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AN INVESTIGATION ON FLUORESCENCE QUENCHING OF Cu(II) COMPLEX AND *IN VITRO* CYTOTOXICITY ASSAY

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ABSTRACT. Fluorescence study of Cu(II) complex of 4-((2-hydroxynaphthalen-1-ylmethylene)amino)-N-(pyridin-2-yl)benzenesulfonamide by alizarin dye has been probed in DMSO. In the fluorescence quenching the electron transfer occurs from Cu(II) complex to alizarin. The structure of the complex was confirmed by EPR and powder XRD techniques. The Cu(II) complex has been amplified by *in vitro* antifungal minimum inhibitory concentration study and also has been submitted for *in vitro* cytotoxicity assay in human cervical cancer cell line (He La). The biological study indicates that the $[Cu(L_2)_2Cl_2]$ has significant antifungal and cytotoxic activity.

KEY WORDS: Cu(II) complex, Powder XRD, Fluorescence quenching, MIC study, *In vitro* cytotoxicity assay

INTRODUCTION

The design and synthesis of new types of Cu(II) Schiff base complexes containing oxygen and nitrogen donor atoms is of significant importance. The increasing interest in transition metal complexes containing a Schiff base ligand is derived from their well-established chemical structure and function in biological systems as well as their pharmaceutical applications [1, 2]. The Schiff bases are broadly used as pigments, colorants, catalysts, intermediates in organic synthesis and polymer stabilizers. They also display a varied spectrum of biological activity, including antifungal, antiproliferative, anti-inflammatory, antiviral, antipyretic and antiparasitic. The pronounced biological activity of the metal complexes of Schiff bases derived from sulpha drugs has led to considerable interest in coordination chemistry [3]. Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. Generally fluorescence quenching of ligands through complexation is a phenomenon which can be explained by means of redox activity, magnetic perturbation, electronic energy transfer [4]. Fluorescence quenching is a common technique used in the detection of gases and metal ions [5, 6]. Literature survey reveals that no work has been done on fluorescence quenching studies of sulpha drug Schiff base complexes. Hence we start fluorescence studies on previously synthesised and characterised Cu(II) complex of Schiff base derived from condensation of 2-hydroxy-1-naphthaldehyde with the well-known sulpha drug sulphapyridine. The bio potency of the complex has been amplified by MIC, this antifungal activity has been tested against fungus: *Candida albicans* by using sabouraud dextrose agar medium. Furthermore, this study make an effort to find out the pharmaceutical applications of this complex.

EXPERIMENTAL

Materials

The ligand and the Cu(II) complex used in this study were synthesized by the reported method. The details of the synthesized procedure and characterization of the complex was described in [7]. Dimethylsulphoxide (Merck) and N,N-Dimethyl formamide (Merck) were used as such.

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Equipment for evaluation

The photoactive potentiality of the ligand and the complex were investigated by fluorescence measurements using Perkin Elmer LS 45 Spectrofluorometer.

The photo induced interaction of Cu(II) complex with anthraquinone dye alizarin was studied by fluorescence quenching measurement and it was carried out on Perkin Elmer LS 55 Spectrofluorometer. For fluorescence quenching experiments the sample was prepared by dissolving complex in DMSO and administering the suitable amounts of alizarin. The sample was deoxygenated by bubbling with pure nitrogen, Quartz cells (4x1x1 cm) with high vacuum Teflon stopcocks were used for bubbling.

Biological evaluation

Antifungal susceptibility test by disc diffusion technique

Disc impregnated with known concentration of antibiotic was placed on agar plate that has been inoculated uniformly over the entire plate with culture of the microbes to be tested. The plate was incubated for 24 h at 298 K. During this period, the antimicrobial agent diffuses through the agar and may prevent the growth of the organism. Effectiveness of susceptibility is proportional to the diameter of zone of inhibition.

In vitro cytotoxicity by MTT assay

The measurement of *in vitro* cytotoxicity was evaluated by using vital dyes by protease biomarkers with MTT assay. The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% foetal bovine serum (FBS) was used for the present study.

Nonlinear regression graph was plotted between % Cell inhibition and log concentration and IC_{50} was determined using Graph Pad Prism software. *In vitro* cytotoxicity assay were carried out in KMCH College of Pharmacy, Coimbatore. Antifungal activity (MIC) was carried out in the Department of Microbiology, Periyar College of Pharmaceutical Sciences, Tiruchirappalli by disc diffusion technique.

RESULTS AND DISCUSSION

The ligand and the Cu(II) complex used in this study were characterized by spectral techniques and reported by the authors in [7].

EPR spectrum

The EPR spectrum is a powerful tool in the study of the structure and environment of species that contain unpaired electron. The X-band EPR spectra of the complex at RT and at 77 K in liquid nitrogen are taken. The $g\| > g\perp > 2.0023$ shows the covalent nature of metal–ligand bonds and also confirms the presence of unpaired electron in the $d_{x^2-y^2}$ orbital of this Cu(II) complex. The spin-orbit coupling constant λ value –462 cm^{-1} calculated using the relations, $g_{av} = 1/3[g\| + 2g\perp]$ and $g_{av} = 2(1–2\,\lambda)/10Dq)$, is less than the free Cu(II) ion (– 832 cm^{-1}) which also supports covalent character of Cu–L bond in Cu(II) complex. The covalency parameter α^2 is calculated using the following equation: $\alpha^2_{cu} = – (A\|/0.036) + (g\| – 2.0023) + 3/7(g\perp – 2.0023) + 0.04$. The observed α^2 value 0.87 is less than unity, which indicates that the complex has some covalent character in the ligand environment. The axial symmetry parameter G is 5.20 which indicates negligible exchange interaction [8, 9] of Cu–Cu and supports the monomeric nature of this Cu(II) complex.

Powder XRD spectra

The X-ray diffractograms of the ligand and Cu(II) complex were scanned in the range $2\theta = 3–60°$ at a wavelength of 1.543 Å as shown in the Figure 1. The powder XRD spectrum of Schiff base (HNSP) was compared with the spectra of the Cu(II) complex (HNSPCuC). Few new peaks appeared in the spectra of complex compared to the spectrum of ligand which indicates the formation of metal chelate.

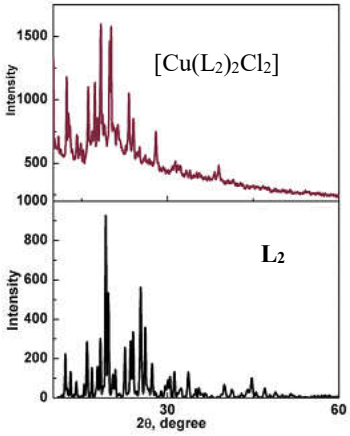

Figure 1. Powder XRD pattern of L_2 and $[Cu(L_2)_2Cl_2]$.

The size D_{XRD} of the Schiff base and its complex are calculated with the help of XRD patterns using Debye Scherrer's formula [10] $D_{XRD} = 0.9\lambda/\beta \cos\theta$, where 'λ' is the wavelength, 'β' is the full width at half maximum and 'θ' is the peak angle. The ligand and the complex have the crystallite size of 73 and 36 nm respectively, suggesting that ligand and its complex are nanocrysalline. The X-ray diffraction pattern of metal chelates have reduced size than ligand due to the increasing values of full width half maximum [11, 12]. The unit cell calculations have been done for cubic symmetry from the important peaks and $(h^2+k^2+l^2)$ values have been calculated for the complex. The Miller indices for the complex are 1, 3, 7, 12, 16, 19, 22, 26 and 39. The presence of forbidden number 7 in complex indicates that the Cu(II) complex may belong to the hexagonal or tetragonal systems. But the spectral and elemental analysis supports the hexagonal systems. The XRD pattern of ligand supports the molecular formula for the Cu(II) complex is $[Cu(L_2)_2Cl_2]$.

Based on the spectral evidences in [7] EPR and powder XRD spectra confirm the correctness of the structure of the complex proposed in [7].

Fluorescence quenching of $[Cu(L_2)_2Cl_2]$

The emission wavelength of Schiff base and its Cu(II) complex is 362 nm and 448 nm with the intensity of 76 and 780 for the corresponding excitation wavelength 338 and 407. Significant increase in the fluorescence emission intensity of Cu(II) complex establish the coordination of the metal ion to the ligand [13].

The fluorescence quenching of $[Cu(L_2)_2Cl_2]$ with alizarin dye have been probed in DMSO by means of fluorescence spectroscopic technique. Upon excitation of the complex, the electron acceptor alizarin dye interact with the complex and preclude the electron hole recombination process, which causes decrease in the emission intensity of complex. The emission spectrum of

$[Cu(L_2)_2Cl_2]$ measured in DMSO was effectively quenched by increasing the concentration of alizarin dye (0-1.5 × 10^{-5} M) (Figure 2). The observed quenching is entirely due to interaction of $[Cu(L_2)_2Cl_2]$ with alizarin and not due to inner filter effect or reabsorption.

The binding constant for this type of interaction was calculated using fluorescence quenching data by equation (1):

$$\frac{1}{(F^0 - F)} = \frac{1}{(F^0 - F')} + \frac{1}{K(F^0 - F)[Q]} \quad (1)$$

where K is the binding constant, F^0 is the initial fluorescence intensity of complex, F′ is the fluorescence intensity of alizarin adsorbed complex and F is the observed fluorescence intensity at its maximum.

The plot of $1/(F^0\text{-}F)$ versus 1/[Q] gives a straight line (Figure 3) and from the slope the calculated binding constant and correlation co-efficient for the curve are found to be 6.666× 10^4 M^{-1} and 0.996, indicating that the interaction between complex with alizarin dye agrees well with the site binding model [14, 15]. The excited state oxidation potential (E_{s^*/s^+}) of complex has been calculated to be –1.73 V Vs SCE, $E_{s/s+}$ is the oxidation potential of complex, 1.25 V Vs SCE and E_s, the excited state energy is 2.98 eV. The conduction band energy level of alizarin is 0.55V Vs SCE. This suggests that the electron transfer from excited state complex to the conduction band of alizarin (Figure 4) is energetically favourable [11-18].

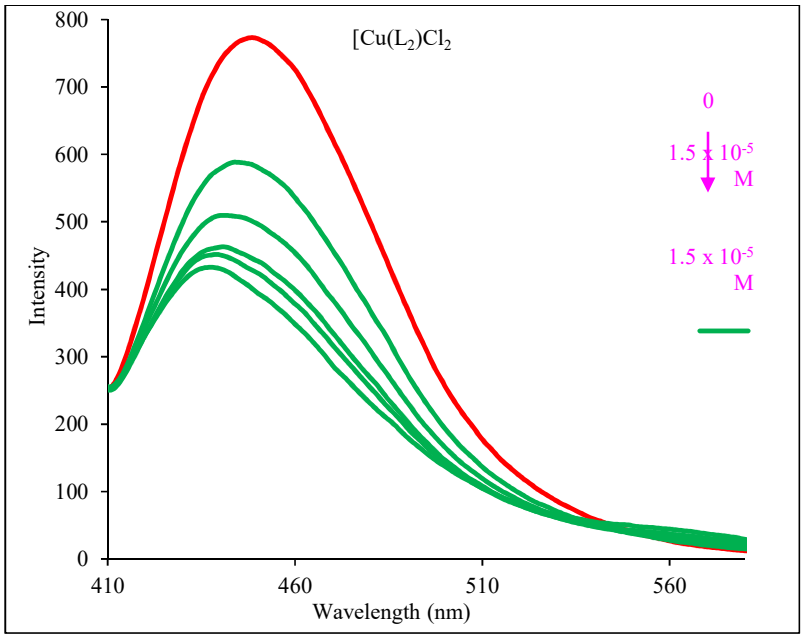

Figure 2. Fluorescence quenching of $[Cu(L_2)_2Cl_2]$.

Calculation of free energy change (ΔG_{et}) for the electron transfer mechanism of $[Cu(L_2)_2Cl_2]$

The thermodynamic feasibility of excited state electron transfer reactions has been calculated by the well-known Rehm-Weller expression [19, 20], $(\Delta G_{et}) = E_{1/2}^{(ox)} - E_{1/2}^{(red)} - E_s + C$, where, $E_{1/2}^{(ox)}$ is the oxidation potential of complexes **6**, $E_{1/2}^{(red)}$ is the reduction potential (conduction band

potential) of alizarin, E_s is the excited state energy of complexes and C is the coulombic term. Since one of the species is neutral and the solvent used is polar in nature, the coulombic term in the above expression is neglected [21] where, $E_{1/2}^{(ox)}$ is the oxidation potential of complex (1.25 V), $E_{1/2}^{(red)}$ is the reduction potential of (conduction band) alizarin. The ΔG_{et} value thus calculated for the electron transfer process, complex to alizarin is negative (–1.18 eV). Hence, the electron transfer process is thermodynamically favourable [22].

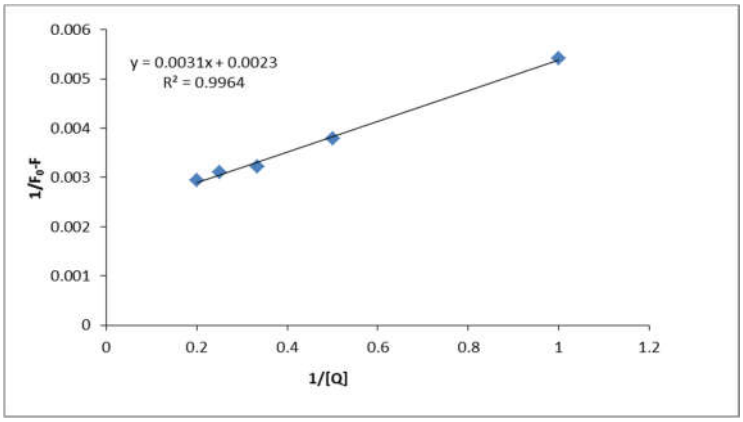

Figure 3. Binding constant plot of $[Cu(L_2)_2Cl_2]$.

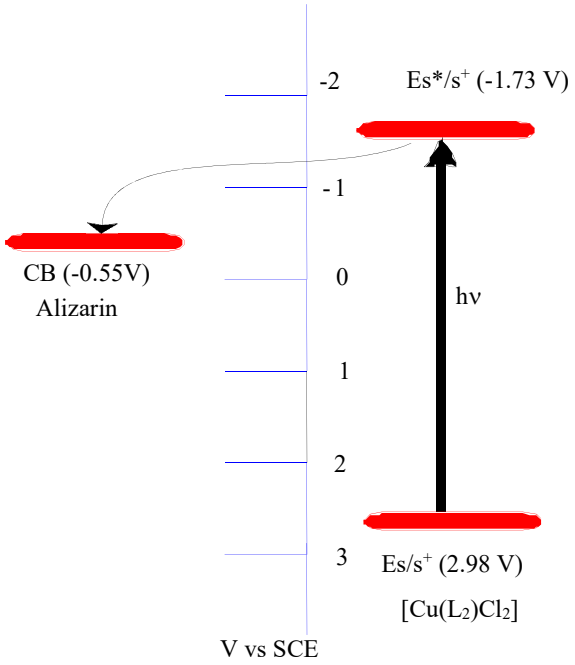

Figure 4. Electron transfer from $[Cu(L_2)_2Cl_2]$to alizarin.

In vitro Antifungal Minimum inhibitory concentration studies

The $[Cu(L_2)_2Cl_2]$ has been screened for *in vitro* antifungal MIC against *Candida albicans* (Figure 5) by using sabouraud dextrose agar medium. Nystatin is used as standard. The test was carried out in DMSO solution at a concentration of 25, 50, 75 and 100 ppm. The result was compared with standard drug Nystatin. Even at very low concentration of 25 ppm the $[Cu(L_2)_2Cl_2]$ has significant activity can be explained on the basis of Overtones's concept [23] and Tweedy's chelation therapy [24]. On chelation, the polarity of Cu(II) ion is reduced to a greater extent due to the overlap of the ligand orbital and positive charge of the zinc ion with donor groups, it increases the delocalization π-electrons over the entire chelate ring and increases the lipophilicity of the complexes, it enhances the penetration of the complexes into lipid membrane of the microorganisms that inhibit multiplication process of the microbes by blocking their active sites [25]. The antifungal activity of the complex increases with increase in concentration of the complex, suggest that the complex having significant antifungal activity.

Figure 5. MIC $[Cu(L_2)_2Cl_2]$ of against *Candida albicans.*

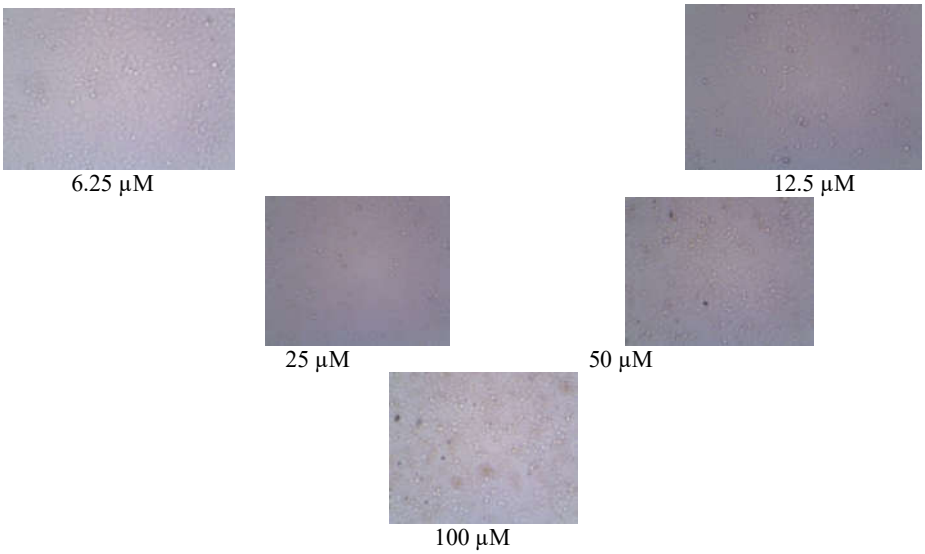

Figure 6. *In vitro* cytotoxic activity of $[Cu(L_2)_2Cl_2]$ in human cervical cancer cell. Line (He La) with the dose 6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM.

In vitro cytotoxicity assay

In the last few decades, human cancer cell lines have aggregated an accessible, easily usable set of biological models to examine cancer biology [26]. The $[Cu(L_2)_2Cl_2]$ has been screened for anticancer activity (Figure 6) according to the MTT assay method. The complex has been submitted for human cervical cancer cell line (He La) with the dose 6.25, 12.5, 25, 50 and 100 μM. The $[Cu(L_2)_2Cl_2]$ complex possesses moderate cytotoxicity with IC_{50} value 79.91 μM.

CONCLUSION

Photoinduced interaction between Cu(II) complex and alizarin was investigated by fluorescence spectroscopy. Based on the above results, it is suggested that the fluorescence quenching involves electron transfer mechanism. The Cu(II) complex act as an electron donor and the dye act as electron acceptor. The calculated value was found to be more negative suggesting the occurrence of electron transfer from excited state Cu(II) complex to alizarin is thermodynamically favourable. We expect this present work of electron transfer reaction might be useful in the fields of environmental pollutant control by waste water treatment. The result of minimum inhibitory concentration study indicates that even at very low concentration the $[Cu(L_2)_2Cl_2]$ has significant activity against *Candida albicans.* The complex also possesses sensible cytotoxicity, compelling us to propose that the electronic effect may be one of the factors in determining the anticancer activities of compounds.

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