the β-diiminato compound caused the dose-dependent decrease of MMP in treated cells and

therefore the observed apoptosis is induced via the intrinsic mitochondrial pathway. In addition, ROS have the potential to induce the collapse of the MMP, and consequently trigger the series of events leading to the mitochondria-associated apoptotic pathway [40, 67]. Consequently, the results confirmed the involvement of ROS and mitochondrial dysfunction in the induction of apoptosis in β-diiminato compound-treated MDA-MB-231 cells. Our results align with previous studies that have demonstrated the potential of Schiff base compounds in inducing apoptosis in cancer cells mediated by ROS generation, disrupting mitochondrial membrane potential, and activating caspases [68-70].

5. Conclusion and future scope of the research

In this study, the novel Schiff base β-diiminato compound demonstrated significant inhibition of cell growth and cytotoxic effects on invasive TNBC cells, specifically MDA-MB-231. The compound exhibited dose-dependent cytotoxicity and induced apoptosis through mechanisms such as G2/M phase cell cycle arrest, phosphatidylserine externalization, mitochondrial membrane depolarization, and increased ROS levels. Its favorable safety profile, combined with its potent anticancer activity, underscores its potential as a promising candidate for further studies in cancer therapeutics.

Given the promising results of the novel β-diiminato compound against TNBC, several avenues for future research are highly encouraging. Further elucidation of specific targets and the molecular pathways involved in the anticancer activity of the β-diiminato compound is warranted. In addition, future studies can investigate the potential synergistic effects of the β-diiminato compound in combination with existing chemotherapeutic agents, which may enhance therapeutic efficacy and reduce drug resistance in TNBC treatment. Further research is also needed to fully assess the long-term safety and optimize the dosage of the β-diiminato compound through chronic toxicity and pharmacokinetic studies. Additionally, its anticancer potential can be evaluated across various cancers, and nanoparticle-based or other targeted delivery systems could also enhance its effectiveness.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Chatgpt to improve the language of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Abbreviation List

ATCC: American Type Culture Collection, USA

Bax: Apoptosis regulator

Bcl-2: Protein encoded in humans by the BCL2 gene

BSA: Bovine Serum Albumin

CDK: Cyclin Dependent Kinase

CO_2: Carbon Dioxide

DAPI: 4',6-Diamidino-2-Phenylindole

DCF: Dichlorofluorescein

DCFDA: 2'-7'-Dichlorodihydrofluorescein Diacetate

DMEM: Dulbecco's Modified Eagle Medium

DMF: Dimethyl Formamide

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FITC: Fluorescein Isothiocyanate

h: hour(s)

HO33342: Hoechst 33342, a fluorescent stain

H&E: Hematoxylin and Eosin

IC_{50}: Half-maximal inhibitory concentration

JC-1: 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

LDH: Lactate Dehydrogenase

MEBM: Mammary Epithelial Basal Medium

MEGM: Mammary Epithelial Cell Growth Medium

min: minute(s)

MMP: Mitochondrial Membrane Potential

mRNA: Messenger Ribonucleic Acid

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

n: Number

P: Probability value

PBS: Phosphate-Buffered Saline

PI: Propidium Iodide

p53: Tumor protein p53

ROS: Reactive Oxygen Species

SD: Standard Deviation

TNBC: Triple-Negative Breast Cancer

TRITC: Tetramethylrhodamine isothiocyanate

VEGFR-2: Vascular Endothelial Growth Factor Receptor 2

Highlights

- LH_3 compound significantly suppresses the viability of MDA-MB-231 cells in a dose-dependent manner.
- LH_3 induces anti-proliferative effects in TNBC cells by causing G2/M phase cell cycle arrest in MDA-MB-231 breast cancer cells.
- LH_3 triggers apoptosis through reactive oxygen mediated mitochondrial dysfunction pathway.
- [illegible] toxicity or nephrotoxicity detected in [illegible] the compound's safety.