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Research Article

ANTI-PROLIFERATIVE EFFECTS OF CU(PHEN)(C-DIMETHYLGLYCINE)NO_3 ON HT-29 AND A2780 CANCER CELL LINES: A POTENTIAL CHEMOTHERAPEUTIC APPROACH

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ABSTRACT

Objectives: This study aimed to evaluate the *in vitro* antiproliferative properties of Cu(Phen)(C-dimethylglycine)NO_3 on human cancer cell lines. Specifically, the study investigated its effects on the proliferation of colorectal carcinoma (HT-29) and ovarian carcinoma (A2780) cells, determined the IC_{50} values, measured caspase-9 activity, and assessed the degree of DNA fragmentation.

Methods: The antiproliferative and apoptotic effects of standardized Cu(phen)(C-dimethylglycine)NO_3 were evaluated at varying concentrations (1, 2, 5, 10, 15, and 20 µM) over 24, 48, and 72 h. Cell viability was assessed using the MTT assay, whereas caspase-9 activity was measured using fluorometric assay kits and a fluorophotometer. DNA fragmentation was analyzed using the Cell Death Detection ELISA Plus kit.

Results: The results demonstrated a time- and concentration-dependent reduction in cell viability for both cell lines. Notably, A2780 cells exhibited a lower IC_{50} (1.76 ± 0.406 µM at 72 h) compared to HT-29 cells (7.03 ± 0.635 µM), indicating greater sensitivity. However, the compound did not significantly alter caspase-9 expression nor induce DNA fragmentation when compared to the control.

Conclusion: Cu(phen)(C-dimethylglycine)NO_3 exerts a significant anti-proliferative effect without triggering apoptosis, suggesting a non-apoptotic mechanism of cytotoxicity that warrants further investigation.

Keywords: Copper(II) complex, Antiproliferative activity, Cancer cell lines, IC_{50}, Apoptosis assay.

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INTRODUCTION

Copper (Cu) plays a critical role in various biological processes, functioning both as an essential trace element and as a component of exogenously administered compounds. As a trace metal, Cu is primarily bound to ceruloplasmin, albumin, and other proteins, regulating its transport and bioavailability in the human body. In exogenous applications, Cu forms coordination complexes with diverse ligands, enabling interactions with biomolecules, particularly proteins and nucleic acids [1,2]. The dual nature of Cu, serving both physiological and pharmacological functions, has attracted significant research interest due to its diverse biochemical actions in human health and disease [3].

Recent studies emphasize the therapeutic potential of Cu complexes in antimicrobial, antiviral, anti-inflammatory, and anticancer therapies [4,5]. Notably, Cu(II) complexes have demonstrated potent anticancer properties by targeting redox homeostasis, DNA damage pathways, and apoptotic mechanisms [5]. The development of Cu-based anticancer agents has been driven by their ability to generate reactive oxygen species (ROS), disrupt mitochondrial function, and interfere with cellular signaling pathways critical for cancer cell survival [3].

A prominent area of research involves Cu(II) complexes with non-steroidal anti-inflammatory drugs (NSAIDs), which have been investigated for their enhanced anti-inflammatory and antiulcerogenic activity compared to uncomplexed NSAIDs [6,7]. These Cu-NSAID complexes exhibit reduced gastrointestinal toxicity and are believed to exert their pharmacological effects through superoxide dismutase-mimetic activity, mitigating oxidative stress-induced damage [4].

Given the increasing interest in Cu-based anticancer agents, this study evaluates the antiproliferative potential of Cu(phen)(C-dimethylglycine)NO_3 against colorectal carcinoma (HT-29) and ovarian carcinoma (A2780) cell lines. The research aims to determine the IC_{50} values, assess caspase-9 activity, and analyze DNA fragmentation to elucidate the cytotoxic mechanisms underlying Cu-based therapy. The findings could provide insights into alternative non-platinum-based chemotherapeutic strategies that may overcome drug resistance and minimize toxicity in cancer treatment.

Furthermore, many have highlighted Cu(II) complexes' antiviral and antibacterial properties. It has been demonstrated that contact with Cu surfaces decreases the influenza A virus's infectivity [8]. Although the exact mechanism is unknown, it has been hypothesized that Cu ions' involvement causes the viral nucleic acid to degrade. The design and manufacture of antiviral and antibacterial materials that may deactivate HIV or H1N1 viruses [9] or antibiotic-resistant bacteria may also benefit from the research and development of Cu complexes. A process for creating Cu-impregnated materials with broad-spectrum antibacterial qualities has been documented as a step in this direction [10].

It has been demonstrated that Cu complexes have been known to cause DNA and chromosome damage [11]. However, Cu complexes with amino acid and amino acid derivatives act as anti-inflammatory, antiulcer, anticonvulsant, anticancer, and/or radiation protection agents [12]. Therefore, this study aimed to evaluate *in vitro* anticancer properties of Cu(phen)(C-dimethylglycine)NO_3 on human cancer cell lines. Study the effect of Cu(phen)(C-dimethylglycine)NO_3 on the proliferation of

human cancer cell lines to study the IC_{50} value, measure the activities of caspase-9, and determine the degree of DNA fragmentation.

METHODS

Cu(phen)(C-dimethylglycine)NO_3 was a kind gift from the Department of Chemistry, Universiti Malaya. The human colon carcinoma cells (HT-29) and ovarian carcinoma cells (A2780) were purchased from the American Type Culture Collection (ATCC), USA. The tissue culture medium (Dulbecco's Modified Eagle Medium [DMEM]) was purchased from Gibco, Life Technology (UK). MTT powder was purchased from CALBIOCHEM® (Germany). Trypsin was purchased from Sciencell™ Research Laboratories. Agarose powder was purchased from Sisco Research Laboratories. Ethidium Bromide and trypan blue solution were purchased from Sigma Aldrich (USA). DNA fragmentation kit was purchased from iNtron Biotechnology INC. Caspase detection kit was purchased from CHEMICON® (USA).

Cell cultures and solutions

A2780 and HT-29 cell lines were cultured as per the manufacturer's instructions in the DMEM medium. Both were incubated in media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (100 IU/mL penicillin and 100 mg/mL streptomycin) in a humidified atmosphere containing 5% carbon dioxide (CO_2) at 37°C [13]. Cell counting was performed before the cell plating to obtain the appropriate number of seeding cells. The number of viable cells was counted under an inverted light microscope.

Cu(phen)(C-dmg)NO_3 was dissolved in purified water to the final concentration of 5 mM. The preparation was performed in laminar flow. This 5 mM stock solution was then diluted in DMEM culture medium (10% FBS and 1% antibiotic) to make 0.2 mM final concentration.

Determination of cytotoxicity

In the MTT assay, A2780 and HT-29 cells were plated at a density of 1 × 10^6 cells/well in 96-well plates. A2780 and HT-29 cells were treated with Cu(phen)(C-dmg)NO_3 ranging from 1, 2, 5, 10, 15, and 20 µM and incubated for 24, 48, and 72 h. Controls without Cu(phen)(C-dmg)NO_3 were also included.

MTT solution (5 mg/mL stock solution in phosphate-buffered saline [PBS]) was added after each incubation time. After 4 h incubation at 37°C, this solution was removed and the produced formazan was solubilized in 100 µL dimethyl sulfoxide in each aspirated well. The plate was gently agitated until the color reaction was uniform [14]. The amount of MTT formazan product was determined by measuring absorbance at 570 nm (630 nm as a reference) using a microplate reader (Infinite M200).

The percent viability of the treated cells was calculated using the formula (OD of treated cells/OD of control cells) × 100% (OD=A570–A630). The concentration that gave 50% inhibition of cell viability (IC_{50}) was determined from the dose- and time-response graph [15].

Expression of caspase-9

Caspase-9 activity was detected using fluorometric assay kits according to the manufacturer's instructions, and fluorescent activity was measured using a fluorophotometer. As specified in the kit, the FAM-VAD-FMK inhibitor was added directly to the cell suspension. The cells were gently mixed and incubated for 1 h at 37°C under 5% CO_2 with tubes protected from light. Cells were washed twice with a wash buffer and followed by centrifugation at 400 × g for 5 min at room temperature after each wash. The supernatant was removed. PBS was added to each tube, and 100 µL of each cell suspension was placed into a well of a black microtiter plate. Fluorescence was read in an Infinite 2000 fluorescence plate reader at 490 nm excitation and 520 nm emission wavelengths.

DNA fragmentation assay

i-genomic CTB DNA Extraction Mini Kit from Intron Biotechnology was used to quantify DNA fragmentation. After treatment, cells were lysed with lysis buffer, and DNA was extracted with ethanol method. The DNA was then electrophoretically separated on a 2.5% agarose gel, and the DNA ladder was visualized under ultraviolet trans-illumination. The duplicate independent experiment was carried out.

Statistical analysis

All the data were expressed in mean±standard error mean. The data were analyzed with one-way analysis variance and Duncan's multiple range test using Statistical Package for Social Science version 24.0. In all statistical analyses, a probability of $p<0.05$ was considered significant.

RESULTS

CytotoxicityofCu(phen)(C-dmg)NO_3

HT-29 and A2780 cells were exposed to different concentrations of Cu(phen)(C-dmg)NO_3. This assay indicated that the cytotoxicity of Cu(phen)(C-dmg)NO_3 was dose- and time-dependent. The IC_{50} values determined by the MTT assay are shown in Table 1.

In Fig. 1, Cu(phen)(C-dmg)NO_3 (over 5 µM/µL) had a slight growth-inhibiting effect on the HT-29 cells after 72 h incubation time. The result indicated that Cu(phen)(C-dmg)NO_3 had a significant growth effect on this cell line in a dose-dependent manner and time-dependent manner. Fig. 2 shows Cu(phen)(C-dmg)NO_3 (over 5 µM/µL) had a significant growth-inhibiting effect on A2780 cells after 48 h incubation time. The result indicated that Cu(phen)(C-dmg)NO_3 had a significant growth effect on this cell line in a dose-dependent manner and time-dependent

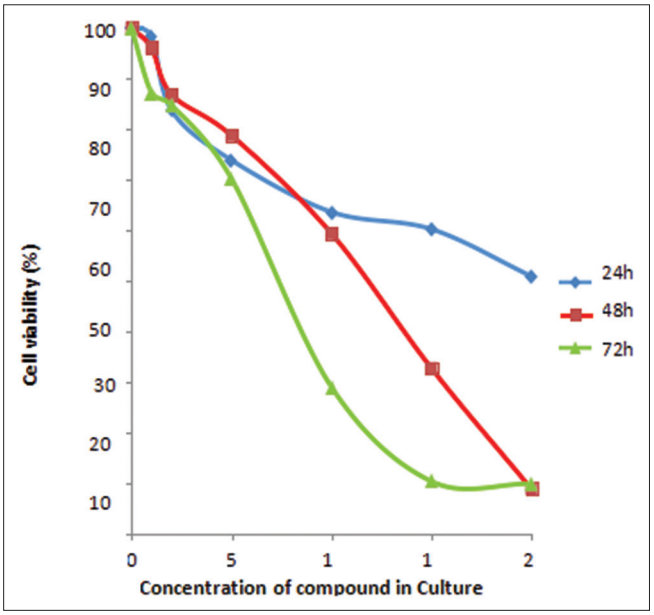

Fig. 1: Dose-dependent antiproliferation against HT-29 cells with Cu(phen)(C-dmg)NO_3 after 24, 48, and 72 h incubation as determined using the MTT assay. The data are presented as mean±standard error mean

Table 1: IC_{50} values of Cu (phen) (C-dmg) NO_3 toward HT-29 and A2780 cells at various incubation times as determined using MTT assay

Incubation time (h)	IC_{50} (µM/µL) HT-29	IC_{50} (µM/µL) A2780
24	18.77±2.14[a]	16.5±0.9[a]
48	10.07±2.05[b]	3.03±0.21[b]
72	7.03±0.64[b]	1.76±0.41[c]

Values were the means of three replicate samples (n=3), and the data were represented as mean±standard error mean. [a-c]Mean with different superscripts differ significantly ($p<0.05$)

manner. Between these cell lines, the A2780 cells were much more sensitive than HT-29 cells. A2780 cells were chosen for the subsequent experiments.

In the dose-response curves obtained from the MTT assay, a significant decrease in the percentage of cell viability was observed after the HT-29 and A2780 cells were treated with Cu(phen)(C-dmg)NO_3 at 1, 2, 5, 10, 15, and 20 µM/µL for 24, 48, and 72 h incubation time. The inhibitory action of the Cu(phen)(C-dmg)NO_3 was also time-dependent. A significant decrease in activity was seen when the compound exposure time was lengthened in the time-dependent antiproliferation assay against the HT-29 and A2780 cells (Fig. 3).

Expression of caspase-9

Cells were incubated for 72 h in the presence (IC_{50} value) or absence of Cu(phen)(C-dmg)NO_3. Caspase-9 activities were measured later using colorimetric assay kits. Values represent mean±standard deviation. As shown in Fig. 4, there is no elevation of expression of caspase-9 from treated cells. In comparison, control cells showed greater value than treated cells.

DNA fragmentation

DNA fragmentation is a characteristic of apoptosis. Therefore, Cu(phen)(C-dmg)NO_3-induced apoptosis was confirmed by the DNA fragmentation assay. However no apparent DNA fragmentation in A2780 cells after treatment with Cu(phen)(C-dmg)NO_3 for 72 h. A typical experimental result of agarose gel electrophoresis is shown in Fig. 5, where the effect of Cu(phen)(C-dmg)NO_3 for 72 h treatment does not produce any DNA fragment ladders.

DISCUSSION

The cytotoxicity of Cu(phen)(C-dmg)NO_3 was tested using an MTT assay. This assay indicated that A2780 cells were more sensitive toward Cu(phen)(C-dmg)NO_3 compared to HT-29 cells. This assay also found that Cu(phen)(C-dmg)NO_3 exhibits potent cytotoxicity against A2780 cells but partially inhibited the viability of HT-29 cells. Table 1 showed that the IC_{50} values determined by the MTT assay had decreased with the increasing incubation time. Figs. 1 and 2 showed the cell growth inhibition of A2780 and HT-29 cells increasing with the increasing concentration of Cu(phen)(C- dmg)NO_3 and incubation time. In short, the cytotoxicity of Cu(phen)(C-dmg)NO_3 toward both cell lines was dose- and time-dependent.

Apoptosis is a process that controls the number and quality of cells. Caspase cascade is a key pathway in the apoptotic signal transduction. Caspases involve two types of subfamilies: Upstream initiators such as caspase-8 and caspase-9, and downstream regulators such as caspase-3 and caspase-6 [16]. These caspases are involved in the regulatory event and lead to the direct response to the cell morphological changes and the cleavage of nuclear protein. However, this study did not find that the treatment from Cu(phen)(C-dmg)NO_3 toward A2780 cells elevated the activity of caspase-9.

Following the caspase activation, there is an increasing number of molecular apoptotic biomarkers, such as DNA fragmentation, which subsequently cause cell death. Cleavage of DNA at the internucleosomal linker sites yields DNA fragments in multiple fragments (180–200 bp), which this appearance results in a ladder formation in a typical experiment of agarose gel electrophoresis. In this study, the absence of apoptosis activity of the A2780 cells when treated with Cu(phen)(C-dmg)NO_3 with the absence of activation of caspase-9 is supported by the non-appearance of a DNA fragment ladder after 72 h incubation time, as shown in **Fig. 5.**

From the previous study, many low-molecular-weight copper complexes with amino acids and amino acid derivatives have been demonstrated

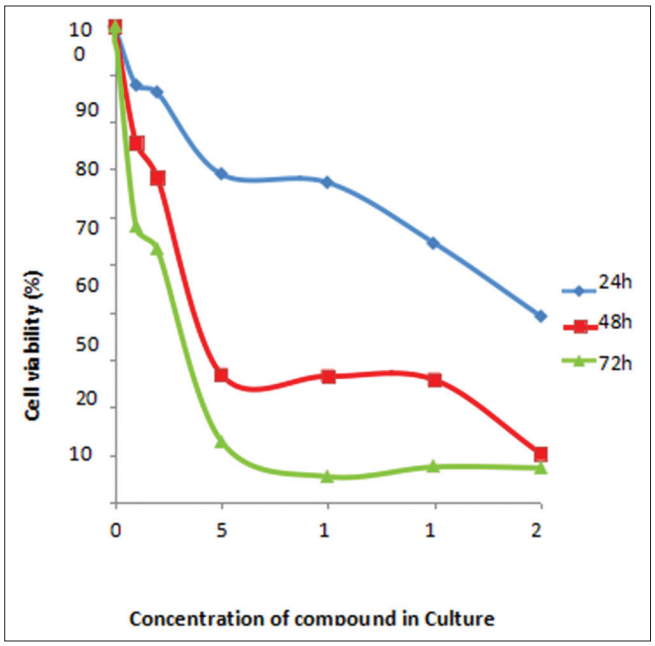

Fig. 2: Dose-dependent antiproliferation against A2780 cells with Cu(phen)(C-dmg)NO_3 after 24, 48, and 72 h incubation as determined using the MTT assay. The data are presented as mean±standard error mean

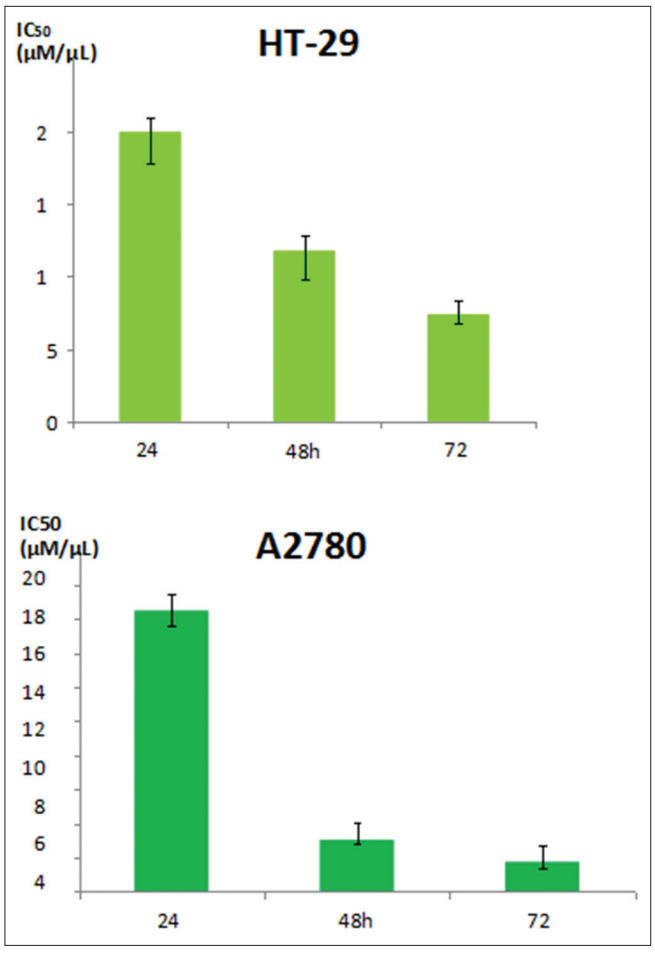

Fig. 3: Time-dependent antiproliferation against HT-29 and A2780 cells as determined by MTT assay at various incubation times. Data are presented as mean±standard error mean

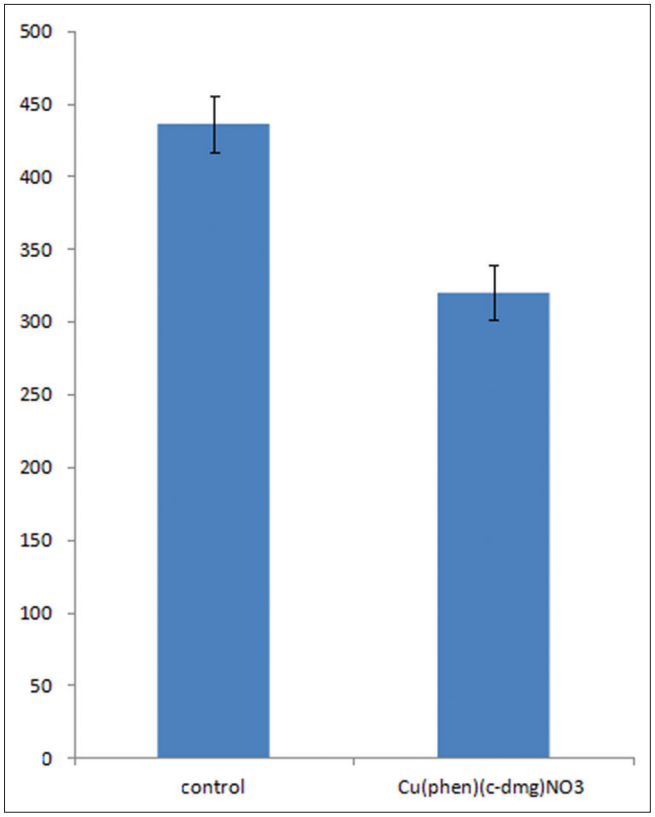

Fig. 4: Effect of Caspase-9 activation in A2780 after treated and untreated with Cu(phen)(C-dmg)NO_3

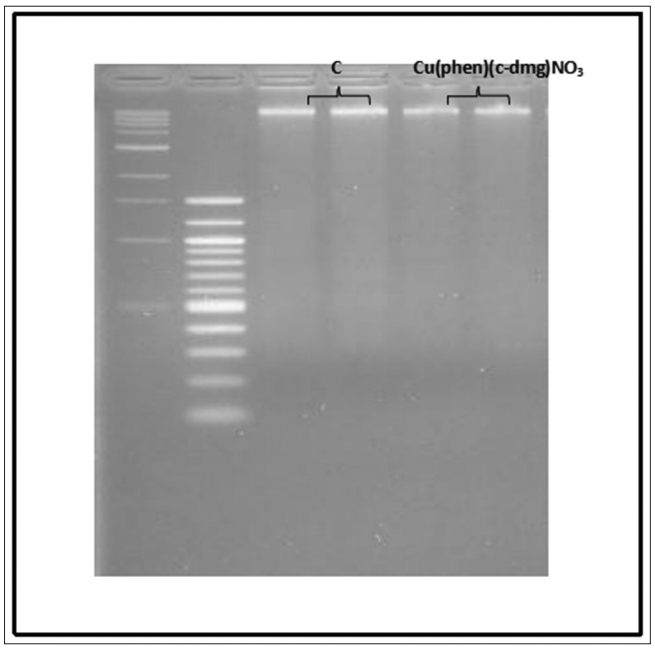

Fig. 5: No apparent of DNA fragment ladder after treatment with Cu(phen)(C- dimethylglycine)NO_3 for 72 h incubation time. Nucleosomal DNA fragments were resolved by electrophoresis in a 2.5% agarose gel and visualized by ethidium bromide staining

to cause DNA and chromatin damage *in vitro* through the production of ROS. ROS are considered to be the major source of spontaneous damage to DNA [17]. An increase in the generation of ROS, or a reduction in their detoxification, results in the induction of apoptosis through depolarization and permeabilization of the mitochondrial membrane. Soluble mitochondrial intermembrane proteins are released, such as cytochrome C and apoptosis-inducing factors, which activate downstream caspases and nucleases, ultimately leading to apoptotic cell death. Oxidative attack on the DNA results in mutagenic structures such as 8-hydroxyadenine and 8-hydroxyguanine, which induces instability of repetitive sequences. The chemical reactions that bring about such mutations are based on the formation of highly reactive and short-lived hydroxyl radicals in close proximity to DNA [18].

In this study, the absence of apoptosis, indicated by the lack of caspase-9 activation and DNA fragmentation, contrasts with previous findings that identify DNA fragmentation as a hallmark of apoptosis. This process is primarily mediated by the caspase-activated DNase (CAD) and its inhibitor CAD (ICAD). Under normal conditions, CAD is inactive due to its association with ICAD, which assists in the proper folding of CAD. Upon apoptotic stimuli, caspase-3 cleaves ICAD, releasing active CAD to degrade DNA. Notably, studies have shown that cells deficient in ICAD or expressing caspase-resistant ICAD mutants can undergo apoptosis without DNA fragmentation, although this fragmentation typically accelerates the apoptotic process [19]. ROS also can indeed cause oxidative stress and contribute to cytotoxicity without triggering apoptosis. ROS can damage various biomolecules, including lipids, proteins, and nucleic acids. This damage can lead to lipid peroxidation, oxidation of amino acids in proteins, and mutations in DNA. Such damage can disrupt cellular function, lead to cell dysfunction, and result in cell death, potentially through mechanisms other than apoptosis [20]. Many *in silico* and *in vitro* methods are available for the detection of cell deaths with or without the involvement of apoptosis [21].

In addition, according to a study done by a group of researchers in Italy, apoptosis was also considered to have occurred although no formation of DNA fragmentation. Instead of classical apoptosis, Tumor necrosis factor can induce extrinsic programmed cell death (PCD) by necrosis-like caspase-independent PCD characterized by absent or marginal chromatin condensation, lack of nuclear fragmentation, and disruption of membrane integrity [22]. Compounds and phytocompounds may target multiple cellular signaling pathways in the prevention or treatment of cancers [23].

In conclusion, Cu(phen)(C-dmg)NO_3 could inhibit the proliferation of the human cancer cell line A2780. The antiproliferation effect was dose- and time-dependent. The data suggest that Cu(phen)(C-dmg)NO_3 may exert an antiproliferative effect on A2780 cells without the involvement of apoptosis activity. It was demonstrated by the absence of caspase-9 activation and DNA fragmentation. However, further investigation is needed using another assay to confirm the exact mechanism involved in the antiproliferation of Cu(phen)(C-dmg)NO_3 because some studies have indicated that apoptosis still occurs, although there is no formation of nuclear fragmentation. Apoptotic cells expressing caspase-resistant ICAD are shielded from DNA fragmentation, will be phagocytosed with the same efficiency as wild-type cells, indicating that DNA fragmentation is not required for phagocytosis. Furthermore, another study is needed to determine whether apoptosis occurred but did not reach completion due to failure of the cellular mechanism or whether it resulted from the inability of apoptosis to progress to the stage that evokes the degradative response.

The antiproliferative effect of Cu(phen)(C-dmg)NO_3 should be tested on normal or healthy cells to ensure that this compound does not cause any toxic effect, and comparison with a commercial drug is also recommended to determine the efficacy of this compound. Assays such as JC-1 will assess mitochondrial membrane potential ($\Delta\psi m$), which is an important indicator of mitochondrial health and functions. The LC3-II/Beclin-1 assay is used to study autophagy, which is a cellular process that degrades and recycles cellular components and will be beneficial in the identification of the mechanism of action of this compound. Furthermore, an *in vivo* study should be conducted to show the antiproliferative effect of this compound.

CONCLUSION

Cu(phen)(C-dimethylglycine)NO3 demonstrates a potent *in vitro* antiproliferative effect against human colorectal (HT-29) and ovarian (A2780) carcinoma cell lines, with a more pronounced sensitivity observed in the A2780 cells. The reduction in cell viability was both concentration- and time-dependent, highlighting the compound's potential as an anticancer agent. Notably, the absence of significant caspase-9 activation and DNA fragmentation suggests that the cytotoxicity induced by this compound does not primarily involve the classical apoptotic pathways. Instead, these findings imply that Cu(phen)(C-dimethylglycine)NO3 may exert its effects through alternative that require further elucidation. Understanding these mechanisms could provide valuable insights into the compound's therapeutic potential and help optimize its application in cancer treatment strategies. Therefore, future studies should focus on detailed mechanistic analyses, including assessments of other cell death pathways and molecular targets, to fully characterize the anticancer properties and safety profile of this compound.

AUTHOR'S CONTRIBUTIONS

The BSc. thesis of Nurfarahdilla Zainudin, who carried out the study and wrote the draft manuscript. The co-supervisors (Ng Chin Theng, Fong Lai Yen, and Yong Yoke Keong) supervised and assisted with the laboratory work. Muhammad Nazrul Hakim edited the manuscript, and Zuraini Ahmad (Supervisor) verified its data.

CONFLICTS OF INTERESTS

All authors have none to declare.

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Nil.

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