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Synthesis, Characterization and *in vitro* Cytotoxicity Studies of Pentadentate Ligand and its Copper(II) Complexes

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A pentadentate ligand was synthesized by reaction of 4-*t*-butyl-2,6-*bis*(chloromethyl)phenol and isonicotinic hydrazide. The ligand was characterized by UV-visible, FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectrascopic techniques. The above ligand was coordinated with various copper precursors to form corresponding copper(II) complexes. The complexes were characterized by UV-visible, FT-IR, molar conductivity and cyclic voltammetry. The *in vitro* cytotoxicity of the ligand and its copper complexes were studied against Ehrlich ascites carcinoma (EAC) cells.

Keywords: Isonicotinic hydrazide, Pentadentate ligand, Copper(II) complexes, *in vitro* cytotoxicity, Ehrlich ascites carcinoma cells.

INTRODUCTION

Transition metals are significant in living organisms to provide appropriate concentrations of metals for use in metalloproteins or cofactors. The redox properties of the metals are important in many of the reactions. Copper and iron proteins participate in many of the biological reactions like binding of dioxygen, *e.g.*, hemocyanin (Cu), hemerythrin (Fe) and hemoglobin (Fe) [1], activation of dioxygen in the synthesis of the hormone epinephrine, *e.g.*, dopamine hydroxylase (Cu), tyrosinases (Cu) and catechol dioxygenases (Fe) [2,3] electron transfer, *e.g.*, plastocyanins (Cu), ferredoxins and c-type cytochromes (Fe) [4] dismutation of superoxide by Cu or Fe as the redox-active metal (superoxide dismutases) [5].

Platinum and ruthenium are explored as potential anticancer agents. However, there is an emerging curiosity in the synthesis of low-cost first-row metal coordination compounds as efficient DNA binders with potential cytotoxic activity. Transition metal complexes exhibit a well-defined coordination geometries and distinct electrochemical or photophysical properties, thereby increasing the functionality of the binding agent. Redox-active metals generally form reactive oxygen species (ROS) and this ROS can be used to induce DNA cleavage [6-8]. The more donor atoms by which a molecule is bound to a metal ion, the stronger will be the assembly. A ligand of such kind was synthesized using 4-*t*-butyl-2,6-*bis*(chloromethyl)phenol and isonicotinic hydrazide which was reacted with copper(II) precursors to form copper(II) complexes.

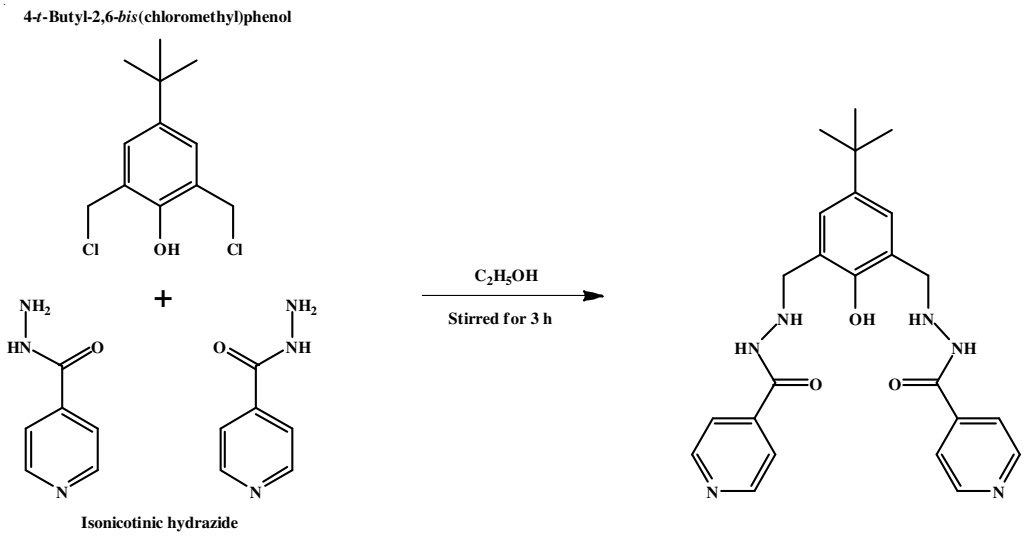
EXPERIMENTAL

All the chemicals and solvents were purchased from SD-Fine Chemicals. UV-visible spectra were recorded using Systronics spectrophotometer operating in the range of 200-800 nm. FT-IR spectra were obtained in Shimadzu IR-Affinity-I spectrometer and sample pellets were prepared using KBr. ^{1}H NMR and ^{13}C NMR spectrum of ligand was recorded from Bruker 400 MHz spectrometer. Conductance of complexes was recorded using Elico conductometer. Cyclic voltammetry was done in HCH Instruments.

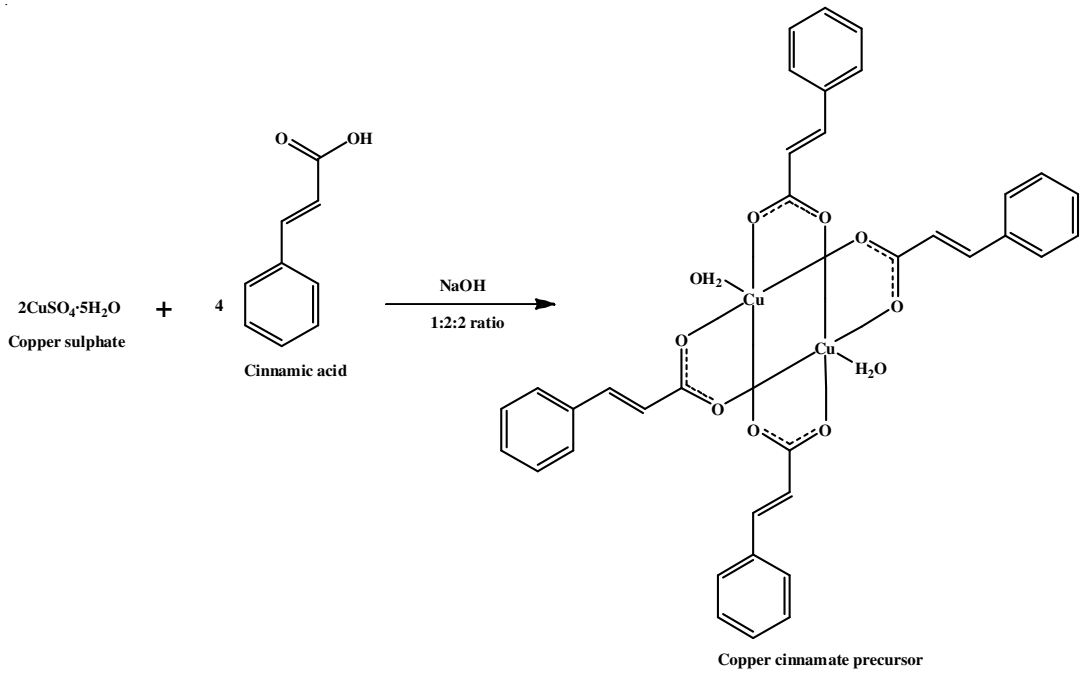
Synthesis of ligand: 4-*t*-Butyl-2,6-*bis*(chloromethyl)phenol was treated with isonicotinic hydrazide in ethanol in 1:2 ratio [9,10]. A yellow solid was obtained, filtered and recrystallized in ethanol (**Scheme-I**).

Synthesis of copper precursors: Cinnamic acid was dissolved in hot water by heating at above room temperature. To this sodium hydroxide was added and stirred in a magnetic stirrer. A solution of copper sulphate ($CuSO_4 \cdot 5H_2O$) in water was slowly added to the mixture in 1:2 ratio [11-13]. A light blue solid obtained, filtered and washed with water (**Scheme-II**).

Synthesis of copper complexes: The above ligand was dissolved in ethanol. Sodium hydroxide was added and stirred for few minutes. A solution of copper precursors in ethanol was slowly added and sodium perchlorate was added in 1:1:1:2 and continued stirring for 5 h [14-16]. A dark green solid was obtained and filtered (**Scheme-III**). Similarly, copper succinate (C6P2) and copper crotonate (C6P3) complexes were synthesized.

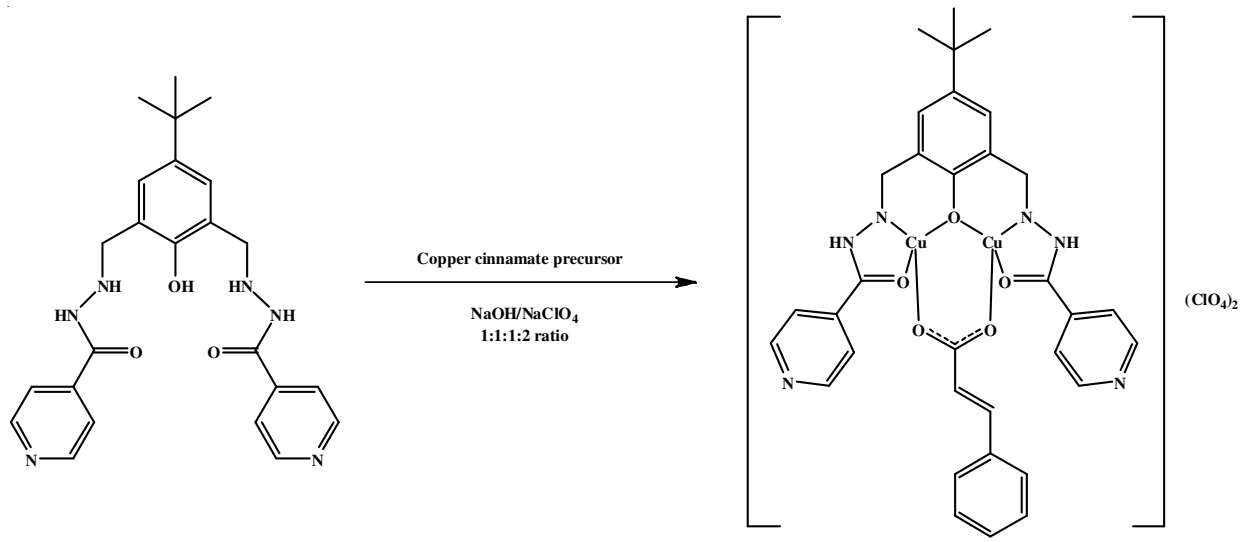


Scheme-I: Synthesis of ligand



Copper cinnamate precursor

Scheme-II: Synthesis of copper precursor



Scheme-III: Synthesis of copper complex (C6P1)

***in vitro* Cytotoxicity study:** The ligand and its Cu(II) complexes were studied for short term *in vitro* cytotoxicity using Ehrlich ascites carcinoma (EAC) cells. The tumor cells aspirated from the peritonial cavity of tumor bearing mice were washed thrice with phosphate buffered saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension (1×10^6 cells in 0.1 mL) was added to tube containing various concentrations of the test compound and the volume was made up to 1 mL using phosphate buffered saline (PBS) [17-20]. Control tube contains only cell suspension. These assay mixture were incubated for 3 h at 37 °C. Further cell suspension was mixed with 0.1 mL of 1 % trypan blue and kept for 2 to 3 min and loaded on hemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The number of stained and unstained cells was counted separately [21,22].

RESULTS AND DISCUSSION

A pentadentate ligand (L6) was characterized by UV-visible, FT-IR, ^{1}H NMR, ^{13}C NMR spectral studies. The UV-visible spectrum of the ligand (Fig. 1) shows π-π* transition at 291 nm, n-π* transition at 329 nm and charge transfer at 387 nm. The FT-IR spectrum (Fig. 2) shows OH stretching at 3329 cm^{-1}, NH stretching at 3247 cm^{-1}, aromatic CH stretching at 2958 cm^{-1}, C=O stretching at 1651 cm^{-1}, CO stretching at 1207 cm^{-1}. The ^{1}H NMR spectrum (Fig. 3) shows aromatic protons adjacent to nitrogen at 9.006, aromatic protons away from nitrogen at 7.804, methylene proton at 4.575. ^{13}C NMR spectrum (Fig. 4) shows hydroxyl carbon at 147.3, *t*-butyl carbon at 31.34, carbonyl carbon at 164.42, aromatic carbon adjacent to nitrogen at 150.27, aromatic protons away from nitrogen at 121.91. The mass spectrum (Fig. 5) shows peaks at *m/e* values 448, 269, 161, *etc.*, shows the presence of molecular ion peak and fragmented ion peaks [23,24].

The copper complexes were characterized by UV-visible, FT-IR spectral studies, conductivity measurements and cyclic voltammetry.

UV-visible: The UV-visible spectra of the complexes were recorded in DMSO solution in the wavelength range 200-800 nm. The band at 291 nm is due to π-π* transition of the benzene ring present in the ligand and it was shifted to higher wavelength (red shift) upon complexation and the band was observed around 300 nm for complexes. Similarly, the band

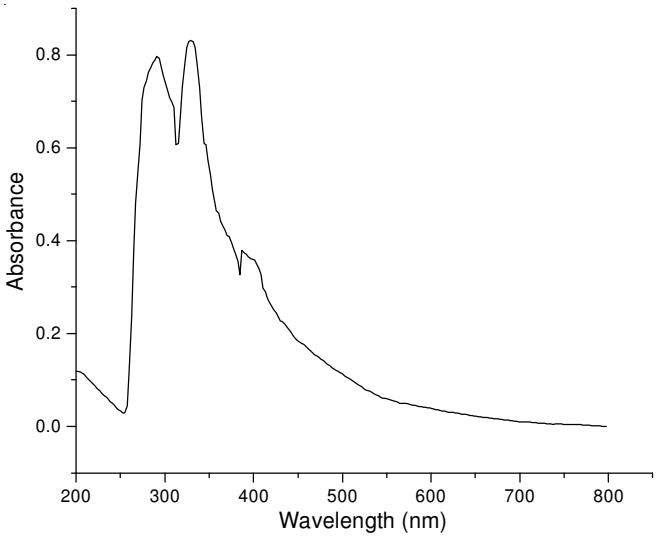


Fig. 1. UV-visible spectrum of ligand

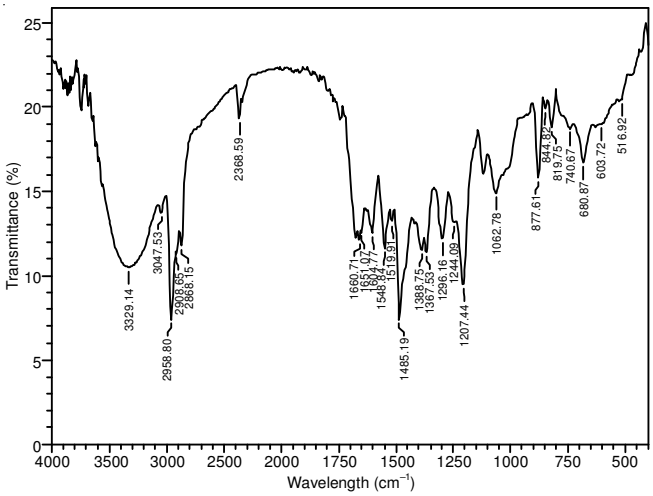


Fig. 2. FT-IR spectrum of ligand

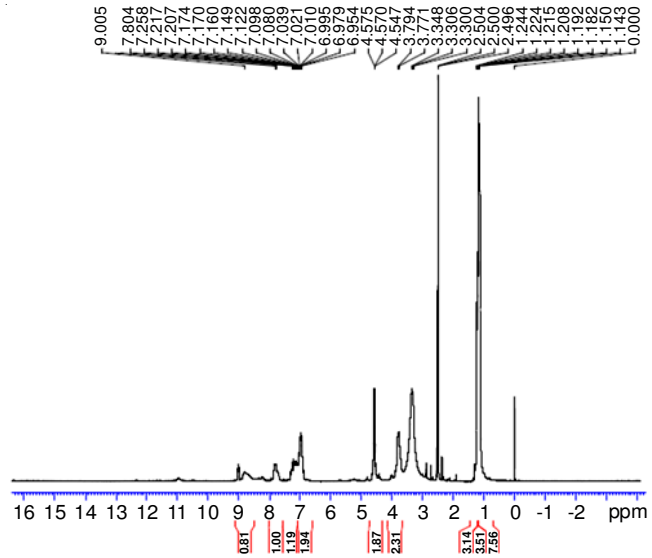


Fig. 3. ^{1}H NMR spectrum of ligand

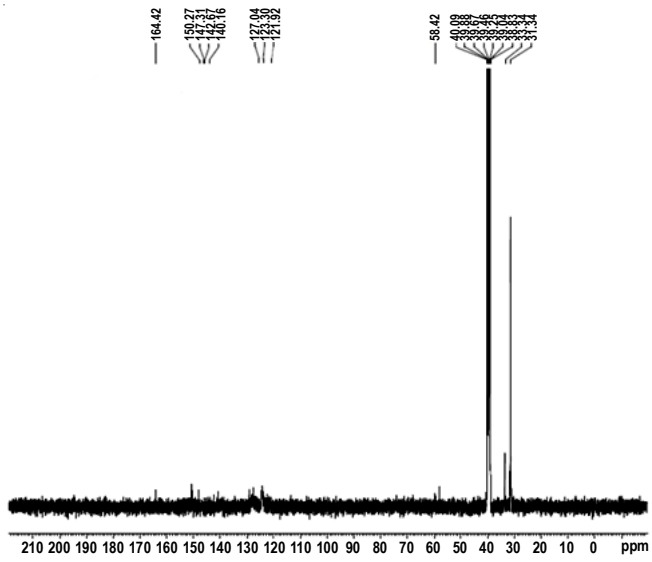


Fig. 4. ^{13}C NMR spectrum of ligand

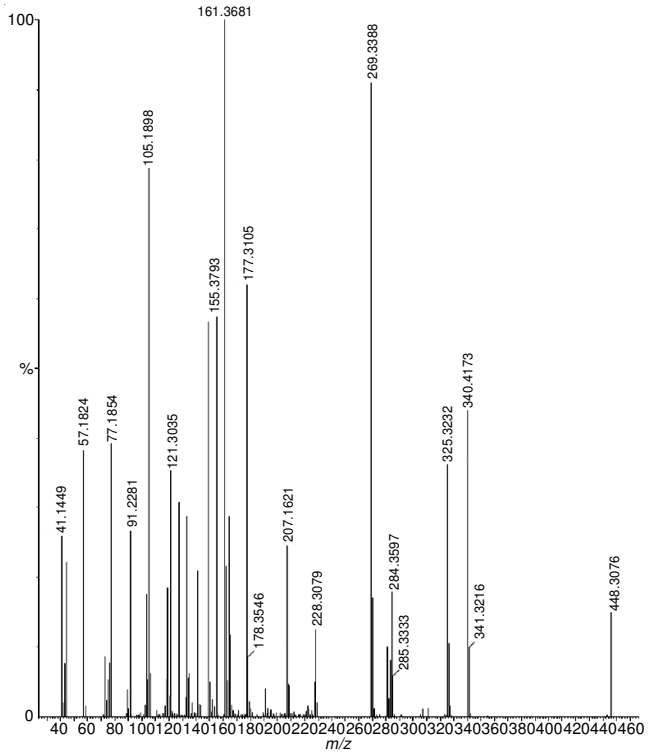


Fig. 5. Mass spectrum of ligand

at 329 nm is due to n-π* transition of nitrogen in the ligand and it was shifted to higher wavelength (red shift) upon complexation and the band was observed around 350 nm for complexes (Fig. 6). The band around 580 nm is due to *d-d* transition (Table-1) [25].

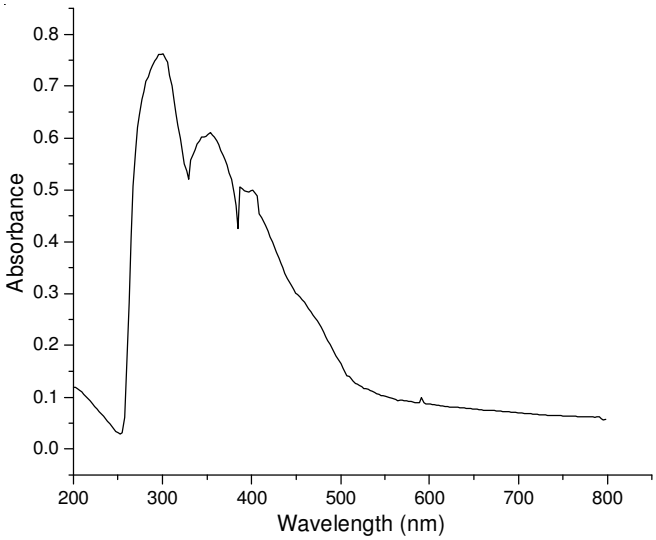


Fig. 6. UV-visible spectrum of complex (C6P1)

TABLE-1
UV-VISIBLE SPECTRAL DATA OF LIGAND AND COMPLEXES

Sample code	λ_{max} value (nm)			
	π-π* transition	n-π* transition	Charge transfer	*d-d* Transition
L6	291	329	387	-
C6P1	296	358	399	591
C6P2	298	346	401	567
C6P3	300	348	401	584

FT-IR spectroscopy: The FT-IR spectrum (Fig. 7) shows OH stretching around 3410 cm^{-1}, NH stretching around 3310 cm^{-1}, CH stretching around 2925 cm^{-1}, OCO stretching around 1260 cm^{-1}, M-N stretching around 550 cm^{-1}, M-O stretching around 450 cm^{-1}. The other key bands of copper complexes are given in Table-2.

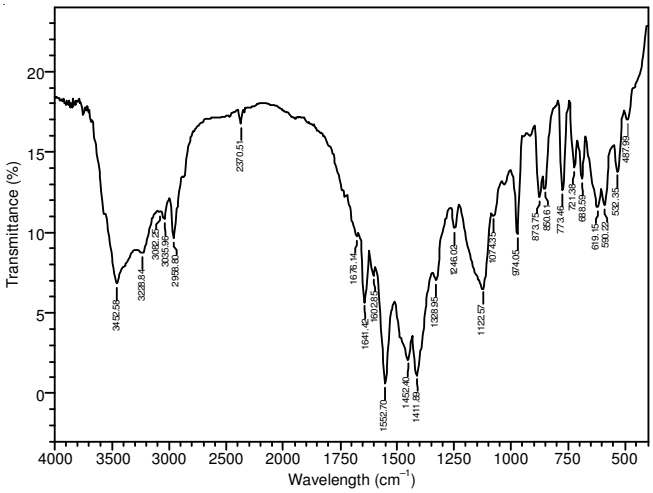


Fig. 7. FT-IR spectrum of complex C6P1

TABLE-2
FT-IR SPECTRAL DATA OF LIGAND AND COMPLEXES

Sample code	Key infrared bands (cm^{-1})						
	OH	NH	Ar-CH	C=O	ClO_4^-	M-N	M-O
L6	3329	3247	2958	1651	–	–	–
C6P1	3452	3228	2958	1641	1122	773	532
C6P2	3444	3239	2960	1624	1118	808	534
C6P3	3436	3243	2939	1589	1112	808	570

Conductance measurements: The molar conductance of the complexes were recorded in dimethyl formamide (DMF). The molar conductance values shows that complexes are 1:2 electrolyte in nature (Table-3).

TABLE-3
MOLAR CONDUCTANCE VALUES OF THE COMPLEXES

Sample code	Molar conductance (Mho cm^2 mol^{-1})
C6P1	124
C6P2	137
C6P3	131

Cyclic voltammetry: The cyclic voltammetry (Fig. 8) reveals that all the complexes exhibit a one electron transfer and the complexes are quasi reversible (Table-4). The electron movement is sluggish.

***in vitro* Cytotoxicity study:** The copper complexes showed the significant increase in activity against Ehrlich ascites carcinoma (EAC) cell (Table-5) when compared to the

TABLE-4
CYCLIC VOLTAMMETRIC DATA OF THE COMPLEXES

Sample code	IPa^{e-6}	IPc^{e-6}	IPa/IPc
C6P1	2.501	4.669	0.535
	4.499	5.669	0.794
C6P2	1.808	2.100	0.861
	3.564	5.872	0.607
C6P3	1.716	2.007	0.855
	1.675	2.582	0.649

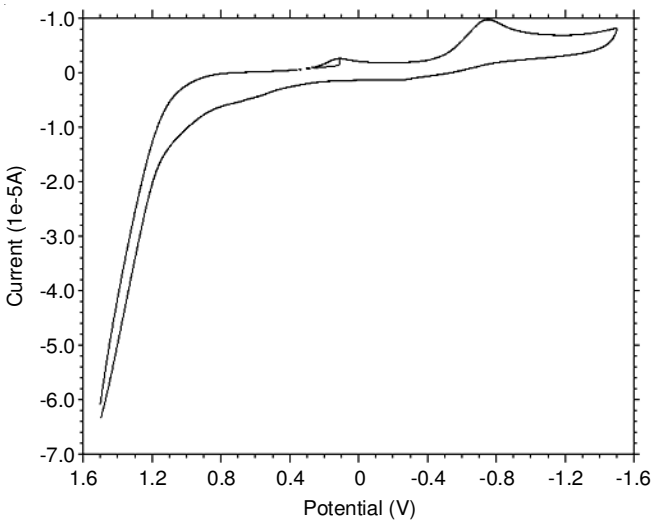


Fig. 8. Cyclic voltammetry of complex C6P1

TABLE-5
CYTOTOXIC ACTIVITY OF LIGAND AND COMPLEXS

Drug concentration (µg/mL)	Percent cell death for EAC			
	L6	C6P1	C6P2	C6P3
200	30	72	70	75
100	17	60	56	58
50	10	52	49	40
20	0	38	32	36
10	0	20	18	25

ligand molecule. In drug concentration 200 µg, about 75 % of the tumor cells were killed by the complexes.

Conclusion

A multidentate ligand was synthesized by using 4-*t*-butyl-2,6-*bis*-(chloromethyl) phenol and isonicotinic hydrazide. It was characterized by UV-visible, FT-IR, ^{1}H NMR, ^{13}C NMR spectral studies. The above ligand was coordinated with various copper precursors to form corresponding copper complexes. The complexes were characterized by UV-visible, FT-IR, conductivity measurements and cyclic voltammetry. The molar conductance values show that all the complexes are found to be 1:2 electrolyte. *In vitro* cytotoxicity study shows that all the complexes were showing activity against Ehrlich ascites carcinoma cell.

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