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ORIGINAL ARTICLE

Synthesis, spectroscopic and anti tumor studies on copper(II) complex of orthohydroxypropiophenoneisonicotinoylhydrazone

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Abstract Orthohydroxypropiophenoneisonicotinoylhydrazone ligand(L) has been synthesized from orthohydroxypropiophenone and isonicotinicacid hydrazide. The Cu(II) complex is prepared and structurally characterized. The structure of the ligand(L) is proposed based on elemental analysis, IR and mass spectra and Cu(II) complex is characterized from the studies of electronic spectra, EPR spectra, powder X-ray diffraction (XRD) and scanning electron microscopy (SEM). On the basis of electronic and EPR studies, rhombically distorted octahedral environment has been proposed for the Cu(II) ion. The powder X-ray diffraction data indicate triclinic structure with the unit cell parameters: a = 9.4734 Å, b = 6.1588 Å, c = 3.9254 Å, and V = 195.6 Å^3 for the complex. The ligand and Cu(II) complex have been tested for *in vitro* antibacterial, antioxidant and cytotoxic activities. The results reveal that the Cu(II) complex exhibits greater activity than the free ligand.

1. Introduction

Interest in the study of hydrazones has been growing because of their antimicrobial, antituberculosis and antitumour activities (Chohan and Sheazi, 1999; Jayabalakrishnan and Natarajan, 2001; Jeeworth et al., 2000; Zhi-hong et al., 2008). Hydrazones play an important role in inorganic chemistry, as they easily form stable complexes with most of the transition metal ions. The development in the field of bioinorganic chemistry has increased the interest in hydrazone complexes, since it has been recognized that many of these complexes

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may serve as models for biologically important species (Wan-yi et al., 2001). Coordination compounds derived from aryl hydrazones have been reported to act as enzyme inhibitors and are useful due to their pharmacological applications (Narang and Singh, 1993; Savanini et al., 2002; Ochiai and Ei-ichiro, 1977). Hydrazones possessing an azomethine —NHN=CH— proton constitute an important class of compounds for new drug development. Therefore, many researchers have synthesized these compounds as target structures and evaluated their biological activities. Hydrazones and their metal complexes are biologically very active compounds. Maria et al. (2007) have reported the antibacterial activities of cobalt(II), copper(II), nickel(II), and zinc(II) complexes of 2-thiophenecarbonyl hydrazone and isonicotinoylhydrazone of 3-(N-methyl)isatin-2-thiophene carbonyl metal complexes exhibit a strong inhibition on the growth of *Haemophilus infrenza* and good antibacterial activity toward *Bacillus subtilis*.

2. Experimental

2.1. Materials

All the chemicals used are of analytical grade. Organic chemicals, such as thiobarbituric acid (TBA), trichloroacetic acid (TCA), α-tocopherol, butylated hydroxy toulene (BHT), 1,1-diphenyl-2-picryl hydrazyl (DPPH), isonicotinicacid hydrazide, and dimethyl formamide (DMF) are procured from Sigma–Aldrich and all metal salts are procured from E. Merck.

2.2. General procedures

The IR spectra of the compounds are recorded on a Nicolet FT-IR 560 Magna spectrometer using KBr (Pellet). Mass spectra are recorded in a Quattro LC, Micro Mass spectrometry. Elemental analysis is obtained from a Vario-Micro Qub elementar analyzer. The electronic spectra of the complexes are recorded on a Perkin Elmer UV/VIS Lambda 950. EPR spectra are recorded on an EPR spectrometer (JEOL FE-1X) operating in the X-band frequencies with a modulation frequency of 100 kHz. Samples of 100 mg are taken in a quartz tube for EPR measurements. The magnetic field is scanned from 2200 to 4200 G, with a scan speed of 250 G min^{-1}. Absorbance is measured using Systronics UV–VIS spectrometer-117. Centrifugation is done using REMI centrifuge. A digital pH meter (model L1-10 Elico, India) is used for measuring pH. X-ray diffractometer (PHILIPSPW3710) using CuK_{α} (1.5418 Å) radiation operated at 45 kV and 25 mA is used in X-ray investigations.

2.3. Synthesis of free ligand(L)

Approximately 15 ml (0.1 M) of orthohydroxypropiophenone is dissolved in 150 ml of methanol and 0.1 M (13.7 g) of isonicotinicacid hydrazide is dissolved in 150 ml of water. The two solutions are taken in a 500 ml round bottomed flask, two pellets of sodium hydroxide are added and refluxed for two hours on a water bath. The resultant product Orthohydroxy propiophenoneisonicotinoylhydrazone(L) is filtered, washed with water and methanol. It is recrystallised using aqueous methanol and dried.

2.4. Synthesis of metal complex

A methanolic solution of copper(II) chloride (0.001 M and 25 ml MeOH) is added to a methanol solution of free ligand (0.001 M and 25 ml MeOH). The reaction mixture is refluxed on water bath for 3–4 h at 70 °C. On cooling the contents, the colored complex is precipitated, filtered, washed with 50% ethanol and dried. The purity of the complex is checked by TLC.

2.5. Antibacterial screening

In vitro antibacterial screening is performed by the agar disc diffusion method (Bauer et al., 1966; Sheikh et al., 2004). The bacterial species used in the screening are gram-negative bacteria such as *Klebsiella pneumoniae* and *Escherichia coli* and gram-positive bacteria such as *Staphylococcus aureus* and *B. subtilis*. Stock cultures of the test bacterial species are maintained on nutrient agar media (Hi-media laboratories, Mumbai) by sub culturing in Petri dishes. The media are prepared by adding the components as per manufacturer's instructions and sterilized in the autoclave at 121 °C and atmospheric pressure for 15 min. Each medium is cooled to 45–60 °C and 20 ml of it is poured into a Petri dish and allowed to solidify. After solidification, Petri plates with media are spread with 1.0 ml of bacterial suspension prepared in sterile distilled water. The wells are bored with cork borer and the agar plugs are removed. To each agar well, 100 ml of the compound reconstituted in DMF of concentration 1.0 mg/ml is added. DMF is used as a negative control and in a similar way, antibiotics, such as ampicillin and tetracycline are used as positive control standards. All the plates are incubated at 37 °C for 24 h and they are observed for the growth inhibition zones. The presence of clear zones around the wells indicate that both the ligand and complex are active. The diameter of zone of inhibition is calculated in millimeters. The well diameter is deducted from the zone diameter to get the actual zone of inhibition diameter and the values are tabulated.

2.6. DPPH scavenging activity

The principle for the reduction in DPPH free radicals is that the antioxidant reacts with stable free radical DPPH and converts it to 1,1-diphenyl-2-picrylhydrazine. The ability to scavenge the stable free radical DPPH is measured by a decrease in the absorbance at 517 nm. Solutions of ligand and Cu(II) complex at 100 μM concentration are added to 100 μM DPPH and kept in ethanol tubes. The tubes are kept at ambient temperature for 20 min and absorbances are measured at 517 nm. For positive control, α-tocopherol is used (Balige et al., 2004). These measurements are run in triplicate. The percentage of scavenging activity is calculated as follows:

$$\text{Scavenging activity } (\%) = [(A_{\text{DPPH}} - A_{\text{TEST}})/A_{\text{DPPH}}] \times 100$$

where A_{DPPH} is the absorbance of DPPH without test sample (control) and A_{TEST} is the absorbance of DPPH in the presence of test sample.

2.7. Inhibition of lipid peroxidation in rat brain homogenate

2.7.1. Preparation of rat brain homogenate

For the present study, Albino Wistar rats (180–200 g) are selected. Prior to decapitation and removal of the brain, the

animals are anesthetized with ether and perfused transcardially with ice-cold normal saline to prevent contamination of the brain tissue with blood. The collected tissues are weighed and their homogenates (10% w/v) are prepared in 0.15 M KCl and centrifuged at 800 rpm for 10 min. The supernatants are used immediately for the study (Bharathi et al., 2008).

2.7.2. Iron(III) induced lipid peroxidation

The incubation mixtures contain a final volume of 1.5 ml brain homogenate (0.5 ml) of 10% w/v), KCl (0.15 M) and ethanol (10 µl) or test compound dissolved in ethanol. Peroxidation is initiated by adding ferric chloride (100 µM) to give the final concentration stated. After incubation for 20 min at 37 °C, reactions are stopped by adding 2 ml of ice-cold 0.25 M HCl containing 15% trichloroaceticacid (TCA), 0.38%. thiobarbituric acid (TBA) and 0.05% butylated hydroxy toulene (BHT). The samples are heated at 80 °C for 15 min, cooled and centrifuged at 1000 rpm for 10 min. The absorbances of the supernatant solutions are measured at 532 nm. Percentage inhibition of thiobarbituricacid reactive substances (TBARS) formed by test compounds is calculated by comparing with the control. Iron(III) and KCl solutions are prepared afresh in distilled water. Since most buffers trap hydroxyl radical or interfere with iron conversion, the reactions are carried out in unbuffered 0.15 M KCl solution (Jayan and Rao, 1994; Braughler et al., 1988).

The inhibition percentages of the selected ligand and Cu(II) complex are evaluated using lipid peroxidation method. The following formula is used in calculating inhibition percentages.

$$\text{Inhibition percentage} = [(A_{\text{CONT}} - A_{\text{TEST}})/A_{\text{CONT}}] \times 100$$

Here, A_{CONT} is the absorbance of the control reaction and A_{TEST} is the absorbance in the presence of the test sample.

2.8. Determination of cytotoxicity of compounds to EAC cells tryphan blue exclusion method (cell viability test)

In vitro short-term cytotoxic activity of drug is determined using EAC cells. The EAC cells that are collected from the animal peritoneum by aspiration are washed repeatedly with PBS to free it from blood. After checking the viability of the cells in a hemocytometer, the cells (1×10^6) in 0.1 ml PBS, 0.01 ml of various concentrations of test compounds (10–100 µg/ml) are made (the test compounds are dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO not exceeding 0.1% of the total volume) and phosphate buffered saline (0.1 mole/l, pH = 7) in a total volume of 0.9 ml are incubated in clean sterile tubes for 3 h at 37 °C. The control tube has 10 µl of solvent. The final volume is made up to 0.9 ml with PBS. To each 100 µl of tryphan blue solution is added. The live (without stain) and dead (with blue stain) cells are counted using hemocytometer and percentage of cell death was calculated using the formula:

$$\%\ \text{Cytotoxicity} = 100 \times (T_{\text{dead}} - C_{\text{dead}})/T_{\text{tot}}$$

where, T_{dead} is the no. of dead cells in the treated group, C_{dead} is that in the control group, and T_{tot} is the total number of dead and live cells in the test compound treated in the group. Cisplatin is used Cisplatin was used as a standard (Devi et al., 1994).

3. Results and discussion

3.1. Characterization of ligand

It is a pale yellow crystalline solid and melts at 248–250 °C. The yield is about 86%. The ligand is analyzed by IR and mass spectroscopy. The IR spectrum of ligand exhibits absorption bands around 1600 cm^{-1} (C═N), 1680 cm^{-1} (C═O) and 3140 cm^{-1}(—NH). Elemental analysis gives C 66.39%, H 5.43%, N 15.34% Calculated C 66.91% H 5.58% N 15.61%. The mass spectrum of ligand (Fig. 1) shows a molecular ion (M^+) peak at m/z value of 269, corresponding to the species $C_{15}H_{15}N_3O_2$. These spectral data confirm the proposed formula of the ligand $C_{15}H_{15}N_3O_2$. These data confirm the formation of ligand Orthohydroxypropiophenoneisonicotinoylhydrazone(L) which is presented in Scheme. 1.

3.2. Electron paramagnetic spectrum of the Cu(II) complex

The EPR spectrum of the Cu(II) complex recorded at room temperature with polycrystalline sample is shown in (Fig. 2), It consists of three lines each in the low field (g_1), mid field (g_2) and high field (g_3) regions. From the peaks' positions and their separations, the g-values evaluated are $g_1 = 2.444$, $g_2 = 2.178$, and $g_3 = 2.031$. From the data, information on the electronic ground state of Cu(II) can easily be inferred. For the g-values $g_1 > g_2 > g_3$, if the quantity $\{R = (g_2 - g_3)/(g_1 - g_2)\}$ is greater than one, the ground state is predominantly $^2A_1$ (d_z^2) and if R is less than unity, the ground state is $^2A_1(d_{x2-y2})$. In the present study the value R is found to be of the order 0.55, i.e. less than unity, indicating that the ground state is $^2A_1(d_{x2-y2})$.

3.3. Electronic spectrum of the Cu(II) complex

The electronic spectrum(optical absorption) of Cu(II) complex shown in (Fig. 3) exhibits four bands at 1284, 1119, 853 and 803 nm, characteristic of rhombic distortion with the general order of the energy levels as $^2A_1(d_{x2-y2}) < {}^2A_1(d_{z2}) < {}^2A_2(d_{xy}) < {}^2B_1(d_{xz}) < {}^2B_2(d_{yz})$. The optical absorption bands observed at room temperature are assigned as follows: 1284 nm (7788 cm^{-1}): $^2A_1(d_{x2-y2}) \rightarrow {}^2A_1(d_{z2})$, 1119 nm (8937 cm^{-1}): $^2A_1(d_{x2-y2}) \rightarrow {}^2A_2(d_{xy})$, 853 nm (11,723 cm^{-1}): $^2A_1(d_{x2-y2}) \rightarrow {}^2B_1(d_{XZ})$ and 803 nm (12,453 cm^{-1}): $^2A_1(d_{x2\text{-}y2}) \rightarrow {}^2B_2(d_{yz})$, respectively. These observations are in tune with those reported earlier and the bands are accordingly ascribed to Cu(II) complex in octahedral coordination with rhombic

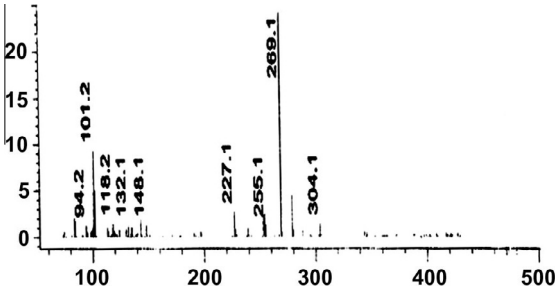
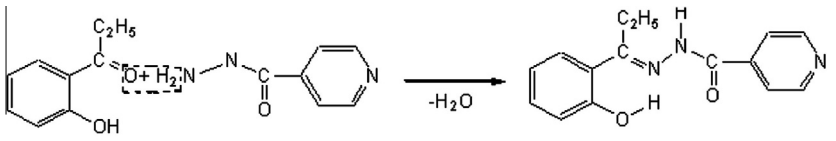


Figure 1 Mass spectrum of the ligand.



Orthohydroxypropiophenone Isonicotinicacid-hydrazide Orthohydroxypropiophenone-isonicotinoylhydrazone(L)

Scheme 1 Synthesis and structure of ligand.

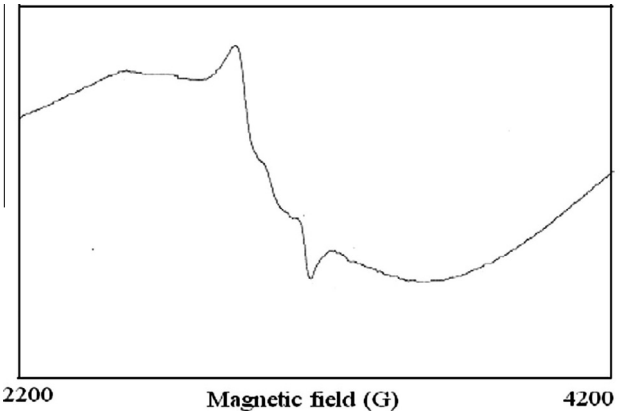
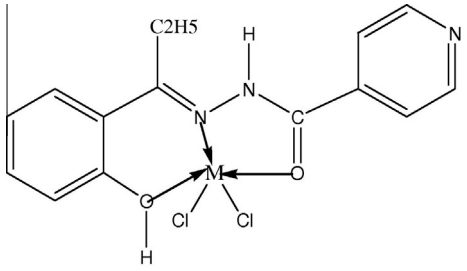


Figure 2 Powder X-band EPR spectrum of Cu(II) complex at room temperature (v = 9.205 GHz).



Scheme 2 The Proposed structure of Cu(II) complex.

(C_{2v}) distortion. Based on the spectral studies the following structure is proposed for the Cu(II) complex(Scheme. 2).

3.4. Surface morphological studies

Powder X-ray diffractograph of Cu(II) complex are depicted in Fig. 4. The observed and calculated diffraction data are given in Table 1. Using the trial and error method the unit cell parameters of Cu(II) complex are found to be a = 9.4734 Å, b = 6.1588 Å, c = 3.9254 Å, α = 109.497°, β = 108.040°, γ = 99.642° and unit cell volume V = 195.6 Å^3. These data of the Cu(II) complex support the triclinic system. The observed X-ray pattern of the free ligand sample studied in the present investigation indicates amorphous nature. To evaluate the crystallite size of the synthesized Cu(II) complex, D is determined using Debye–Scherer formula (Warren, 1990) given by

$$D = \frac{0.94\lambda}{\beta \cos\theta}$$

where β is the full width at half maximum of the predominant peak and θ is the diffraction angle and λ is the wave-

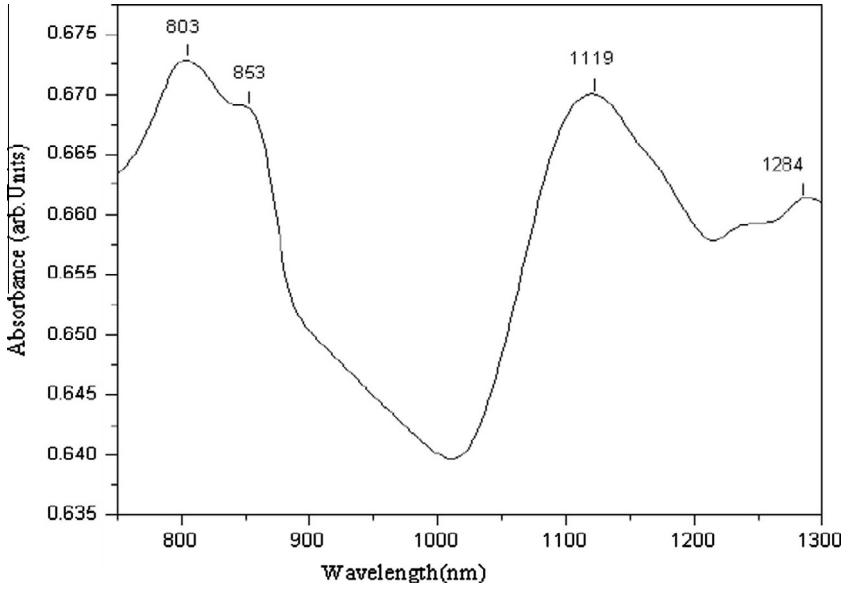


Figure 3 Electronic spectrum (nm) of Cu(II) complex.

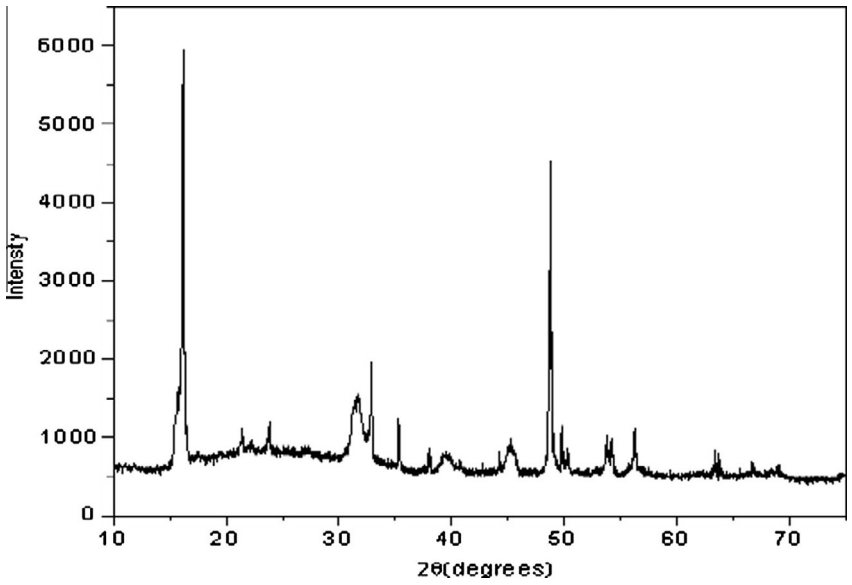


Figure 4 XRD spectrum of Cu(II)complex.

Table 1 Powder X-ray diffraction data of Cu(II) complex.

d-spacing (Å)		2θ values		$\Delta 2\theta$	(*hkl*)
Observed	calculated	Observed	calculated		
5.5342	5.5342	16.00	16.00	0.000	(0 1 0)
5.4640	5.4640	16.21	16.21	0.000	($\bar{1}$ 1 0)
4.1193	4.1193	21.55	21.55	0.000	(1 1 0)
3.7003	3.7003	24.03	24.03	0.000	(*bar*1 0 1)
2.8072	2.8072	31.85	31.85	0.000	(1 0 1)
2.7050	2.7050	33.09	33.09	0.000	($\bar{1}$ 1 1)
2.5364	2.3518	35.36	35.28	0.77	(3 $\bar{1}$ 1)
2.3523	2.3514	38.23	38.24	−0.015	($\bar{2}$ 2 1)
2.2587	2.2606	39.88	39.84	0.036	($\bar{4}$ 0 1)
2.0378	2.0410	44.42	44.34	0.072	(3 $\bar{1}$ 1)
1.9947	1.9940	45.43	45.45	−0.016	(1 $\bar{3}$ 1)
1.8582	1.8586	48.97	48.97	0.011	(3 $\bar{1}$ 2)
1.8236	1.8241	49.97	49.95	0.014	(4 1 0)
1.8028	1.8015	50.59	50.59	−0.039	($\bar{5}$ 1 0)
1.6944	1.6922	54.08	54.15	−0.076	(4 $\bar{1}$ 1)
1.6314	1.6327	56.35	56.30	0.050	($\bar{1}$ $\bar{3}$ 2)
1.4627	1.4617	63.55	63.60	−0.049	($\bar{4}$ 3 1)
1.3983	1.3984	66.85	66.84	0.005	(0 3 1)
1.3578	1.3536	69.12	69.37	−0.250	(1 1 2)

length of light. The sizes of the crystallites of the Cu(II) complex are found to be of the order of 60 nm. The SEM micrographs of the Cu(II) complex are shown in (Fig. 5). The SEM image of Cu(II) complex consists of a cauliflower shaped particle.

3.5. Antibacterial activity

The *in vitro* antibacterial properties of the free ligand and Cu(II) complex are evaluated against gram-negative and gram-positive bacteria. The obtained results are reported in Table 2. The Cu(II) complex shows higher antibacterial activity than that of free ligand. It is evident that Cu(II) metal chelate exhibits effective antibacterial activities. The increased activity of the metal chelate can be explained on the basis of chelation theory (Sengupta et al., 1998). It is known that chelation tends to make the ligand more powerful and exhibits more antibacterial activity than the ligand. It is observed that in a complex, the positive charge of the metal is partially shared with the donor atoms present in the ligand and there may be π-electron delocalization over the whole chelating.

The synthesized ligand and Cu(II) complex have been screened for reduction in DPPH free radicals and inhibition of iron(III) induced lipid peroxidation at 100 μm concentration. The Cu(II) complex shows good activity in DPPH scavenging (33%) and ferric ion induced lipid peroxidation (45%) as seen in the case of standard antioxidant α-tocopherol, but free ligand shows low activity against DPPH scavenging and ferric ion induced lipid peroxidation. Relevant data are presented in Table 3.

3.6. Tryphan blue exclusion method (cell viability test)

The compounds are tested using the short term *in vitro* cytotoxicity toward EAC (Ehrlich Ascites Carcinoma) cells as a preliminary screening technique of tryphan Blue exclusion method (cell viability test) for their cytotoxic potential (Devi et al., 1998). This is one of the methods to assess cytotoxicity of anticancer compounds. This test is based on the principle that living cell membrane has the ability to prevent the entry of a dye. Hence, they remain unstained and can be easily distinguished from dead cells, which take the dye. Thus the percentage of viable cells can be determined. Results of the short term *in vitro* cytotoxicity of the compounds are shown in Table 4. These preliminary experiments are carried out mainly with three different concentrations of the compounds. At 100 μg/ml concentration the standard (Cisplatin) shows 80% cell death. At that same concentration the Cu(II) complex shows (61%) cell death.

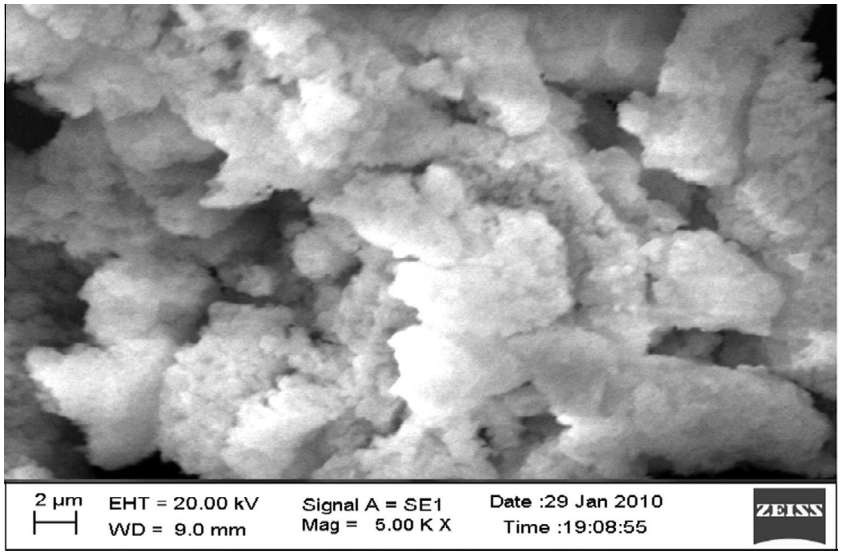


Figure 5 The SEM image of Cu(II) complex.

Table 2 Antibacterial screening data of the ligand and Cu(II) complex (Diameter of Zone of Inhibition in mm).

Compound	*K. Pnuemoniae*	*E. Coli*	*B. Subtilis*	*S. aureus*
Ligand(L)	06	14	08	–
Cu(II) complex	22	25	18	20
Ampicillin	43	40	43	42
Tetracycline	32	33	30	32

Table 3 Effect of ligand and Cu(II) complex on scavenging of DPPH and Fe^{3+} induced lipid peroxidation at 100 μM concentration.

Compound	DPPH scavenging (%)	Fe^{+3} induced lipid peroxidation
Ligand(L)	22	30
Cu(II) complex	33	45
α-Tocopherol	53	65

Table 4 Short-term *in vitro* Cytotoxicity of ligand and its Cu(II) complex toward EAC cells.

Compound	Percentage of cell-death at different concentrations after 3 h			
	10 μg/ml	50 μg/ml	100 μg/ml	LC_{50} μg/ml
Ligand(L)	15	14	31	2342.57
Cu(II) complex	35	45	61	49.47
Cisplatin	14	25	80	42.45

The Cu(II) complex is found to have considerable cytotoxicity in the cell viability test.

4. Conclusions

In the present work, coordination chemistry of ligand, obtained from the reaction of orthohydroxypropiophenone and isonicotinicacid hydrazide is described. The Cu(II) complex has been synthesized using the ligand and characterized on the basis of analytical and spectral data. The EPR and electronic spectral studies suggest that Cu (II) complex has a rhombically distorted octahedral environment for Cu(II) with $^2A_{1g}$ ($dx^2 - y^2$) as the ground state and powder X-ray diffraction suggests triclinic structure of the complex. Upon complexation with metal ions particularly for Cu(II), the antibacterial activity of the ligand is enhanced against four bacteria (*B. subtilis*, *S. aureus*, *E. coli* and, *K. pneumonia*). The synthesized ligand and Cu(II) complex are screened for reduction of DPPH and inhibition of iron induced lipid per oxidation at 100 μM concentration. Among them, Cu(II) complex shows good activity in DPPH scavenging (33%) and ferric ion induced lipid per oxidation (45%) as seen in the case of standard antioxidant α-tocopherol, but free ligand shows low activity against DPPH scavenging and ferric ion induces lipid peroxidation. The Cu(II) complex shows more *in vitro* cytotoxic activity than free ligand. However, its *in vitro cytotoxic* activity can be further confirmed by *in vivo* methods.

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