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Research

A Prospective Drug for Anticancer Activity

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Abstract

A new Schiff base ligand (fluorene di imino benzene, FDIB) was synthesized and characterized corresponding ternary Cu(II) complex [Cu(FDIB) Phen]$^{+2}$ was synthesized, characterized and studied its biological properties. Complex exhibits distorted square planar geometry where Schiff base and act as N N donar ligands. The in-vitro cytotoxicity of the complex towards human cervical carcinoma (HeLa) cells was assayed by MTT method. IC_{50} values of the complex reveal that the complex has potent cytotoxic effect against the cancer cell lines, and the inhibitory rate of complex is higher than that of standard drug doxorubicin.

Keywords: Cu (II) Schiff Base Complex; Cytotoxic Studies; Nuclear Localization Studies

Introduction

DNA is the primary intracellular target of anti-cancer drugs, so the interaction between small molecules and DNA can cause DNA damage in cancer cells, blocking the division of cancer cells, and resulting in cell death [1-4]. The development of compounds cleaving DNA under physiological conditions is of current interest, due to their potential applications in genomic research and as foot printing and therapeutic agents [5-7 & 16-19]. Copper is an endogenously biocompatible metal ion, and its complexes are also known to play a significant role both in naturally occurring biological systems and as pharmacological agents. Cu(II) complexes could tune the molecules for specific interaction with the targets and thereby, reduce the toxicity due to chelation effect. Schiff bases bearing heterocyclic residues possess excellent biological activities, therefore attracted many researcher's attention in recent year [8-12]. Cu(II) Schiff base complexes could inhibit *in vitro* tumour cell growth and in human tumour cell cultures and it was found that the cytotoxic activities of Cu(II) complexes were higher than that of free ligands [13]. Copper (II) complexes are also regarded as the most promising alternatives to cisplatin as anticancer drugs. Fluorene (2-carbaldehyde fluorene) compounds have medicinal and pharmaceutical applications. Among the wide chemical derivatives are a heteropolymer which have activity and effectiveness against cancer [14].

Hence it was thought important to design and synthesize molecules that bear an intercalating moiety; ligands with N-heterocyclic aromatic ring in which the heteroatom can coordinate to the metal ion are expected to induce a good extent of planarity to the complexes because of rigidity introduced in the ligand framework by the coordinating heterocycle. we report here the syntheses, characterization, and cytotoxic studies of [Cu (FDIB) phen]$^{+2}$ at physiological conditions.

Experimental Section

Materials and Instruments

General: 1, 10 Phenanthroline (phen), flourene-2-carbaldehyde, and $CuCl_2.2H_2O$ (99.99% purity), were obtained from Sigma, USA. All other chemicals and solvents (spectroscopic grade) were purchased from commercial sources and were used without further purification.

Elemental analyses were obtained from the micro analytical Heraeus Carlo Erba 1108 elemental analyzer. Copper content was determined on Shimadzu AA-6300 atomic absorption spectrophotometer. ESI mass spectra of ligand and complex were recorded using a quattro Lc (Micro mass, Manchester, UK) triple quadrupole mass spectrometer with Mass Lynx software. The molar conductivity was measured on a Digisun Digital conductivity bridge (model: DI-909) with a dip type cell. Infrared spectra were recorded on a Shimadzu -1600 IR spectrometer, in KBr pellets in the 4000-400 cm^{-1} range. A UV-vis spectrum of the complex was recorded on Shimadzu (160A) UV-visible spectrophotometer using 1-cm quartz micro-cuvettes (800-200nm). Magnetic susceptibilities of the complex was recorded at room temperature on a Faraday balance (CAHN-7600) using $Hg[Co(NCS)_4]$ as the standard. Diamagnetic corrections were made by using Pascal's constants [15]. The Thermo Gravimetric Analysis (TGA), were performed on the Metler Toledo (TGA/SDTA 851^e) thermo analyzer.

Synthesis of Schiff Base ligand (FDIB)

To a methanolic solution (10 mL) of flourene -2-carbaldehyde (0.388 g, 2 mmol) 1,2 di amino benzene [added in a methanol (10 mL) solution, (0.108 g, 1 mmol)] was added and refluxed for 3h. Completion of the reaction was monitored by TLC. The resulting

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mixture was cooled, filtered and recrystallized from MeOH which gave a dark yellow product.

Synthesis of [Cu(FDIB) Phen]$^{+2}$

To a MeOH solution (10 mL) of Schiff base (0.460 g, 1mmol) an aqueous solution of copper chloride (0.199 g, 1 mmol) was added drop wise and stirred for 3h.at r.t. Heterocyclic base (0.198 g phen, 1 mmol) dissolved in methanol (10 mL) was added drop wise to a methanolic solution (10 mL) of above reaction mixture with constant stirring for 5 h at room temperature. A green precipitate was separated by filtration and air dried.

Analytical data: FDIB

(Yield: 80%). M.P: 192°C. IR (KBr phase, cm^{-1}): υ = 1608s, 1530m, 1454s, 1333s,1296m, 952m, 924m, 879m, 836s, 767s, 739s. ^{1}H NMR (400 MHz, CDCl3) δ= 8.55 (s, 2H), 8.15 (s, 2H), 7.88 (d, J = 8.1 Hz, 2H), 7.84 (d, J = 4.4 Hz, 2H), 7.82 (d, J = 3.9 Hz, 2H), 7.56 (d, J = 7.3 Hz, 2H), 7.40 (t, J = 7.0 Hz, 2H), 7.35 (d, J = 7.4, 1.2 Hz, 2H), 7.29 – 7.27 (m,2H), 7.24 – 7.20 (m, 2H), 3.97 (d, J = 22.4 Hz, 4H). Elemental Analysis, Calculated: C, 88.67; H, 5.25; N, 6.08. Found: C,88.69; H, 5.25; N, 6.06. ESI-MS in MeOH: *m/z* = 460 $[M]^+$

Data for [Cu(FDIB) Phen]$^{+2}$

(Yield: 75%). M.P: 293°C. UV-Vis in MeOH [λ_{max}/nm (ε/$M^{-1}cm^{-1}$)]: 207 (4.44x10^5), 241 (1.86x10^5), 275 (1.34x10^4), 572 (538). IR (KBr phase, cm^{-1}): υ = 1625s, 1581s, 1514s, 1421m, 1346ms,1143s, 1105s, 1045m, 854m,779m,719s,644m, 520s, 508m, 491s,430s. ESI-MS in MeOH: *m/z* = 704 $[M]^+$ Elemental Analysis, Calculated: C, 78.83; H, 4.72; N, 7.94; Cu,9.01 Found: C, 78.23; H, 4.70; N, 7.28; Cu, 8.98. Λ_M [$\Omega^{-1}Cm^2M^{-1}$, using 3x10^{-3}M solution at r.t.] - (a) in MeOH: 269. (b) in H_2O-MeOH (10:1): 266. μ_{eff} = 1.82 BM at r.t.

***in vitro* Cytotoxicity Assay**

To study the effect of the Cu(II) complex on cell growth, HeLa (human cervical carcinoma) cells growing exponentially were added to 96-well plates (Tarson Ltd) at a density of 3 × 103 per well after counting on Bright Line Haemocytometer (Thermo Scientific). Cells were grown according to the American Type Culture collection (ATCC) instructions. The number of cells were selected to avoid potential over confluence of the cells. Cells were maintained at 37 oC and 5% CO2 in a humidified incubator till cells adhered and reached 70-80% confluency. The cells were treated with the Cu (II) complexes (5-20μM) at three different concentrations for 24 hrs and 0.5% (v/v) DMSO employed as control ensuring an equal volume of 200 μL Complete media (RPMI1640+10% FBS) across the plate. The MTT (Tetrazolium bromide salt) compound was used for the measurement of cytotoxicity due to mitochondrial activity of viable cells by the reduction of Tetrazolium bromide salt and metabolically active cells to form insoluble Formazon crystals which is in yellow colour and solubilized by the addition of detergent. After incubation of 96 well plates with Cu (II) complex, MTT reagent (Sigma Aldrich) was added to each well working concentration of 0.4 mg/mL prepared in complete medium, and the plates were placed in the incubator for 3 hrs. After MTT reagent treatment, the medium was discarded, and added 100 μL of DMSO (Cell Culture Grade Sigma Aldrich) followed by the agitation of plates for 5 mins before measuring. The colour was quantified by Bio-Rad Model 680 X ELISA plate readers at optimized absorbance is 570nm. This data from cancer cell lines were acquired from triplicate independent cell passages and the IC50 values were calculated from the plot of cell viability with different concentration of complex. Student's t-test was applied for the statistical analysis of the results.

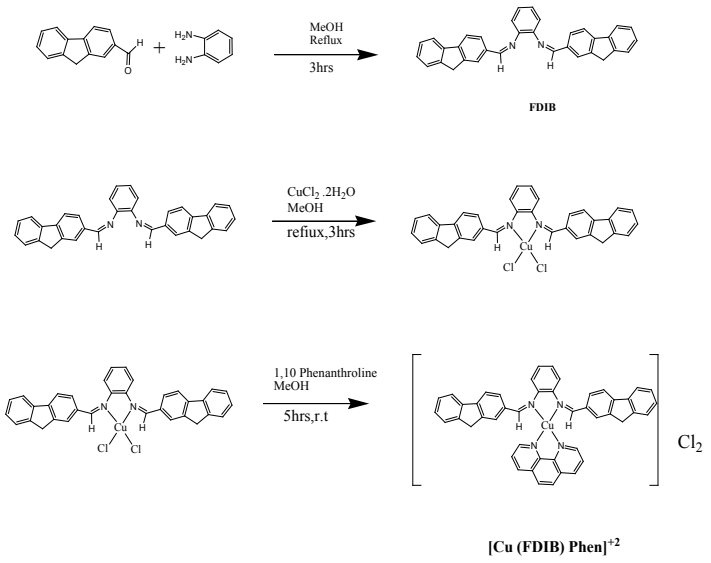
Cells Preparation for Confocal Microscopy

He La cells were grown in a phenol red RPMI1640 medium with 10% FBS fetal bovine serum and 1% Pen Strep glutamine (Hi Media) in an atmosphere of 5% (v/v) CO_2-enriched air at 37˚C. For microscopic studies, cells were cultured on cover slips (Corning 22X22 mm) incubated till reached 80% confluency for Cu (II) complex treatment, and cells were treated with 10 μM complex for 24 hrs, the cells were rinsed with cold PBS (Phosphate Buffer Saline) buffer solution before stained with DAPI (Fluorescent dye). For solvation of dye, dye was diluted with double distilled water. For microscopic studies cells were stained with 50 μM dye and then incubated for about 10 mins. Confocal imaging studies was carried out on one set with DAPI and one set with unstained used as control. Here DAPI was used for nuclear localisation in live cells.

Results and discussion

Syntheses and Characterization

The ligand was synthesised and charecterised by elemental analysis, ESI-MS, IR, H^1-NMR. The Cu(II) complex was synthesized (Scheme 1) and characterized by elemental analysis, ESI-MS, IR, UV-Vis, Magnetic moment, Molar conductance. The complex was highly soluble in CH_2Cl_2, DMSO, MeOH, EtOH, H_2O-MeOH mixture. The complex is non-hygroscopic and stable both in solid and solution phases. The molar conductance values for the complex in pure MeOH and H_2O-MeOH mixture (10:1) were similar and the values indicate that the complex is non-electrolyte. The analytical



Scheme 1. Syntheses of ligand (FDIB) and complex.

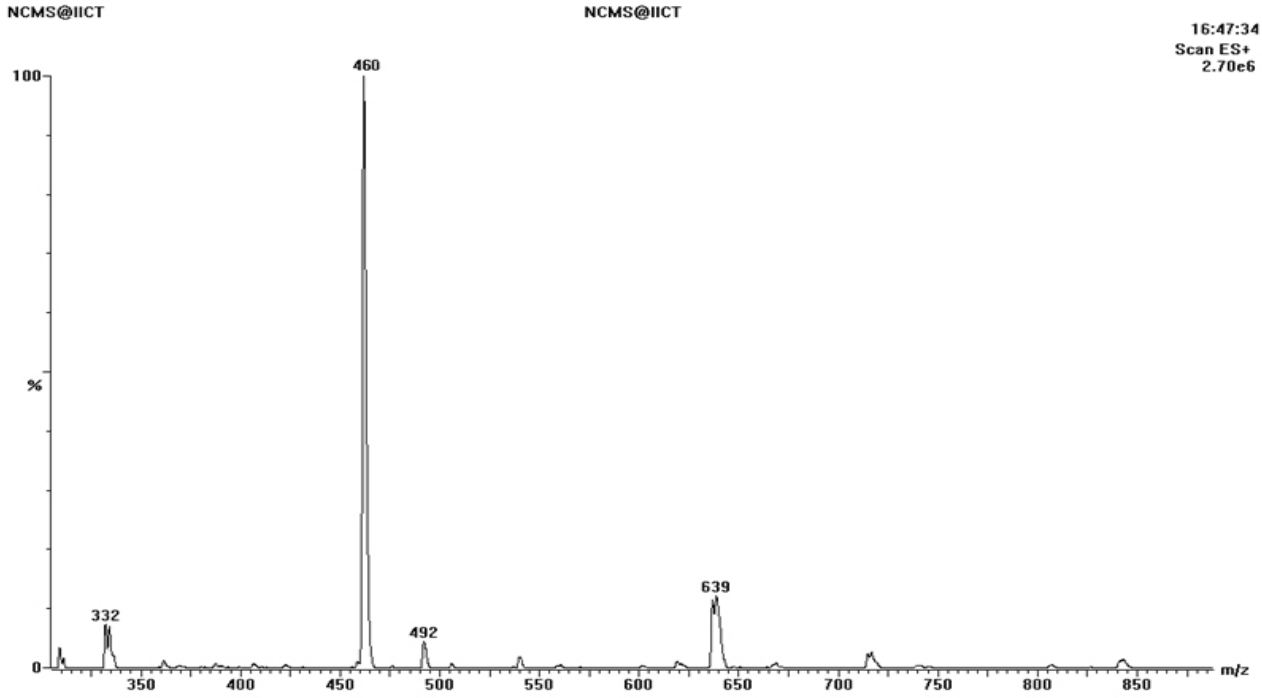
data for the Cu(II) complex are in good agreement with the molecular formulae of the complex.

ESI-MS spectra

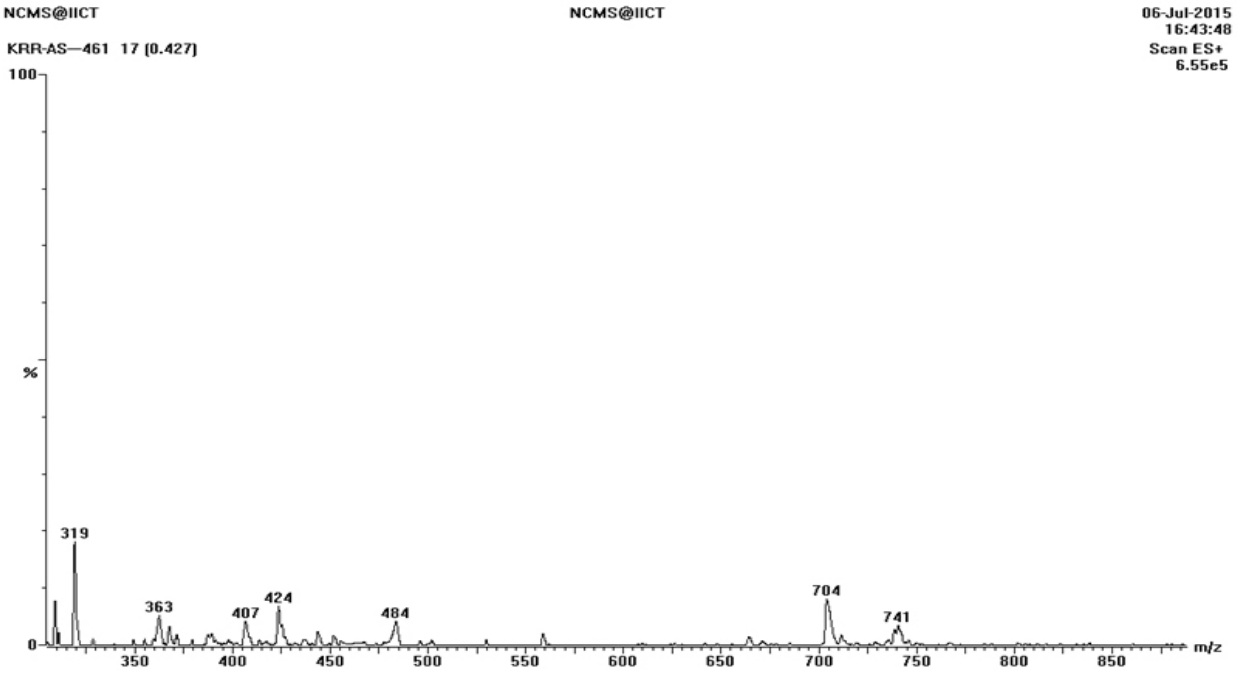
The mass spectra of the ligand and the complex were studied in positive mode, the mass spectrum of ligand shows a molecular ion peak at m/z = 460. Complex shows a molecular ion peak at m/z = 704 due to the absence of the two chloride ions [Cu (FDIB) Phen]$^{+2}$ which is equivalent to its molecular weight. The mass spectrometry results are in good agreement with the proposed molecular formulae of the complex. (Fig.1S; ESI).

Infrared spectra

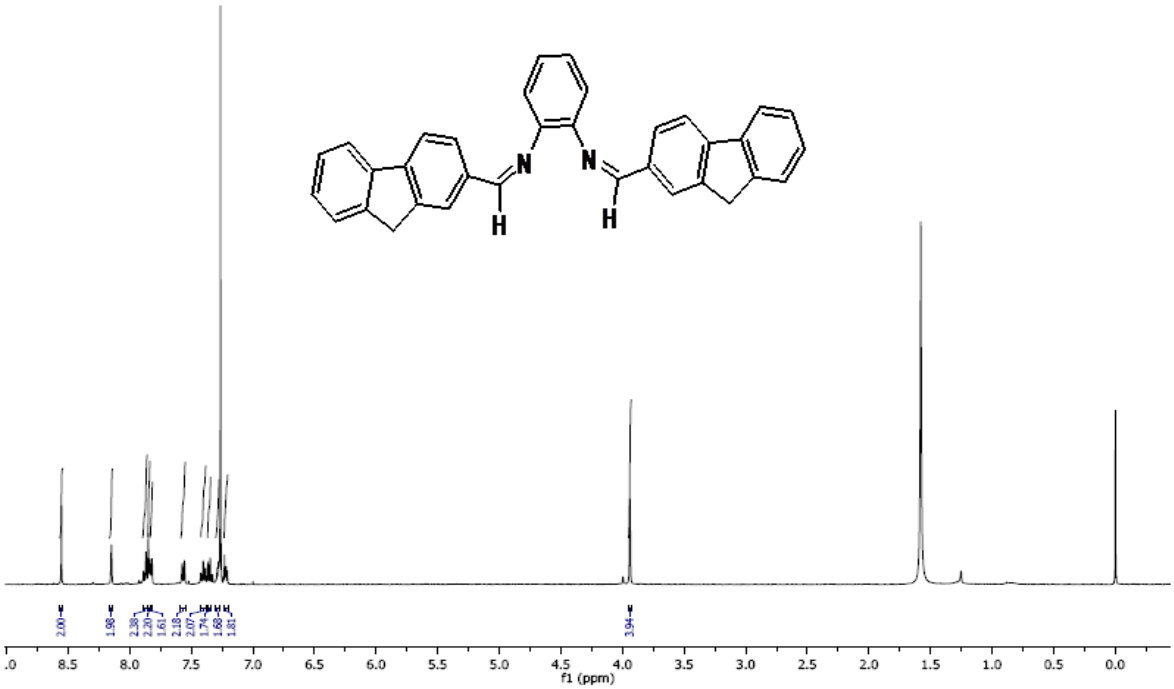
In the IR spectra, of Schiff base ligand (FDIB), a strong band at



ESI mass spectra of ligand FDIB (1)



ESI mass spectra of [Cu(FDIB) Phen]$^{+2}$ (2)



^{1}H NMR (400 MHz, CDCl3) δ= 8.55 (s, 2H), 8.15 (s, 2H), 7.88 (d, J = 8.1 Hz, 2H), 7.84 (d, J = 4.4 Hz, 2H), 7.82 (d, J = 3.9 Hz, 2H), 7.56 (d, J = 7.3 Hz, 2H), 7.40 (t, J = 7.0 Hz, 2H), 7.35 (d, J = 7.4, 1.2 Hz, 2H), 7.29 – 7.27 (m, 2H), 7.24 – 7.20 (m, 2H), 3.97 (d, J = 22.4 Hz, 4H). At 1.5 to 2.0, range moisture peak is obtained.

Fig. 2S. ^{1}H NMR spectra of the ligand FDIB**.**

1608 cm^{-1} was due to the azomethine **ν(C=N)** linkage, indicating that condensation between aldehyde group of Fluorene (2-carbaldehyde fluorene) and 1, 2 di amino benzene resulting in the formation of Schiff base ligand. A Strong band, typical of **ν(C=N)** stretching, was detected at 1625 cm^1 in the spectra of complex. The observed shift in the **ν(C=N) stretch after complexation** confirms the formation of a coordinate bond from imine N-atoms to the metal ion (Fig. 3S; ESI). The peaks corresponding to the ring stretching frequencies $\nu_{(C=C)}$ and $\nu_{(C=N),}$ at 1504**cm^{-1}**, 1419 cm^{-1}, of free phen were shifted to higher frequencies upon complexation, 1514**cm^{-1}**, 1421 **cm^{-1},** for the complex. suggesting the coordination

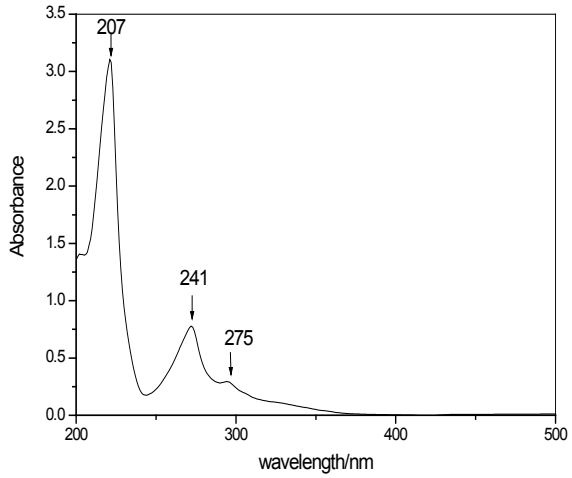


Fig. 3S. Electronic absorption spectra of [Cu(FDIB) Phen]$^{+2}$ complex

of the heterocyclic Nitrogen atoms to the metal ion. The characteristic out of plane hydrogen bending modes of free phen observed at 854 **cm^{-1}**, 734 **cm^{-1},** were shifted to 848 **cm^{-1}**, 719 **cm^{-1}** for complex upon metalcomplexation. The non- ligand peaks at and at 492**cm^{-1},** for complex was assigned to **ν (Cu-N)** stretching vibrations respectively **[20].**

Uv-Vis spectra

Uv-vis absorption spectra of the complex in pure MeOH (Fig. 4S; ESI), shows a broad band centered at 572 nm (17,482 cm^{-1}) corresponding to (^{2}B$_1$g→^{2}Eg transition) which is consistent with a distorted square planar geometry, around Cu(II) complexes [21]. In the uv region, band at 275 nm (36,363 cm $^{-1}$), for the complex is due to n→π* transitions of azomethine (C=N) function of Schiff base were assigned to LMCT transitions and the

remaining bands in the Uv region (200-270 nm) were assigned to the π-π* transitions of intra ligand & coordinated 1,10 phen.

Magnetic Moment

The magnetic moment values of the metal complex at room temperature is 1.82 BM which indicates the monomeric and paramagnetic nature of Cu(II) complexes with spin (1/2) system [22].

Molecular Structures and Molecular modeling studies

The minimum-energy structure of the complex was determined with semi empirical PM3 Hamiltonian as implemented in HYPERCHEM 8.0 software program package. The optimized structures are presented in Fig: 1. In the absence of crystal structure for the

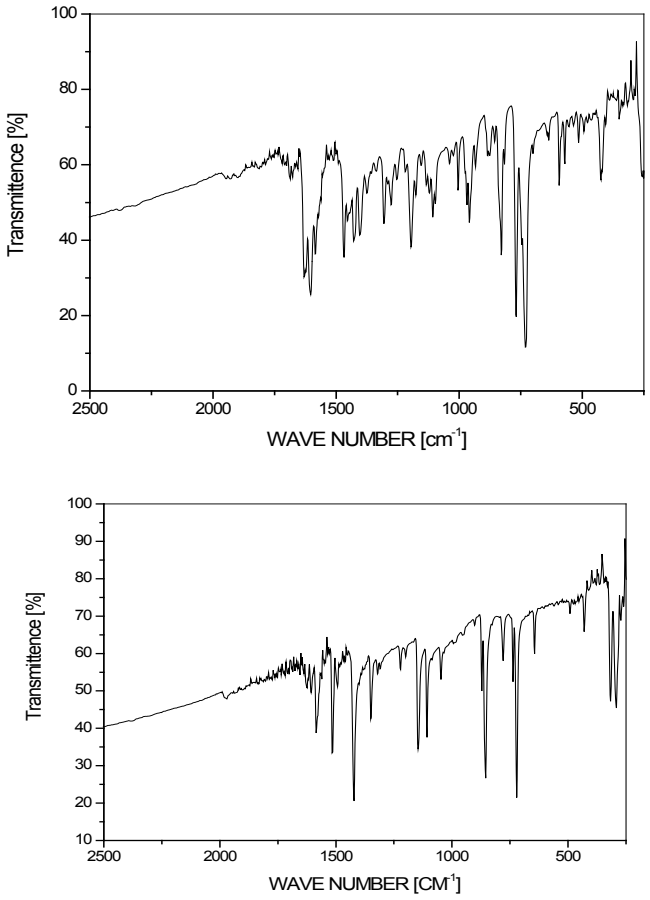


Fig. 4S. IR Spectra of the ligand FDIB **(1)**
IR Spectra of [Cu(FDIB) Phen]$^{+2}$ complex **(2)**

complex, the structure is proposed based on the molecular modelling studies. The conclusions are based on comparison of parameters i.e. bond lengths, bond angles and energy. They are presented in Table 1.

Table 1: Bond Angles (^{0}) for [Cu(FDIB) (phen)]$^{+2}$

[Cu(FDIB) (phen)]$^{+2}$
N_1-Cu-N_2 = 86.97
N_1-Cu-N_4 = 91.14
N_2-Cu-N_3 = 94.89
N_4-Cu-N_3 = 88.54

Thermo Gravimetric Analysis (TGA)

To examine the presence of water molecules the complex subjected to thermo gravimetric analysis. It is well known that water molecules present in the crystal lattice of the complex are of two types, lattice water and coordinated water. The lattice water will be lost at low temperatures (50-120 ^{0}C) and coordinated water molecules at high temperatures (120-250 ^{0}C) [23]. In the present complex (Fig. 5S; ESI) there was no weight loss corresponding to the water mol-

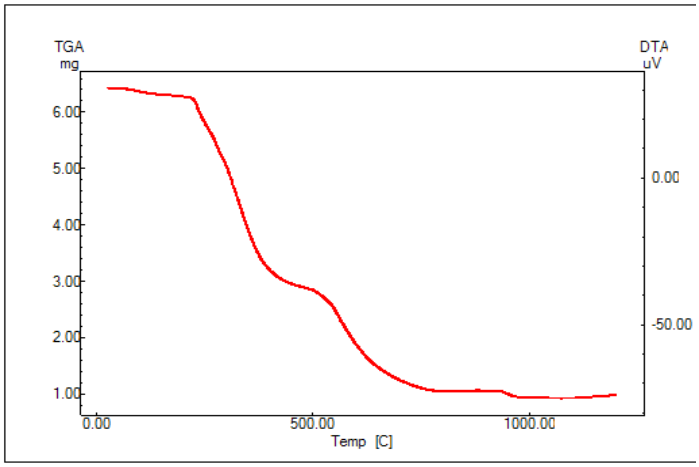
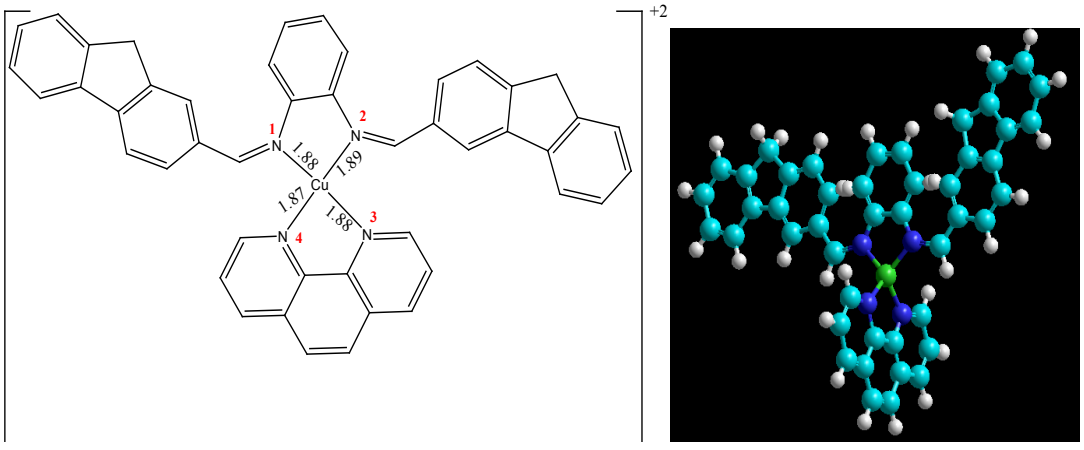


Fig. 5S. TGA Spectrum of [Cu(FDIB) Phen]$^{+2}$ complex.



TOTAL ENERGY: -9612.8589 Kcal/mol

[Cu (FDIB) (phen)]$^{+2}$

Fig. 1. Energy-minimized molecular structures with relevant bond lengths, bond angles and energy for the complex [Cu(FDIB) Phen]$^{+2}$.

ecules in the temperature ranges mentioned above. This indicates that the complex does not have water molecules neither in the lattice nor in the coordination sphere. Based on the electronic spectra and TGA data a distorted square planar geometry was conformed for the complex.

Cytotoxicity against HeLa cell lines

The *in vitro* cytotoxic activity of the complex was investigated against human cervical carcinoma (HeLa) cells by using the MTT assay for cell viability (Fig.2). The HeLa cells were incubated with an increasing concentration (5-20 μM) of compex separately for 24 h in comparison with the well known drug Doxorubicin.The complex was found to be active against cancer cell lines and their IC_{50} values (Table 2) were obtained by plotting the cell viability against the concentrations of the complex the results reveal that both the complex and the drug have potent cytotoxic effects against the cancer cell lines, and the inhibitory rate of complex is higher than that of the drug Doxorubicin, this confirms that the statistical significance between the two groups is due to the strong hydrophobic forces interaction of the complex and chelation of the ligand with the copper(II) ion are the solely responsible factors observed for cytotoxic property of the complex. The cytotoxicity of the complex

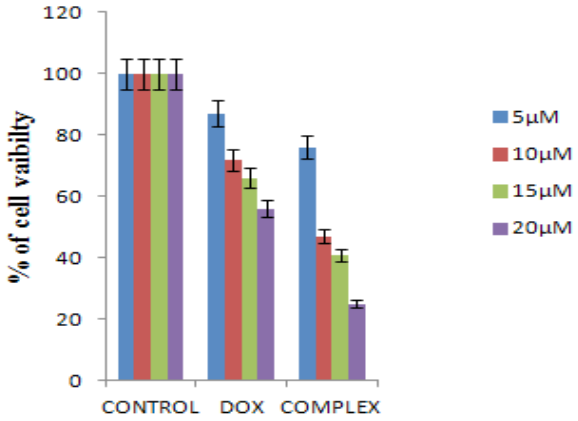


Fig.2. In vitro cell viability of HeLa cells.

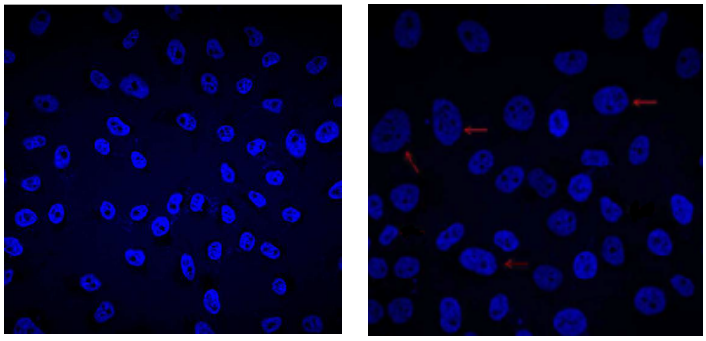
was found to be concentration dependent and the cell viability decreased with increasing concentration of copper(II) complex [24-26]. This is a clear indication that the copper complex acts very specifically on cancer cells.

Table 2: IC_{50} (μM) values for [Cu (FDIB) Phen] $^{+2}$ with HeLa cell lines

COMPLEX	IC_{50} (μM)
DOXORUBICIN	4.5
[Cu(FDIB) Phen]$^{+2}$	2.3

Confocal Microscopy

In order to assess the intracellular localization of the complex inside the HeLa cancer cells, confocal microscopic experiments were performed as an initial investigation of its applicability as a fluorescent probe for live cell applications. The molecular mechanism of cell death studied by treating the HeLa cancer cells with 10 μM complex concentration for 24 h and then observed their cytological changes adopting DAPI staining. The representative morphological changes observed for copper complex such as cytoplasmic blebbing, nuclear swelling and late apoptosis indication of dot-like chromatin condensation are shown in Fig.3. The numbers of abnormal cells are increased at 10 μM concentration of the complex. Hence the live imaging studies revealed that Cu(II) complex act as a future potential chemotherapeutic agent. The present work has thus demonstrated that the ternary complex of copper (II) with nitrogen donors enhance apoptosis in cancer cells [27] which could be a useful strategy for designing more effective drugs.



Control Untreated Treated with [Cu(FDIB)(phen)]$^{+2}$

Fig.3. Nuclear localization studies of Cu(II) complex in HeLa cells, the treatment concentration is 10 μM, cells were stained with DAPI.

Conclusions

A New Cu(II) Schiff base complex was synthesized , characterized and its biological properties were studied. The complex exhibits distorted square planar geometry. From the *in vitro* cytotoxicity studies it was inferred that the complex exhibits higher activity than thestandard drug doxorubicin and this may be due to the strong chelation and increased electron delocalization in six and five membered chelate rings, which in turn increases the permeability of the copper ion into the cells. The results obtained from the present copper (II) complex is of importance for the development of metal based agents for anticancer applications.

Acknowledgements

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Appendix A. Supplementary material (SI)

ESI Mass spectra (Fig.S1), ^{1}H NMR spectra of the ligand (Fig.S2), Electronic absorption spectra of the complex in MeOH (Fig.S3). IR spectra (Fig.S4), and TGA specta of the complex (Fig.S5).

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