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Anticancer Activity of New Di-Nuclear Copper (I) Complex

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Abstract
In-vitro biological activities of the free new H_4L (indole-7-thiocarbohydrazone) ligand and its Ni(II), Pd(II) , Pt(II), Cu(II), Ag(I), Zn(II) and Cd(II) complexes are screened against two cancerous cell lines, that revealed significant activity only for $[Cu_2Cl_2(H_4L)_2(PPh_3)_2]$ after 72 h treatment by the highest tested concentrations. The Copper(I) complex was characterized by X-ray Crystallography and the NMR spectra, whereas it has been confirmed to have momentous cytotoxicity against ovarian, breast cancerous cell lines (Caov-3, MCF-7). The apoptosis-inducing properties of the Cu(I) complex have been investigated through fluorescence microscopy visualization, DNA fragmentation analysis and propidium iodide flow cytometry.
Keywords: Cu(I) complex, biological investigation, anticancer activity & DNA fragmentation analysis.

Introduction:

Cancer is an imperative area of interest in the life sciences as it has been a prime assassin disease throughout human history. It is not one disease, but a bulky group of diseases characterized by uncontrolled growth and spread of abnormal cells. Heterocyclic molecules are distinguished to play a critical role in health care and pharmaceutical drug design [1,2]. In the relevant annual reviews are to be found examples of metal ions in biological systems and coordination chemistry for the series. Inorganic chemistry useful in the medical field can be divided into two main categories: firstly, as metal ions to a target protein is free or whether the drugs as ligands; and secondly, metal-based drugs and imaging agents of central metal ion is usually significant of the mechanism of action [3-5]. The choice of the coordinated ligand(s) seems to be as vital as the choice of metal(s) because being the integral part of biologically active complexes, in addition these organic molecules (ligands) can exert a biological activity of their own [6-11]. Purine, thiosemicarbazone, imidazole, benzohydroxamic ligands as nitrogen donor ligands include various types of anti-cancer activities of the range of simple copper complexes have been studied when some metal-based antitumor drugs in vitro and in vivo studies have demonstrated greater antineoplastic power than Cisplatin[12] . Copper complexes are behaved as the most promising option anticancer drugs as cisplatin, when this idea supported by a number of research articles describing the synthesis, thus DNA binding and cytotoxic activities of many copper complexes [13,14]. There are only few complexes of Copper(I) in the literature, whereas they also be evidence for a very sturdy cytotoxic activity against tumor cells *in vitro* [15,16]. Anticancer activity of Cu(I) complex is related to their ability to produce *reactive oxygen species* (ROS). Copper(I) ions can reduce hydrogen peroxide to hydroxyl radical. Copper(II) ions may in turn be reduced to Cu(I) by superoxide anion($O_2^{\bullet-}$). Consequently, it can be terminated that the production of reactive oxygen species such as $OH^{\bullet}$ are driven by the Copper, in spite of the form in which it is initially introduced into the body Cu^+, or Cu^{2+} [17,18]. The hydroxyl radical ($OH^{\bullet}$) is supposed to be the main factor causing DNA damage in cells under oxidative stress [19-21]. Hence, we emphasis to report the anticancer activity of new Copper(I) indole-7- thiocarbohydrazone complex .

$Cu^{2+} + O_2^{\bullet-} \rightarrow Cu^+ + O_2$

$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^-$

Experimental

The ligand (H_4L) and its Cu(I) complex which were prepared and characterized according to the previously described procedure [22]. The ligand(H_4L) and its Cu(I) complex were evaluated for their *in vitro* cytotoxicity towards human ovarian adenocarcinoma and breast cancer cell lines (Caov-3, MCF-7) respectively .

- *Cell Culture*

The cell lines (Caov-3, MCF-7) were provided by Department of Pharmacy, Faculty of Medicine, University of Malaya. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma Aldrich) and L-glutamine at 37 °C in a 5% CO_2 humidified atmosphere.

- *MTT Cytotoxixity Test*

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out as described by Mosmann [23] with some modifications. The cells were seeded into 96-well plates (5000 cells/well) and allowed to adhere overnight. Copper complex was pre-dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentrations (eight concentrations, 0.39-50 µg/mL), such that the final concentrations of DMSO did not exceed 0.5%. Each cell was treated with the test compound solutions (three wells on a plate for each concentration) for 24, 48 and 72 h. Treated and untreated cells were inspected qualitatively using an inverted light microscope (100

X). Then, 10 µl of MTT (5 mg/mL) was added to each well and the plates were incubated at 37 °C for 4 h. The media was then gently aspirated, and 100 µl DMSO was added to dissolve the formazan crystals. The amount of formazan product was measured spectrophotometrically at 570 nm using a microplate reader (Power Wave X 340). The concentration of the test compound required for 50% inhibition of cell growth (IC_{50})was determined by interpolation of regression analysis.

- *Apoptosis Detection with Fluorescence Microscopy AO/PI Staining Assay*

The cancer cell lines were seeded in a 25 mL culture-flask (1×10^6 cells/mL) and treated with the Copper complex at the corresponding IC_{50} concentrations for 24, 48 and 72 h. The cells were washed with phosphate buffered saline (PBS) and suspended in 500 µL of PBS followed by addition of a 1:1 mixture of Acridine orange (AO) 10 µg/ml and propidium iodide (PI) 10 µg/ml. A drop of the suspension was placed on a glass slide and covered with a cover slip. Images of the cells were taken by a UV-fluorescence microscope within 30 min.

- *DNA-Fragmentation Analysis*

The cancer cell lines were seeded in a 25 mL culture-flask (1×10^6 cells/mL) and treated with the respective IC_{50} concentrations of the test compound for 24, 48 and 72 h (control cells were treated with 0.5% DMSO vehicle). The cells were then washed with PBS.The treated and control cells were washed with PBS and harvested. Cellular DNA was extracted using the apoptotic DNA ladder detection kit (Chemicon International Inc., Palo Alto, CA, USA). The DNA fragments were then separated by a 1.5% agarose gel electrophoresis at 50 V for 3 h.The Gels were then stained with ethidium bromide and visualized on a UV-illuminator.

- *Cell cycle analysis*

A flow cytometry analysis was carried out to determine the cell cycle distribution in treated ovarian adenocarcinoma (Caov-3) cell line with $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ [24]. In brief, (Caov-3, MCF-7) cell lines (5×10^4 cells/ml) were treated with $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ at IC_{50} concentration for 24, 48 and 72 h. After fixation with cold ethanol, cells were washed with PBS and stained with PI (50 µl, 10 mg/ml) for 1 h at 37 ˚C. In addition, RNase A (10 mg/ml) was also used to limit the ability of PI to bind only to DNA molecules. The stained cell was analyzed for DNA content using flow cytometer (BD FACSCanto TM II).

Results and discussion

- *Structure of the Copper(I) complex*

The reaction of H_4L with equimolar amount of CuCl and PPh_3 afforded the Cu(I) complex of $[Cu_2Cl_2(H_4L)_2(PPh_3)_2]$ (Scheme 1). The crystal structure of the complex is shown in (Figure 1). The thiocarbohydrazone is almost planar (r. m. s. deviation = 0.151 Å) and adopts an *anti* geometry. Two neutral thiocarbohydrazones, acting as μ_2-S-donor ligands, doubly bridge pairs of the Cu(I) atom into a centrosymmetric dimer. The Cu centers within the $Cu_2(\mu_2\text{-}S)_2$ core are separated by 3.0681(6) Å, which is larger than sum of their van der Waals radii (2.80 Å). One Cl atom and one PPh_3 group complete a distorted tetrahedral geometry around each metal center, with coordination angles being *ca*. 96-119°.The geometric parameters of the parallelogram and those pertaining to the metal centers are compatible with the reported values for similar structures [25-27]. The Cl atom is intramolecularly hydrogen bonded to N3, and intermolecularly H-bonded to a methanol solvate molecule. Theoretical calculations on similar dinucler Cu(I) structures suggested that the hydrogen bonding between the halogen ligands and the solvent molecules plays a crucial role in the formation of S-bridged dimers *vs*. halogen-bridged or monomeric structures [22,25] .

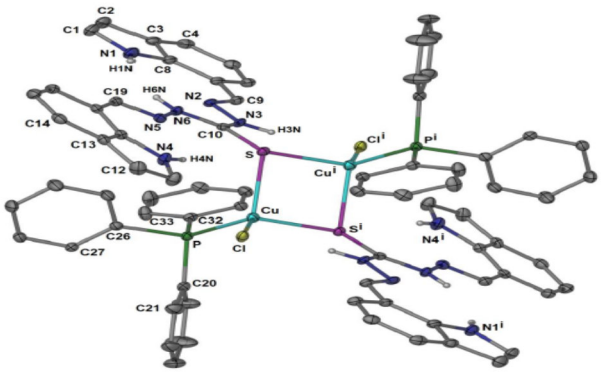
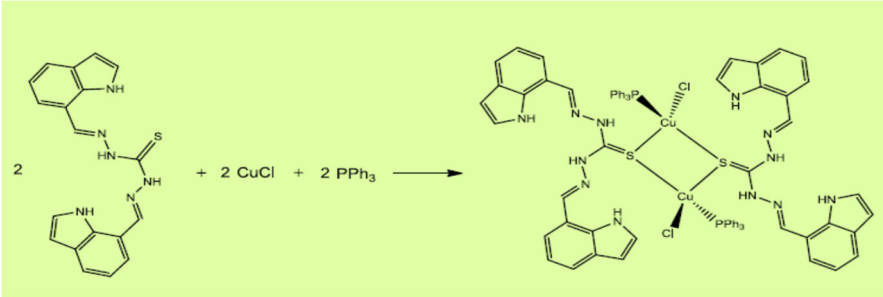


Figure 1 Molecular structure of $[Cu_2Cl_2(H_4L)_2(PPh_3)_2]$ with thermal ellipsoids drawn at the 30% probability level. C-bound H atoms and methanol solvent molecules are omitted for clarity. Symmetry code: i = -*x*+1, -*y*+1, -*z*+1.



Scheme 1. Synthesis of the Cu(I) complex

The Cu complex also characterized by ^{1}H , ^{13}C NMR and HSQC spectra in DMSO-d_6 that in agreement with the crystal structure, indicating the stability of the structure in the solution . The ^{13}C NMR spectrum shows an upfield shift of the CS signal (~3 ppm) from that in the spectrum of H_4L. Yield 0.38 g, 53%. Anal. Calc. for $C_{74}H_{62}Cl_2Cu_2N_{12}P_2S_2$: C, 61.57; H, 4.33; N, 11.64. Found: C, 61.44; H, 4.08; N, 11.98%. ^{1}H NMR (DMSO-d_6): δ 6.59 (2H, br.s, Ar-*H*); 6.65 (2H, br.s, Ar-*H*); 6.99 (2H, br.s, Ar-*H*); 7.16 (2H, t, J = 6.8 Hz, Ar-*H*); 7.24-7.48 (34H, Ar-H); 7.58 (2H, br.s, Ar-*H*); 7.69(4H, m, Ar-*H*); 7.77 (2H, d, J = 7.7 Hz, Ar-*H*); 8.55 (2H, br.s, *H*CNN); 8.94 (2H, br.s, *H*CNN); 11.30 (2H, s, indole-N*H*); 11.33 (2H, s, indole-N*H*); 12.32 (2H, s, NN*H*); 12.55 (2H, s, NN*H*) ppm. ^{13}C NMR (DMSO-d_6): δ 102.86, 116.82, 117.92, 119.75, 119.84, 123.96, 124.14, 124.70, 126.44, 126.96, 127.14, 128.75-129.37, 130.48, 131.57, 133.71-133.96 (*Ar*); 149.89, 150.29 (H*C*NN); 172.43 (*C*S) ppm.

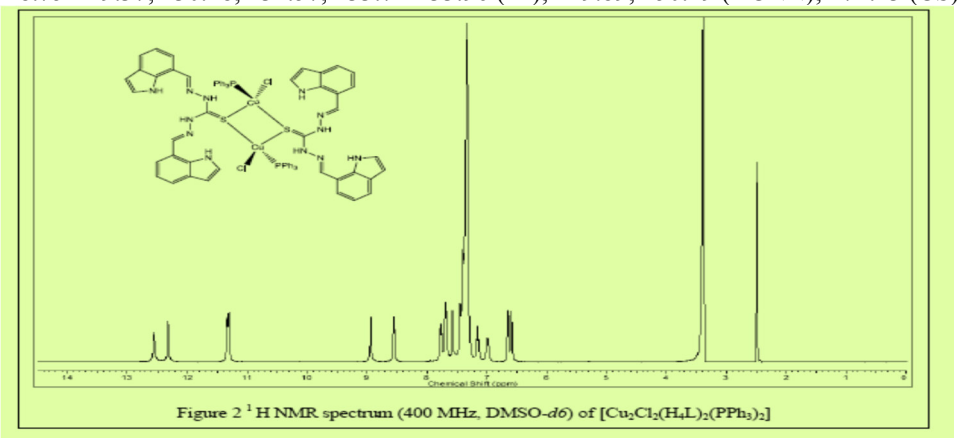


Figure 2 ^{1}H NMR spectrum (400 MHz, DMSO-*d6*) of $[Cu_2Cl_2(H_4L)_2(PPh_3)_2]$

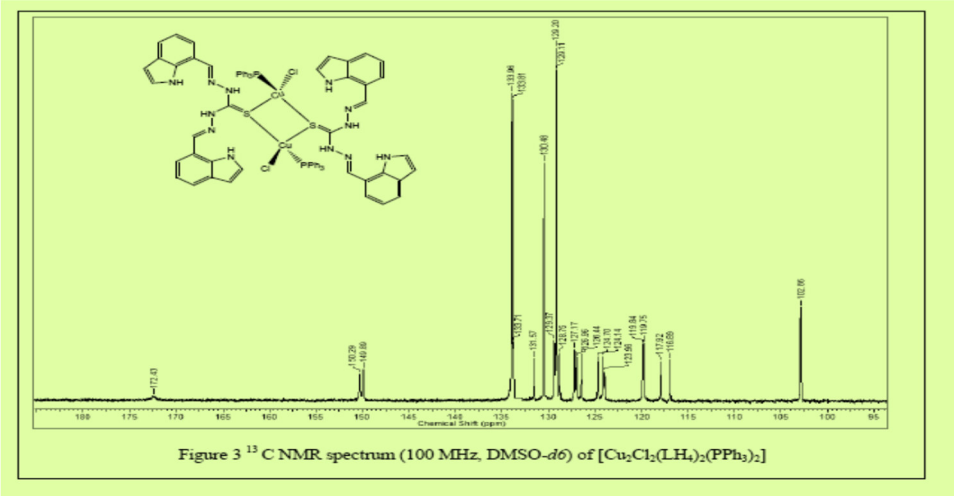


Figure 3 ^{13}C NMR spectrum (100 MHz, DMSO-*d6*) of $[Cu_2Cl_2(LH_4)_2(PPh_3)_2]$

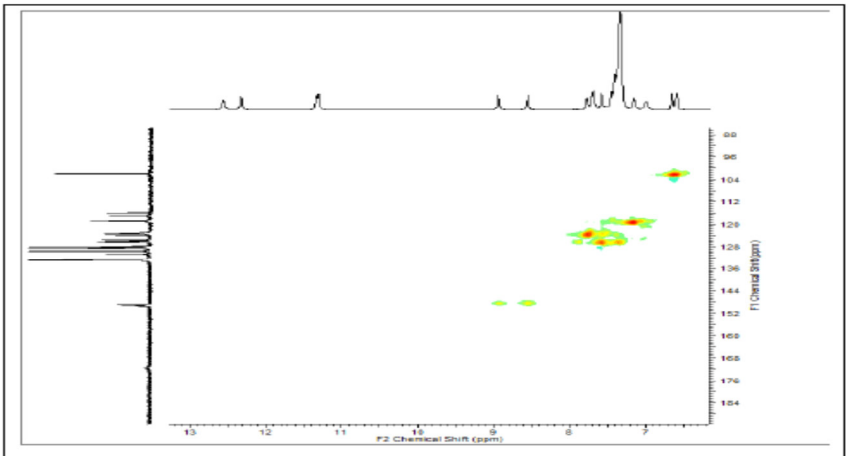


Figure 4 HSQC spectrum (in DMSO-*d6*) of $[Cu_2Cl_2(H_4L)_2(PPh_3)_2]$

- *Cytotoxicity assay for Cu*(I) *complex*

(Table1) lists the IC_{50} values of the Copper(I) complex after treating the (Caov-3, MCF-7) cancer cell lines for 24, 48 and 72 h. Based on outcomes that, the IC_{50} values were (13, 25.75) µM for 24 h treatment; however, the value declined, in case of prolonged treatment, where, IC_{50} values were (10, 4.25) µM for 48 h treatment, and further dropped to (4, 2.5) µM for 72 h treatment; which obviously signifies that, the prolonged treatment considerably decreased the IC_{50} values, and elevated the toxic activity. For the purpose of comparability, ovarian adenocarcinoma and breast cancer cell lines were also treated with cisplatin a clinical anticancer drug (Figure 5, Figure 6), and the results presented in (Table1) that $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ revealed higher cytotoxicity than cisplatin against the (Caov-3, MCF-7) cancer cell lines .

Table 1 IC_{50} (μM) values of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ and cisplatin at different time (24, 48 and 72) h

Time	IC_{50} values (µg/ml) of Caov-3	IC_{50} values (µg/ml) of MCF-7
24 h	13±0.61 $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$	25.75±1.25 $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$
	18±0.45 Cisplatin	14.76±0.58 Cisplatin
48 h	10±0.25 $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$	4.25±0.25 $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$
	12.5±0.31 Cisplatin	11.89±0.58 Cisplatin
72 h	4±0.22 $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$	2.5±0 $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$
	4.5±0.21 Cisplatin	9.13±0.62 Cisplatin

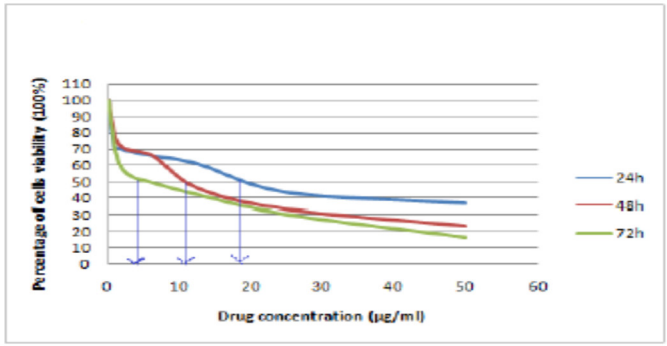


Figure 5 ovarian adenocarcinoma (Caov-3) treated with cisplatin at 24,48 and 72h treatment

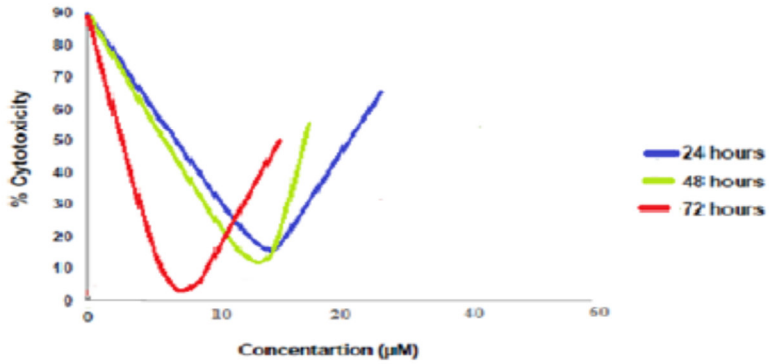


Figure 6 Breast cancer (MCF-7) treated with cisplatin at 24,48 and 72h treatment

- *Apoptosis assay*

Apoptosis initiation is an anti-proliferative mechanism by which the cancer cells go through programmed death. Cells undergoing apoptosis are characterized by morphological and biochemical changes including cell shrinkage, chromatin condensation and DNA fragmentation. Based on the results of the in vitro cytotoxicity assay for $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$, it was considered valuable to explore the apoptotic properties of the compound. Apoptosis in the ovarian adenocarcinoma (Caov-3) and breast (MCF-7) cancer cell lines were screened through fluorescence microscopy visualization and DNA fragmentation analysis and propidium iodide flow cytometry.

- *Fluorescence microscopy visualization*

Apoptotic cells display increased plasma membrane permeability to certain fluorescent dyes. Acridine orange (AO)/propidium iodide (PI) is a fluorochrome mixture for nuclear staining which permits uniqueness between viable, apoptotic and necrotic cells. In this work, the ovarian adenocarcinoma (Caov-3) plus breast cancer (MCF-7) were subjected to AO/PI staining after treatment with respective IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ for 24, 48 and 72 h. Fluorescence microscopy exposes that in the (Caov-3, MCF-7) cancer cell lines the Copper complex encouraged cell death fundamentally *via* apoptosis especially at 72h when the apoptotic cell clearly observed (Figure 7, Figure 8).

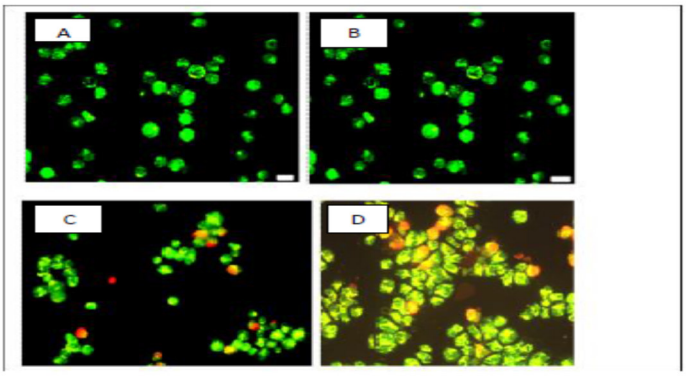


Figure 7 A. Control (untreated cells), B. 24h treatment with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$, C. 48h treatment with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$, D.72h treatment with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ at Caov-3

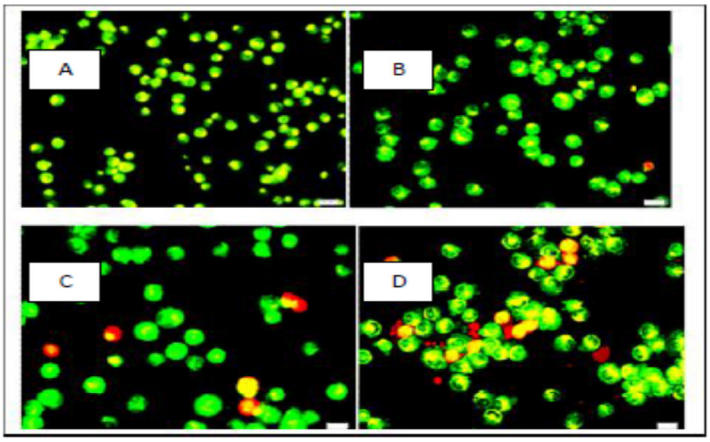


Figure 8 A. Control (untreated cells), B. 24h treatment with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$, C. 48h treatment with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$, D.72h treatment with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ at MCF-7

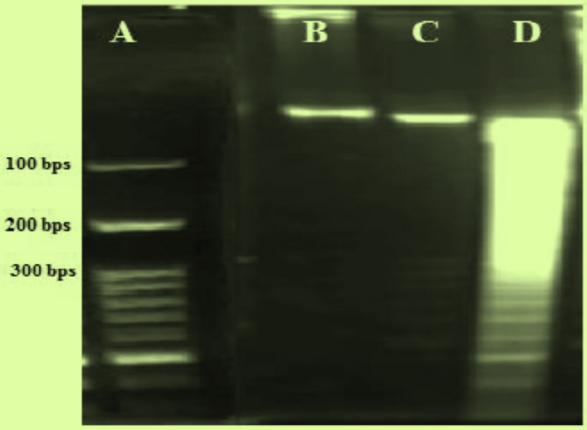


Figure 9 Gel electrophoresis of apoptotic DNA fragmentation. Lane A: 1000 bp Molecular weight marker , Lane B: Untreated Caov-3 , Lane C: treated Caov-3 at 48h, Lane D: Treated Caov-3 at 72h

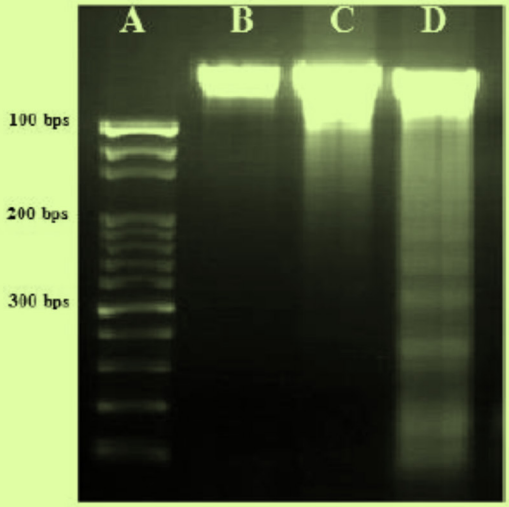


Figure 10 Gel electrophoresis of apoptotic DNA fragmentation. Lane A: 1000 bp Molecular weight marker , Lane B: Untreated MCF-7 , Lane C: treated MCF-7 at 48h, Lane D: Treated MCF-7 at 72h

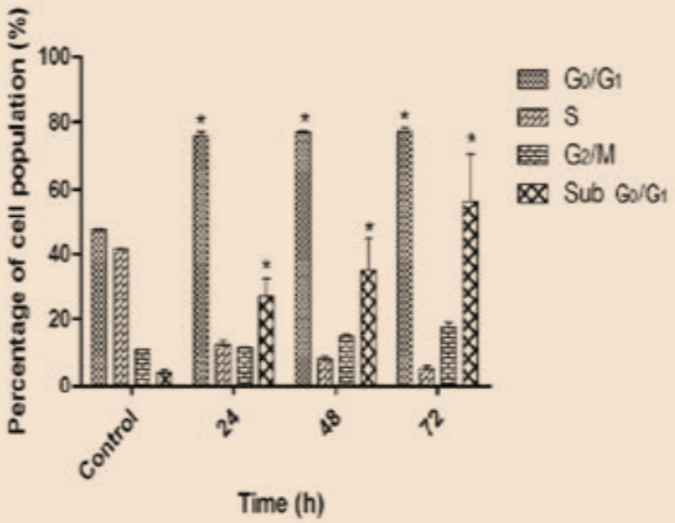


Figure 11 Flow cytometric analysis of cell cycle distribution in the Caov-3 cell line treated with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ for 24, 48 and 72 h. *($p < 0.05$) when compared with the control.

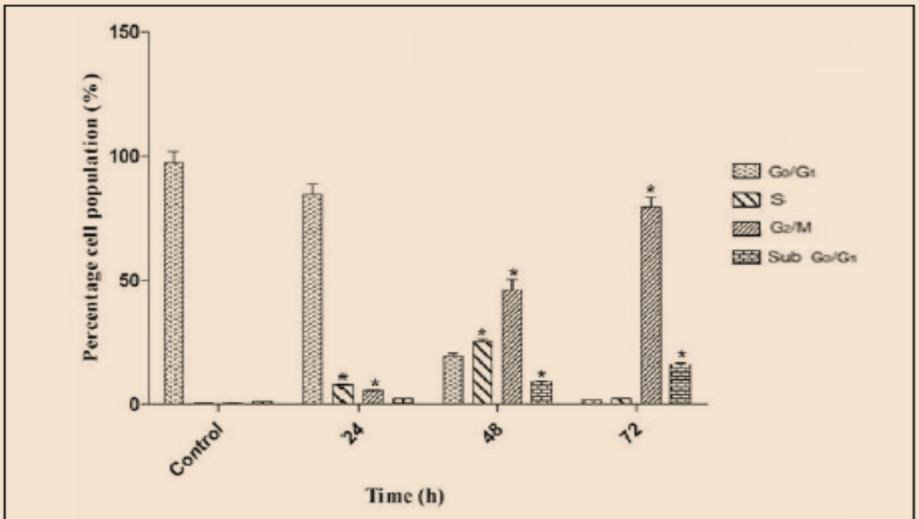


Figure12 Flow cytometric analysis of cell cycle distribution in the MCF-7 cell line treated with IC_{50} concentrations of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ for 24, 48 and 72 h. *($p < 0.05$) when compared with the control.

- *DNA Fragmentation analysis*

The degeneration of nuclear DNA into nucleosomal units is a biochemical feature of apoptosis. To evaluate the apoptotic DNA fragmentation in the cancer cells, the ovarian and breast cancer cell lines were incubated with the correspond IC_{50} value of $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ for 48 and 72 h. The cells were then lysed, the nuclear DNA was extracted, and subjected to gel electrophoresis. Inspection of the electrophoretic profiles discovered a ladder formation detected only at 72h at both of ovarian and breast cancer cells (fragments range from 400 to 1000 bp), which signify the incidence of apoptosis (Figure 9, Figure 10).

- *Cell cycle analysis*

The DNA content of cells duplicates during the S phase, therefore cells in the G_0 and G_1 phases (before the S phase) have unreplicated DNA, while those in the G_2 and M phases (after the S phase) have replicated DNA. Analysis of cell cycles by flow cytometry enables to distinguish and quantify the cells in different phases of the cell cycle. On the flow cytometry DNA histograms cells with degraded and thus hypodiploid DNA are represented in so-called "sub-G_0/G_1" peaks therefore, sub-G_0/G_1 is a specific marker of cell death by apoptosis [28,29]. Flow cytometry analysis of the Caov-3, MCF-7 cancer cell lines after treatments with respective IC_{50} concentrations of the $[Cu_2(H_4L)_2(PPh_3)_2Cl_2]$ for 24, 48 and 72 h was carried out (Figure11, Figure12). Moreover, the histogram of Caov-3 illustrates gradual increases in the populations of sub-G_0/G_1 phase during the incubation periods whereas the histogram of MCF-7 illustrates gradual increases in the populations of G_2/M phase In general, flow cytometry suggests the induction of apoptosis by the Copper(I) complex in the Caov-3 cancer cell through G_0/G_1 phase while through Sub-G_0/G_1 phase for MCF-7 cell cycle arrest.

Conclusion

As it was presented in this article, Copper(I) coordination complex has notable and cancer-selective cytotoxicity against the Caov-3, MCF-7 cancer cell lines as inferred from the MTT-based IC_{50} values and different apoptotic assay methods in consequence, Intensive research possibly will enable to apply it as anticancer drug.

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