Synthesis, Characterization and Application of Copper Complexes in Bio-inorganic Chemistry

M.Sc. Thesis

By

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Synthesis, Characterization and Application of Copper Complexes in Bio-inorganic Chemistry

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Submitted in partial fulfillment of the requirements for the award of the degree

of

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by

ARUN KUMAR

DISCIPLINE OF CHEMISTRY

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Synthesis, Characterization and Application of Copper Complexes in Bio-inorganic Chemistry** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF CHEMISTRY, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2017 to June 2018 under the supervision of Dr. Suman Mukhopadhyay, Professor, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

ARUN KUMAR

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Professor SUMAN MUKHOPADHYAY

ARUN KUMAR has successfully given his/her M.Sc. Oral Examination held on _____

Signature of Supervisor
Date:

Convener, DPGC
Date:

Signature of PSPC
Date:

Signature of PSPC
Date:

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Abstract

Present work of this thesis stems from our interest in exploring structural and biological properties of copper – Schiff base complexes and copper non-steroidal anti-inflammatory drug compounds (NSAID). Copper (II) complexes of four non-steroidal anti-inflammatory drugs (NSAID) *i.e.* aspirin, ibuprofen, naproxen, and diclofenac with NNO donor ligand *(E)*-2-(1-((2-(diethylamino) ethyl)amino)ethyl)phenol (HL) have been synthesized. All the complexes are characterized with the help of ESI-MS, IR spectroscopy. X-ray crystallography studies done for complex **1**, **$[Cu_2(L)_2(asp)]ClO_4$** and revealed that complex **$[Cu_2(L)_2(asp)]ClO_4$** is monoclinic in nature with square pyramidal geometry around copper centers. Interaction of all the complexes with proteins (BSA, HSA), and DNA was studied to establish their potent role as metal-drug system. Cytotoxicity assay was performed for all the complexes on cancer cell lines A549 and MCF7 using MTT dye.

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NOMENCLATURE

Å	Angstrom
ºC	Degree Centigrade
$\bar{\upsilon}$	Wavenumber
%	Percentage
θ	Angle
α	Alfa
β	Beta
γ	Gamma
Δ	Delta
mmol	Milimole
M	Molar
Mm	Milimolar
µM	Micromolar
L	Litre
mL	Millilitre
µL	Microliter
K	Kelvin

ACRONYMS

C	Carbon
H	Hydrogen
N	Nitrogen
O	Oxygen
His	Histidine
Cu	Copper
Zn	Zinc
Ni	Nickel
BSA	Bovine Serum Albumin
HSA	Human Serum Albumin
a.m.u	Atomic mass unit
KBr	Potassium Bromide
DMSO	Dimethylsulphoxide
H_2O_2	Hydrogen peroxide
UV-Vis	Ultraviolet-visible spectroscopy
Cl	Chlorine
NSAID	Non-steroidal anti-inflammatory drugs
Na	Sodium
DAPI	4′,6-diamidino-2-phenylindole
MeOH	Methanol
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloride
ORTEP	Oak Ridge Thermal Ellipsoid Plot Program
ESI-MS	Electron Spray Ionosation- Mass Spectrometry

IR	Infrared spectrometry
NMR	Nuclear Magnetic Resonance
Asp	Aspirin
Dicl	Diclofenac
Ibu	Ibuprofen
Nap	Naproxen

Chapter 1

Introduction

1.1 Role of metals in biological systems

Metals are present throughout the Earth's crust as constituents of important natural sources such as water, land and within living organisms. Around 33% of every single protein that contain metals, for example, iron, zinc and copper as cofactors plays a critical part in biological processes such as dioxygen activation, protein structure stabilization, enzymatic catalysis and critical reactions (**Table 1.1**)[1–3].

Table 1.1: Some of essential biomolecules containing metals, examples and their role in biological systems[4].

Category	biological function	Examples (metal ion involved)
Nonprotein	Metal transport and structural	siderophores (Fe); Skeletal (Ca, Si)
Proteins	photo-redox	Chlorophyll (Mg).
	oxygen transport	hemocyanin (Cu); haemoglobin(Fe)
	Structural	Zn fingers (Zn)
	electron transfer	cytochromes (Fe); Azurin (Cu)
Enzymes	oxidation of phenol	catechole oxidase (Cu)
	oxido- reductases	phenoxazinone synthase (Cu); nitrrogenases (Fe, Mo, V)
	Isomerases and synthesases	vitamin B12 (Co)

1.2 Role of copper in biological systems

Copper (Cu) is one of the essential elements of life that can be classified as a micronutrient[4]. It is additionally known as the third most abundant transition metal in biology, after iron and zinc[5]. More than thirty enzymes are known containing copper ions as cofactor in human and animals for maintaining cellular activities, potential synergetic activity with drugs and other specific function such as metabolism[6,7]. The standard concentration of copper ion in human blood is one ppm[8].

Deficiency of copper causes certain diseases such as Parkinson diseases[11], anemia[12], cardiovascular disease[10], diabetes[11], Menke's disease[12], and others. Also, high concentrations of copper can cause toxicity, and specific diseases such as oxidative-stress related disorders[13], Wilson's diseases, Alzheimer's disease (AD) and Atherosclerosis during aging[14].

1.3 Copper containing metalloproteins

Copper-containing metalloproteins and enzymes makes copper the third most abundant transition metal, after iron and zinc, in the human body[4]**.** It is an essential bio-element in biological systems and required for many metabolic reactions[15,16]. Copper present as Cu (I) or Cu (II) in these proteins/enzymes and coordinate to sulfur, oxygen and/or nitrogen donor ligands such as, cystine, histidine, imidazole and tyrosine as shown in **Figure 1.3**.

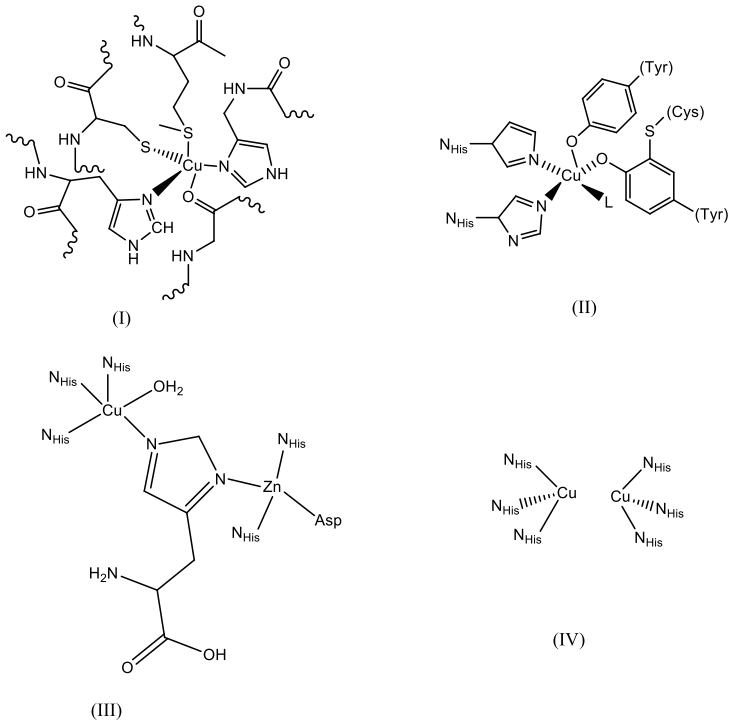


Figure 1.1: Some copper containing metalloprotein active sites: (**I**) azurin[17],(**II**) galactose oxidase[18], (**III**) Cu, Zn SOD in the oxidizedform[19–21], (**IV**) dicopper(II) center of catechol oxidase in the deoxy state[22,23].

It's also known that copper enzymes play a major role as catalysts or electron transporter in oxidation reactions of different substrates such as aromatic amines and phenols, and in various biological processes such as electron transport, oxygen carrier and superoxide dismutase, and many other biological activities (**Table 1.2**).

Table 1.2 Some protein/enzyme having Cu at their active sites, their biological functions, and catalyzed reactions[17,18,20,23–25].

Copper containing protein/enzyme	**Biological function**	**Catalysed reaction**
Azurin	Electron transfer	$Az(ox) + e^- \rightleftharpoons Az(red)$
Catechol Oxidase	Oxidation of o-catechol to o-quinone	o-Catechol ⟶ o-Quinone

Cu, Zn- SOD	Free radical scavenging	$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$
Galactose Oxidase	Galactose oxidation (oxidation of primary alcohols to aldehydes in sugars)	$RCH_2OH + O_2 \longrightarrow RCHO + H_2O_2$
Hemocyanin	O2 transport	$Hc + O_2 \rightleftharpoons Hc.O_2$
Nitrus oxide Reductase	Reduction of N2O to N2	$N_2O \longrightarrow N_2$
Plastocyanin	Electron transfer	$Pc(ox) + e^- \rightleftharpoons Pc\ (red)$

1.4. Targeting copper over other transition metals

Copper ions forms complexes with various ligands with different stoichiometry, geometry and stereochemistry, commonly present as mononuclear, binuclear and polynuclear species[26,27]. Copper ions possesses unique chemical properties that include; various stereochemistry in complexes,[28,29] the extensive ability bind with various ligands, especially with oxygen and nitrogen donor types[29,30], redox chemistry due to different oxidation states. Additional attention has been paid to the medicinal chemistry of copper in light of the fact that blood plasma has high affinity for copper binding, and therefore contribution of ceruloplasmin and albumin represent quite seventy seven percent of the overall copper content binding[31,32]. Complexes of Copper(II) with biologically active ligands have been synthesized previously and found to exhibit varied pharmacological effects such as anti-diabetic[33], anti-amoebic[34], anticonvulsant[35,36], anticancer, antitumor[37], anti-inflammatory[38], antiulcer[38], and antimicrobial activities[39,40].

1.5 Metal carboxylate

Carboxylates ($RCOO^-$) as ligands are one of the important classes of compounds in inorganic and bioinorganic chemistry with varied chemical and physical properties, which depends on the nature of R group. Amino acids for example, cysteine (Cis), aspartates (Asp), histidine (His), are classified as one of the most biologically important carboxylate ligands in various metalloprotein[41]. HSAB (hard soft acid base) principle is extremely useful to determine the stability of metal complexes, reaction mechanisms and pathways. According to HSAB principle, hard acid prefer to bind with hard base and soft acid prefer soft base. Negatively charged oxygen of carboxylate ligands acts as hard bases, and has a high tendency to react with hard acid metals. Numerous reports are available of synthesis and characterization of stable metal-carboxylate complexes. The biological role of metal carboxylate complexes has been reported and displayed various activities such as anti-inflammatory and anti-cancer activities[42,43].

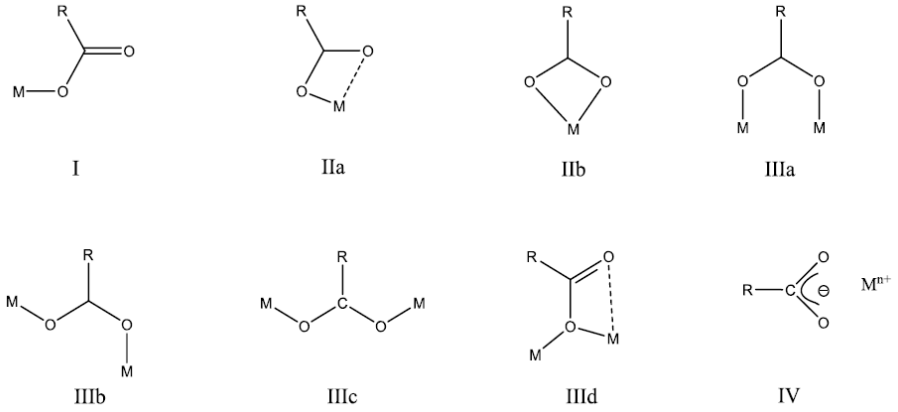


Figure 1.2: Metal-Carboxylate binding modes: (I) monodentate, (IIa) bidentate asymmetrical mode, (IIb) bidentate symmetrical mode, (IIIa) *syn-syn* mode, (IIIb) *syn-anti* mode, (IIIc) *anti-anti* mode, (IIId) monodentate bridging, (IV) ionic mode[44].

The mode of carboxylate binding to metals has been studied by Deacon and Phillips[44] by comparing the difference of the IR stretching frequencies between *anti*-symmetric υ_{asym}(COO-) and symmetric υ_{sym}(COO-) stretching vibrations, $\Delta_{(COO-)}$, for a great deal of metal carboxylate complxes. Based on the results of their studies the following guidelines can be used to identify

the coordination mode of the carboxylate group to metal ions: $\Delta_{(COO-)}$ symmetrical chelate coordination < $\Delta_{(COO-)}$ ionic coordination < $\Delta_{(COO-)}$ bridging coordination = $\Delta_{(COO-)}$asymmetric chelate coordination < $\Delta_{(COO-)}$ monodentate coordination.

1.6 Copper carboxylate complexes

Coordination chemistry of carboxylate groups, like those in amino acids and in other carboxylate containing ligands as in the NSAIDs, to Cu ion is quite interesting and have been studied for their unique chemical properties and biological activities[6]. A large number of mononuclear and binuclear copper(II) carboxylate complexes have been synthesized previously[45,46], characterized and studied for their biological activities and biomimetic models for the copper containing enzymes; SOD, catechol oxidase and phenoxazinone synthase [47,48].

1.7 Copper complexes with NSAIDs

The first used NSAID was acetylsalicylic acid, which was first synthesized in 1897 by Felix Hoffmann and marketed by Bayer as aspirin. Several analgesic and antipyretic NSAIDs such as naproxen, diclofenac, aspirin and ibuprofen were used as coordinated ligands to copper (II) ions and reported in literature[45,49]. It is well known that copper (II) contain non-steroidal anti-inflammatory drugs enhance the activity and minimized the side effects as compared to NSAID's in humans and animals[50,51]. By taking this thought into consideration various complexes with NSAID's have been synthesized previously, the biological role and activity of copper and its complexes as well as the significance of the NSAIDs in medicine, binary and ternary of copper(II)–NSAID complexes have been synthesized explored for various biological properties[46],[22].

1.8 Copper Schiff base complexes

Schiff base (named after Hugo Schiff) compounds are formed by condensation reaction between primary amine with aldehyde or ketone under specific temperature and pressure. Schiff bases (likewise called

imine) are structurally nitrogen analogues of ketone or aldehyde where carbonyl group is replaced by imine group. Schiff base ligands are easy to synthesize and they have tendency to form complexes with various metal ions. Over the past few years, there have been several reports on biological application of metal Schiff base complexes including antibacterial[52,53],antifungal[54], anticancer[55], anti-oxidant[42], anti-inflammatory[56], and antimalarial[57] and several catalytic reactions[30,58,59].

1.9 Organization of thesis:

The aim of this project was to synthesize the copper complexes in combination of Schiff base and NSAIDs ligands and to study their applications in biochemistry. This was to be achieved by reproducing a Schiff base ligand and carrying out its complexation reaction with copper and different NSAIDs.

Chapter 2: This chapter includes literature survey and motivation of the project.

Chapter 3: This chapter includes instruments, materials, and the experimental procedures used to synthesize the copper complexes. It also includes experimental techniques employed to study BSA, HSA, DNA binding properties and cytotoxic properties against A549 and MCF7 cell lines using MTT dye.

Chapter 4: In this chapter results have been discussed which were obtained after the synthesis and application study of the metal complexes.

Chapter 5: This chapter concludes the described work and also looks for possible future scope and applications.

Chapter 2

Review of past work and project motivation

Metal coordination complexes have shown various applications ranging from catalysis to antitumor medicines. In these compounds, metal ion itself could have a variety of roles supported by its oxidation states, coordination geometry, electronic, magnetic, and photochemical behaviors. Schiff base plays an important role in coordination chemistry as a ligand by their complex formation ability owing to the presence of different donor atoms.

Developing new chemotherapeutical agents with high efficiency and low toxicity is a challenge in antitumor drug development. Within the past, metal based compounds were widely utilized in the treatment of diseases; however lack of clear distinction between therapeutic and toxic doses is a motivational challenge. In 1960 Barnett Rosenberg discovered cisplatin, which witnessed a new era of metal based anticancer drugs. The broad success of cisplatin within the clinical treatment of varieties of neoplasia has placed coordination chemistry of metal based medications on leading edge in battle against cancer.

Additional new Platinum based medicines like carboplatin, and oxaliplatin are important compounds in cancer treatment. Platinum-based medicine broadly utilized in the clinical treatment of varied kinds of cancer is still limited by their toxic side effects like nephrotoxicity, neurotoxicity and acquired drug resistance[60–64]. Bioinorganic and medicinal chemists have since then focused on the design and synthesis of new metal-based anticancer agents with a better biological activity, better selectivity, lower toxicities and different mechanisms of action to overcome the unresolved clinical problems of cisplatin analogues drugs.

In this area, complexes containing copper metal ion showed encouraging results[37,65–67]. Copper based complex are explored expecting that, endogenous metals might prove less toxic for normal cells with respect to cancer cells. The biological properties of copper complexes have been

extensively studied because some complexes have been able to exhibit antibacterial[52], antifungal[68], anticancer[37], anti-oxidant[42], anti-inflammatory[38], and antimalarial[57] activities. One of the most investigated non-Pt compounds as potentially attractive anticancer agents were copper complexes[37].

An anticancer drug may work by binding to carrier proteins in the blood which increases its solubility in the blood plasma and results in deliver of drugs to its target cells. Cu compounds have high tendency to bind with blood plasma, as proteins are one the main cellular targets for anticancer drugs so the study of drug–protein interactions are very important to understand the activity and toxicity of drugs. Being motivated from these reports, four new complexes of Cu have been synthesized by utilizing NSAIDs with the Schiff base ligand 2-(1-((2-(diethylamino)ethyl)imino)ethyl)phenol (HL) reported by Chaitali Biswas *et al*. (2010)[69], and their BSA, HSA, DNA binding and cell cytotoxicity activity have been explored.

Chapter 3

Experimental Section

3.1 Reagent and Chemicals:

All of the chemicals [except copper perchlorate and salts of aspirin and naproxen] were of analytical grade and used as received without further purification. These chemicals included N^1,N^1-diethylethane-1,2-diamine (Sigma Aldrich), 1-(2-hydroxyphenyl)ethanone (Sigma Aldrich, assay 95%), trimethylamine (Avantor, assay 99.5%), Copper perchlorate hexahydrate $Cu(ClO_4)_2.6H_2O$ was prepared in laboratory. Salts of ibuprofen and diclofenac are purchased from sigma Aldrich (assay 98%). Sodium salt of naproxen and potassium salt of aspirin was prepared in laboratory from aspirin and naproxen (sigma Aldrich, assay 98%).

3.2 Instrumentation:

Infrared spectra (4000 to 500 cm−1) were recorded with a BRUKER TENSOR 27 instrument in KBr pellets. NMR spectra were recorded on an AVANCE III 400 Ascend Bruker BioSpin machine at ambient temperature. Mass spectrometric analyses were done on Bruker-Daltonics, microTOF-Q II mass spectrometer. Spectrophotometric measurements were performed on a Varian UV-Vis spectrophotometer (Model: Cary 100) (for absorption) and a Fluoromax-4p spectrofluorometer from Horiba JobinYvon (Model: FM-100) (for emission) using a quartz cuvette with path length of 2 cm. Single-crystal X-ray structural studies were performed on an Agilent Technology Supernova CCD diffractometer equipped with a low-temperature attachment.

3.3 Synthesis of Schiff-base Ligand and its Metal complexes:

3.3.1 Synthesis of Schiff-base ligand (E)-2-(1-((2-(diethylamino) ethyl)amino)ethyl)phenol*) (HL)*

The tridentate Schiff base HL was prepared according to the previously reported procedure[69] by mixing 5 mmol of 1-(2-hydroxyphenyl) ethanone (0.37g) with 5 mmol of N^1,N^1-diethylethane-1,2-diamine (0.86 g) in 20 mL of methanol. The reaction mixture was stirred under reflux condition for 4 h at 70˚C. After evaporating the solvent, clear bright yellow solution obtained, that is used further for complex formation.(400.13 MHz, 298 K, $CDCl_3$) δ:0.98 (t, 6H), 2.21 (s, 3H), 2.52 (q, 4H), 2.72 (t, 2H), 3.52 (t, 2H), 6.64 (t, *J* = 8 Hz, 1H), 6.82 (d, 1H J = 8 Hz), 7.16 (t, *J* = 8 Hz 1H), 7.38 (d, *J* = 8 Hz, 1H), 13C NMR (100.61 MHz, CDCl3) δ: 164 (C attached to –OH), 171 (C of -C=N-), ESI-MS (+ve mode): $[M + H]^+$: 235.19, IR spectroscopy: ῡ(C=N): 1614 cm^{-1}.

3.3.2 Synthesis of $[Cu_2(L)_{2(}asp)]ClO_4$ 1

A methanolic solution (5mL) of Schiff-base HL (0.11g, 1 mmol), was added to solution of $Cu(ClO_4)_2.6H_2O$ (0.27g, ,1 mmol) dissolved in methanol(10mL), then triethylammine (130µL, 1 mmol) was added to solution. Further potassium salt of aspirin (0.109g, 0.5 mmol) in 5mL methanol was added to above mentioned solution drop wise. The green precipitate formed upon complexation was removed *via* filtration, and the resulting filtrate was allowed to stand for crystallization at room temperature. Upon very slow evaporation of the solvent, green colored block shaped crystals were obtained after 8-10 days. ESI-MS (+ve mode): $[M - ClO_4]^+$: 771.2, IR spectroscopy: ῡ(C=N): 1594 cm^{-1}, ῡ(ClO_4): (1077, 620 cm^{-1}).

3.3.3 Synthesis of Complex $[Cu_2(L)_{2(}diclo)]ClO_4$ 2

To a methanolic solution (10 mL) of $Cu(ClO_4)_2.6H_2O$ (0.27g ,1 mmol), HL (0.11g, 1 mmol) dissolved in 5mL is added and then triethylammine (130µL, 1 mmol) was added to solution. Further sodium salt of diclofenac 0.16g (0.5 mmol) in 5 mL methanol added drop wise, reaction mixture was kept on stirring at room temperature for 4 hrs. The green precipitate formed upon complexation was removed *via* filtration and the resulting filtrate was allowed to stand for crystallization at room temperature. Upon very slow evaporation of the solvent, dark green colored block shaped crystals were obtained after 6-7 days.

ESI-MS (+ve mode): $[M - ClO_4]^+$:888.2, IR spectroscopy: $\bar{\upsilon}$(C=N): 1585 cm^{-1}, $\bar{\upsilon}(ClO_4)$: (1081, 621 cm^{-1}).

3.3.4 Synthesis of Complex $[Cu_2(L)_{2(}ibu)]ClO_4$ 3

Schiff-base HL (0.11g, 1 mmol) was dissolved in methanol (5 mL), added to solution of $Cu(ClO_4)_2.6H_2O$ (0.27g, ,1 mmol) dissolved in methanol(10 mL), then triethylammine (130µL, 1 mmol) was added to solution. Further sodium salt of ibuprofen (0.114g, 0.5 mmol) in 5mL methanol was added to above mentioned solution drop wise. The reaction mixture was stirred at room temperature for 4 hours. The green precipitate formed upon complexation was removed by filtration, resulting dark green colored compound was collected after evaporating the solvent and characterized by ESI-MS and IR spectroscopy. ESI-MS (+ve mode): $[M - ClO_4]^+$: 797.3, IR spectroscopy: $\bar{\upsilon}$(C=N): 1581 cm^{-1}, $\bar{\upsilon}(ClO_4)$: (1081, 621 cm^{-1}).

3.3.5 Synthesis of Complex $[Cu_2(L)_{2(}nap)]ClO_4$ 4

A methanolic solution (5mL) of Schiff-base HL (0.11g, 1 mmol), was added to solution of $Cu(ClO_4)_2.6H_2O$ (0.27g, ,1 mmol) dissolved in methanol(10mL), then triethylammine (130µL, 1 mmol) was added to solution. Further potassium salt of naproxen (0.13g, 0.5 mmol) in 5mL

methanol was added to above mentioned solution drop wise. The green precipitate formed upon complexation was removed *via* filtration, resulting dark green colored compound was collected after evaporating the solvent and characterized by ESI-MS and IR spectroscopy. ESI-MS (+ve mode): $[M - ClO_4]^+$: 823.32, IR spectroscopy: $\bar{\upsilon}$(C=N): 1583 cm^{-1}, $\bar{\upsilon}(ClO_4)$: (1077, 620 cm^{-1}).

3.4. BSA and HSA binding study

The protein binding studies of these complexes were investigated using human serum albumin (HSA) and bovine serum albumin (BSA) by means of fluorescence spectroscopy recording excitation at 295 nm and the corresponding emission at 340 nm. The excitation and emission slit widths and scan rates were kept constant throughout the experiment. A 10 μM stock solution of BSA/HSA was prepared using Tris-HCl buffer (pH ~ 7.4) solution and stored at 4°C for further use. Stock solutions of complexes **1**-**4** (1 mM in strength) were also prepared in Tris-HCl buffer and 2% DMSO. Fluorescence intensity of 2 mL stock solution of BSA/HSA was measured and recorded as blank. Thereafter it was titrated by successive additions of 10 μL of the respective stock solution of complexes (upto 100 μL). The fluorescence data was further analyzed by the Stern–Volmer equation, and Scatchard equation.

3.5. DNA binding study

The DNA binding studies of these complexes were investigated using ct-DNA by means of fluorescence spectroscopy, recording excitation at 391 and emission at 457 nm. Stock solutions of complexes **1**-**4** (1 mM in strength) were prepared in Tris-HCl buffer and 2% DMSO. Firstly we have taken 2 mL Tris-HCl buffer (pH ~ 7.4) in a cuvette then 135μL Tris-HCl was discarded from cuvette, 110μL of ct-DNA (1mg/mL) and 25μL DAPI (1mg/mL) was added to it to make 2 mL of stock solution. Fluorescence intensity of 2 mL stock solution was measured and recorded as blank. The

excitation and emission slit widths and scan rates were kept constant throughout the experiment. Thereafter it was titrated by successive additions of 10 μL of the respective stock solution of complexes (upto 100 μL). The fluorescence data was further analyzed by the Stern–Volmer equation, and Scatchard equation.

3.6. Cell culture and cell lines

A549, MCF7 Cell lines were purchased by NCCS (The National Centre for Cell Science). A549 were maintained in RPMI media containing 10% of heat inactivated fetal bovine serum (FBS) (US origin), 1% of penicillin streptomycin solution. MCF7 was maintained in DMEM with 10% FBS and 1% penicillin streptomycin solution. Cells were incubated in 37°C with 5% CO_2 the cells were trypsinized on reaching 70-80% confluency and centrifuged at 200 g for A549 and 130 g for MCF7 cells and were reseeded in T25cm^2 and then were cryopreserved after second and third passages. These preserved cells were revived and used for further experiment.

3.7. MTT assay for the study of cell cytotoxicity

To investigate the cytotoxicity effect of copper Schiff base complexes on A549 and MCF7 cells, an *in vitro* colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has been performed. 1,2,3,4 compounds stock solutions were made using DMSO and then were further diluted in various concentration (160, 80, 40, 20μL) using media and were kept for 24 hours, for both MCF7 and A549 cells. For cytotoxicity, the MTT colorimetric assay was used. Cultures in the exponential growth phase were trypsinized and diluted in culture medium to achieve a suspension of 1×10^6 cells/mL. Then, 100μL of the suspension was added to the appropriate wells of a sterile 96- well flat-bottomed microtiter plate (Nest; USA) after keeping for 24 hours in incubator at 37°C and 5% CO_2 , the cells were treated with above mentioned concentration of the drugs. Each drug dilution was assessed in triplicate. Three wells containing only complete medium were used as blank controls for nonspecific dye

reduction. And three well containing 1% DMSO was used to determine the effect of dmso on the cell lines. The plates were then incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 hr. Cell viability was determined by using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. After 24 hours of incubation, 10 μL of MTT (5 mg/mL) was added to each well. The plate was again incubated for another 4 h to allow reduction of MTT. Subsequently, the medium containing MTT was carefully aspirated from the wells. Approximately 100 μL of DMSO was added to the wells to dissolve the formazan crystals. The plate was then incubated for another 5 min. The cell viability was determined by the optical density reading of formazan solution using the SYNERGY H1 microplate reader. The IC_{50} value for each compound was calculated as the concentration of the compound tested which inhibited the growth of 50% of the cells relative to the untreated control cells.

3.7 X-ray crystallography:

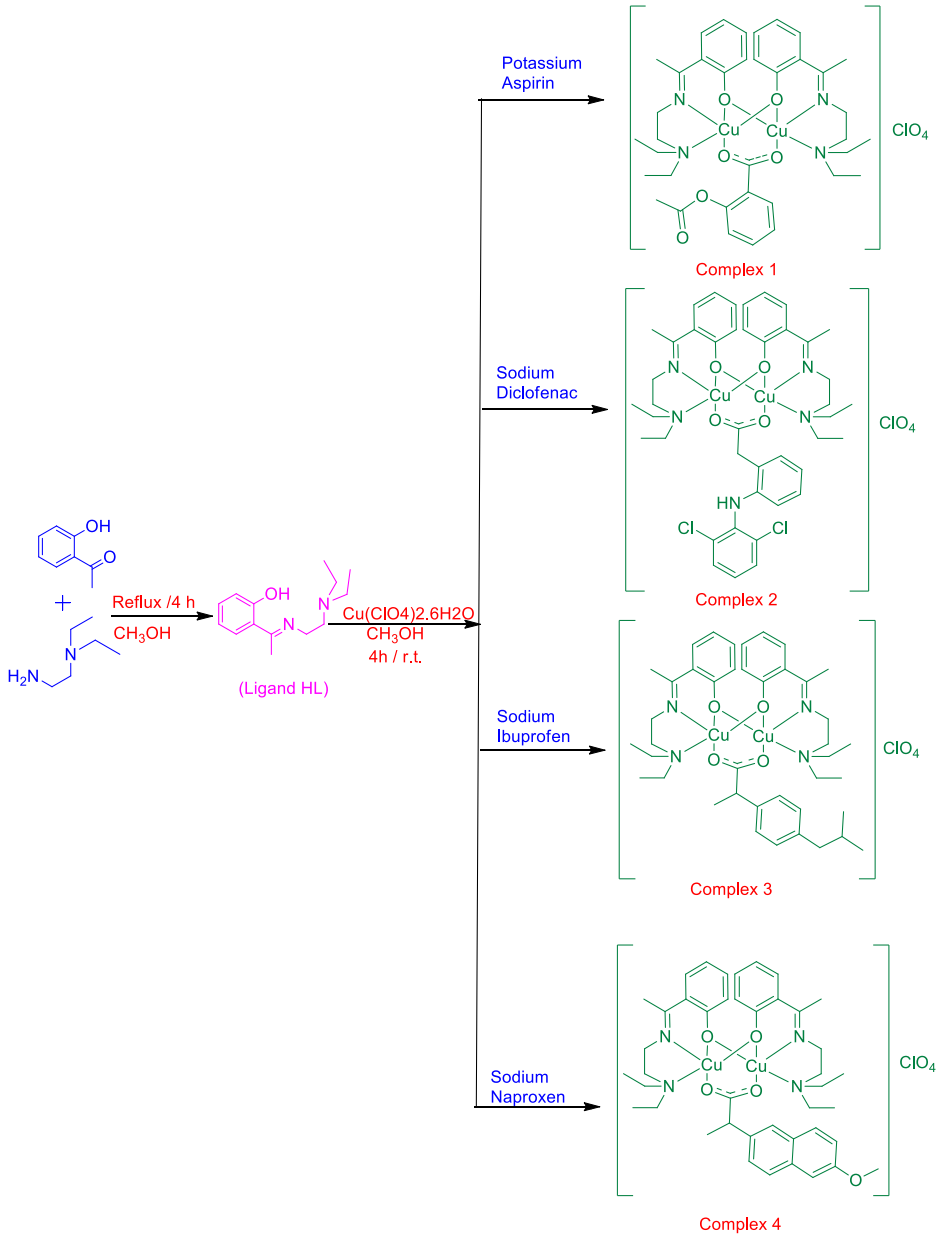
A dark green needle like specimen of complex **1** with approximate dimensions 0.330 x 0.260 x 0.210 mm was used for the X-ray crystallographic analysis. The crystals of other complexes could not be obtained. The X-ray structural studies were performed on a CCD Agilent Technologies (Oxford Diffraction) SUPER NOVA diffractometer. Data for all the complexes were collected at 293K using graphite-monochromated CuKα radiation (λα = 1.54184 Å). The strategy for the data collection was evaluated by using the CrysAlisPro CCD software. The data were collected by the standard 'phi-omega' scan techniques and were scaled and reduced using CrysAlis- Pro RED software. The structures were solved by direct methods using SHELXS-97 and refined by full matrix least squares with SHELXL-97, refining on F^2. The positions of all the atoms were obtained by direct methods. All non-hydrogen atoms were refined anisotropically. The remaining hydrogen atoms were placed in geometrically constrained positions and refined with isotropic temperature factors, generally 1.2Ueq of their parent atoms.

Chapter 4

Results and Discussion

4.1 Syntheses and characterization:

Schiff base ligand HL is formed by reacting 1-(2-hydroxyphenyl)ethanone with N^1,N^1-diethyethane-1,2-diamine in 1:1 molar ratio in methanol (Scheme **1**). The ligand HL was reported previously[69]. HL is reacted with copper perchlorate in 1:1 molar ratio in presence of methanol and trietylamine, which gives green colored solution; further methanolic solutions of aspirin salt is added drop wise which yields more dark green colored solution ,which upon further concentration and slow evaporation furnished green needle shaped crystals of $[Cu_2(HL)_2(asp)]ClO_4$ (Complex **1**) (Scheme **1**). With similar procedure three more complexes were synthesized by using salts of diclofenac, naproxen, and ibuprofen in place of potassium salt of aspirin. The ligand HL has been characterized by ^1H and ^{13}C NMR and ESI-MS spectroscopy. Complexes **1** has been characterized by IR and ESI-MS spectroscopy, single crystal X-ray crystallography and **2, 3** and **4** have been characterized by IR and ESI-MS spectroscopy.



Scheme 1: Reaction scheme for synthesis of Schiff base ligand **HL** and complex **1-4.**

4.1.1. FT-IR spectra:

The IR spectra of ligand and all the complexes (**1- 4**) have a prominent band around 1600 cm^{-1} assignable to $\bar{\upsilon}$(C=N) imine stretching mode (Figure **4.1** – **4.5**). The free ligand has the imine stretching band at $\bar{\upsilon}$ = 1614 cm^{-1} whereas for complexes **1-4**, the same band occurs at $\bar{\upsilon}$= 1594, 1585, 1581, and 1583 cm^{-1} respectively. The shift of this band towards lower frequency

suggests coordination of –C=N- group to the metal ion through imine nitrogen atom [70]. Band near 1580 and 1470 cm^{-1} appears due to carboxylate groups from NSAIDs binding to copper centre, band near 640 cm^{-1}, and a strong band 1075 cm^{-1} occur due to non-coordinated perchlorate group[71].

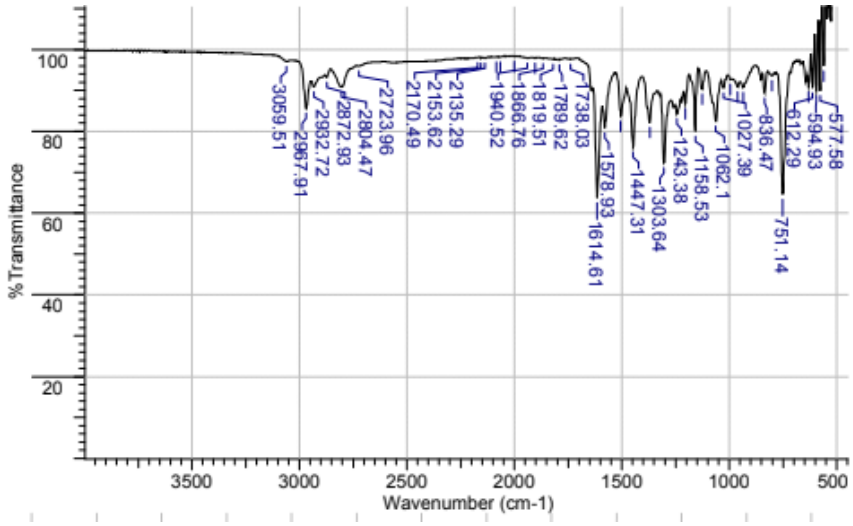


Figure 4.1: IR spectrum of ligand **HL**.

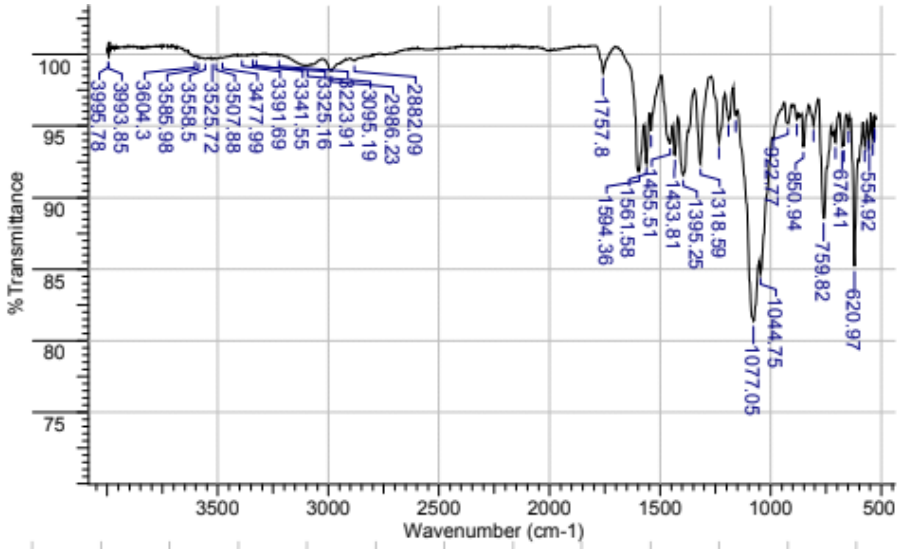


Figure 4.2: IR spectrum of Complex **1**.

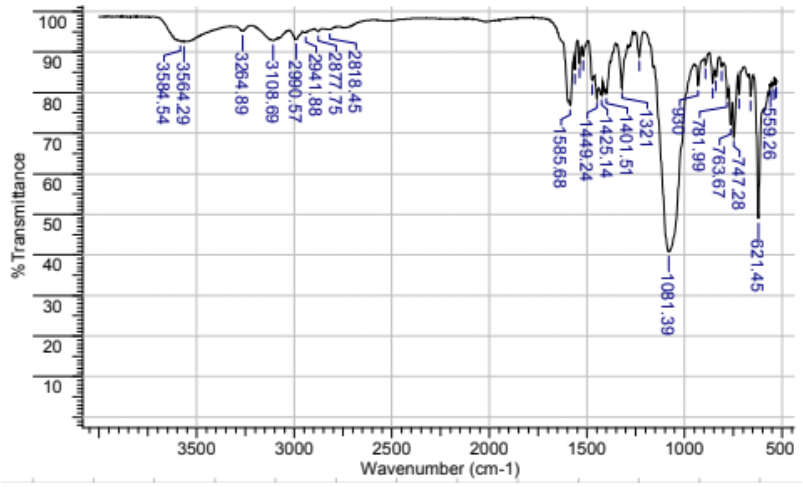


Figure 4.3: IR spectra of complex **2**

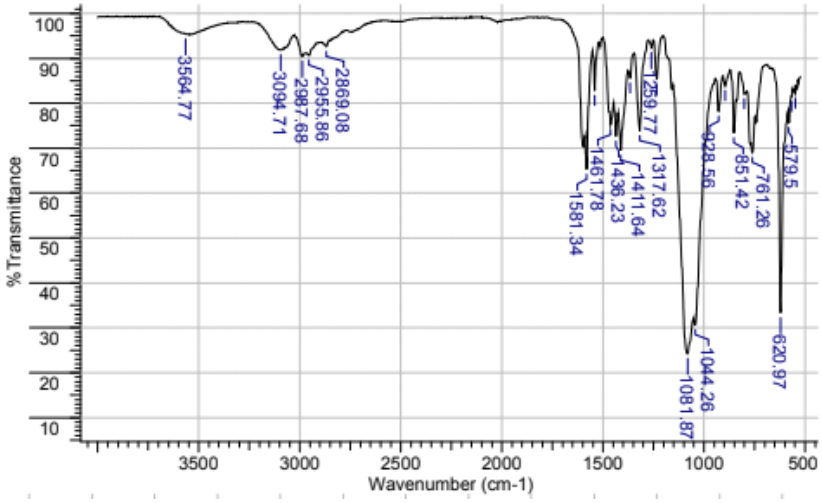


Figure 4.4: IR spectrum of Complex **3**.

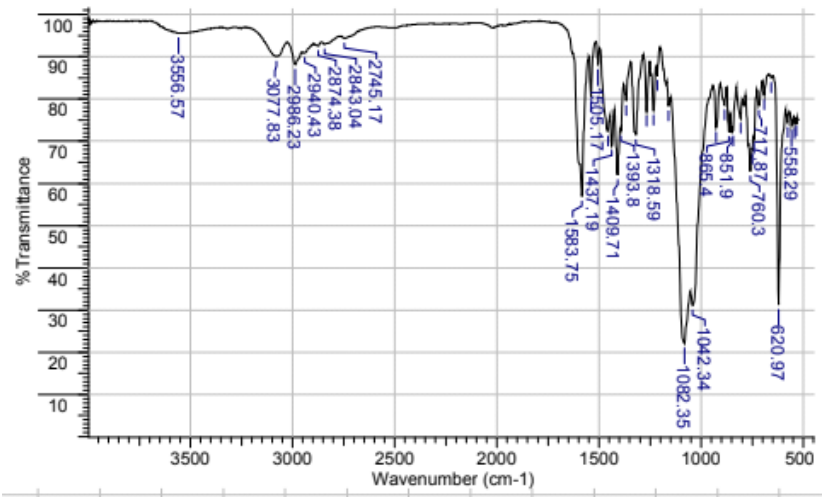


Figure 4.5: IR spectrum of Complex **4**.

4.1.2. NMR spectra:

^{1}H NMR and ^{13}C NMR data were found to be in good agreement with the structure proposed for the ligand H_2L (Figure 4.6 – 4.7).

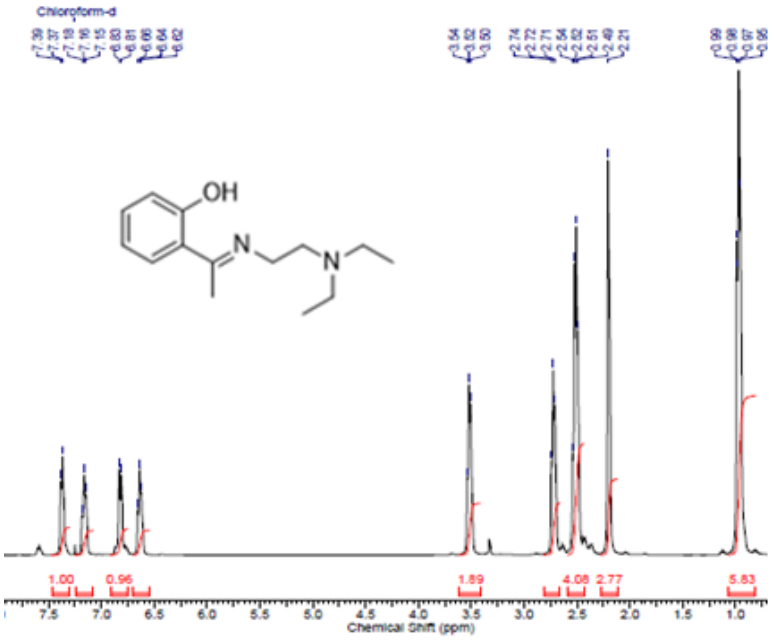


Figure 4.6: ^{1}H NMR data of ligand **HL**.

For ^{1}H NMR, all the four aromatic protons lie in the range of δ = 6.6 to 7.4 ppm. Five Peaks corresponding to seventeen aliphatic protons observed from 0.9 ppm to 3.54 ppm. The chemical shift values for all the proton are given as: *δ*ppm: 0.98 (t, 6H), 2.21 (s, 3H), 2.52 (q, 4H), 2.72 (t, 2H), 3.52 (t, 2H), 6.64 (t, *J* = 8 Hz, 1H), 6.82 (d, 1H J = 8 Hz), 7.16 (t, *J* = 8 Hz 1H), 7.38 (d, *J* = 8 Hz, 1H).

In ^{13}C NMR, a total of 11 peaks were obtained. All of the benzene carbon were observed in the range of δ = 118 to 135 ppm, except for the carbon directly attached to –OH group, which was observed at δ = 164 ppm.

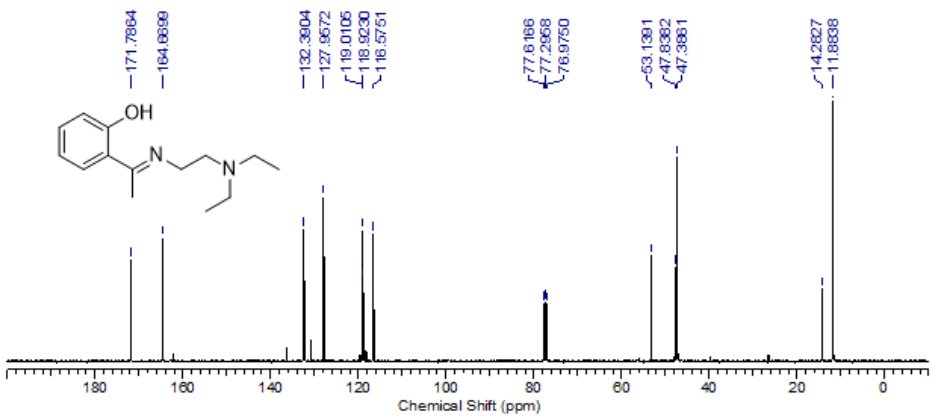


Figure 4.7: ^{13}C NMR data of ligand H_2L

4.1.3. Mass spectra:

The electrospray ionization mass (ESI-MS positive) spectra of Schiff base ligand and all the metal complexes provide reliable evidence for the formation of the suggested structures (Figure **4.8** – **4.12**). The spectra of complex **1-4** suggest that all the complexes are binuclear. Complex **1-4** show $[M\text{-}ClO_4]^+$ molecular ion peak at m/z = 771.2, 888.2, 797.3 and 823.3 respectively, ligand HL shows $[M + H]^+$ molecular ion peak at m/z = 235.92. However in the spectra of complex **3** and **4**, peak at m/z = 296.1

was observed which can be assigned to monocationic specie with formula $[Cu(L)]^{+}$.

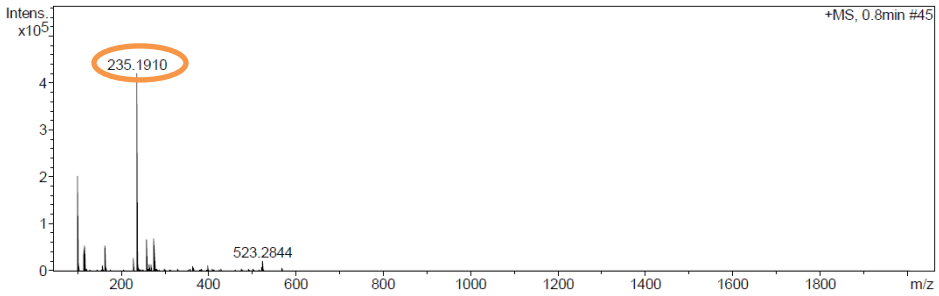


Figure 4.8: ESI-MS spectra of **HL**

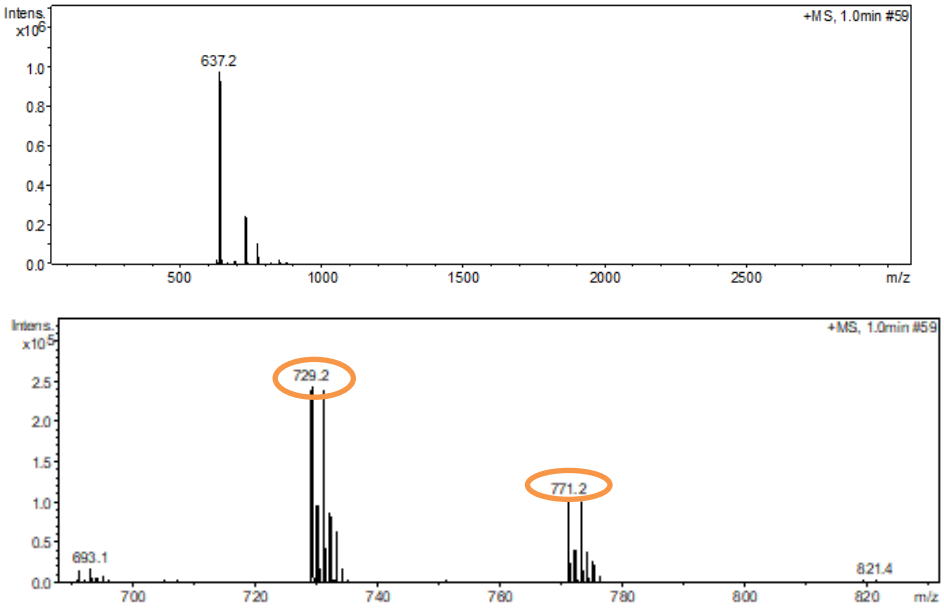


Figure 4.9: ESI-MS spectra of complex **1**.

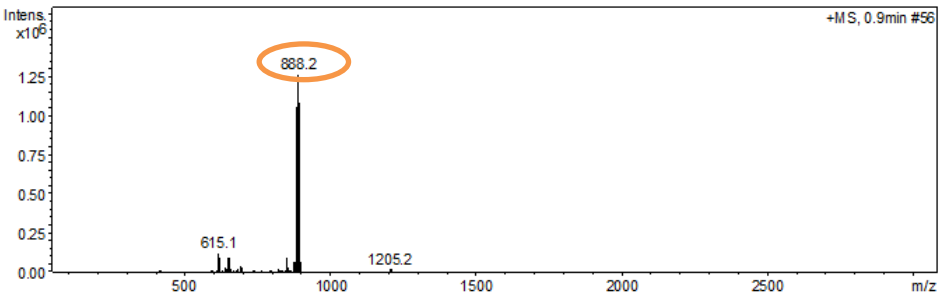


Figure 4.10: ESI-MS spectra of complex **2**.

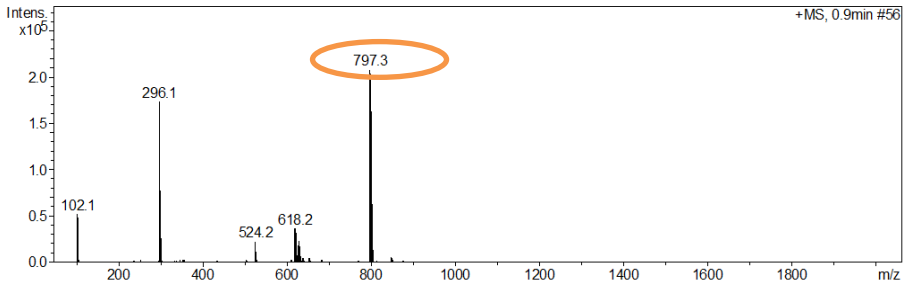


Figure 4.11: ESI-MS spectra of complex **3**.

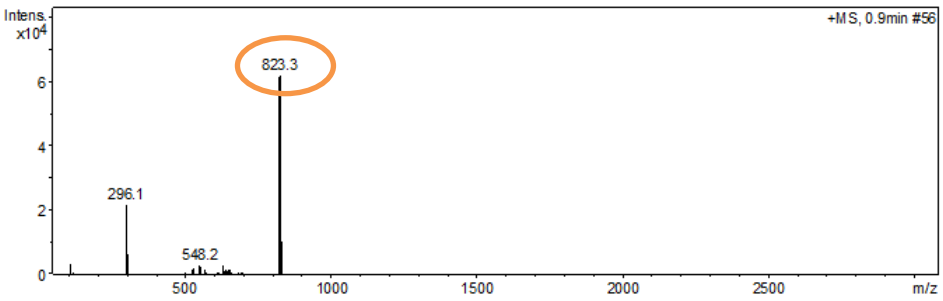


Figure 4.12: ESI-MS spectra of complex **4**.

4.1.4. X-ray crystallography:

Complex **1** has been structurally characterized by X-ray crystallography. The selected bond lengths and angles are given in Table **4.1** and the details of data collection conditions and parameters of refinement process are given in Table **4.2**.

Table 4.1: Selected bond lengths (Å) and bond angles (˚) for complex **1**

Cu(1)-O(1)	1.894(3)	O(2)-Cu(2)-N(4)	170.5(16)
Cu(1)-N(1)	1.941(4)	N(3)-Cu(2)-N(4)	87.04(18)
Cu(1)-O(3)	1.976(3)	O(4)-Cu(2)-N(4)	88.99(16)
Cu(1)-N(2)	2.065(4)	C(1)-O(1)-Cu(1)	124.1(3)
Cu(2)-O(2)	1.901(3)	C(29)-O(3)-Cu(1)	29.3(3)
Cu(2)-N(3)	1.923(4)	C(29)-O(4)-Cu(2)	129.9(3)
Cu(2)-O(4)	1.947(3)	C(7)-N(1)-Cu(1)	126.5(4)
Cu(2)-N(4)	2.062(4)	C(9)-N(1)-Cu(1)	109.9(3)
O(1)-Cu(1)-N(1)	93.91(17)	C(10)-N(2)-Cu(1)	105.4(4)
O(1)-Cu(1)-O(3)	90.58(14)	C(13)-N(2)-Cu(1)	107.8(4)
N(1)-Cu(1)-O(3)	175.50(16)	C(11)-N(2)-Cu(1)	111.7(4)
O(1)-Cu(1)-N(2)	165.73(17)	C(21)-N(3)-Cu(2)	127.7(4)
N(1)-Cu(1)-N(2)	85.15(18)	C(23)-N(3)-Cu(2)	110.1(3)
O(3)-Cu(1)-N(2)	90.43(16)	C(25)-N(4)-Cu(2)	113.3(3)
O(2)-Cu(2)-N(3)	93.41(17)	C(24)-N(4)-Cu(2)	103.4(3)
O(2)-Cu(2)-O(4)	90.06(15)	C(24)-N(4)-Cu(2)	110.6(4)
N(3)-Cu(2)-O(4)	175.21(15)		

Table 4.2: Crystal refinement data for complex **1**

Empirical formula	C_{37} H_{49} Cu_2 N_4 O_6	Z	8
Formula weight	772.88	T (K)	293
Temperature	293(2) K	μ (mm^{-1})	1.103
Wavelength	0.71073 A	F(0 0 0)	3240
Crystal system	Monoclinic	Crystal size (mm^3)	0.330 x 0.260 x 0.210
space group	C 2/c	θ ranges (°)	3.142 - 29.102
a (Å)	13.1433(5)	h/k/l	-17,17/0,24/0,4

			5
b (Å)	18.1562(5)	Reflections collected	9670
c (Å)	34.1701(15)	T_{max} and T_{min}	1.00000 and 0.65488
α (°)	90	Data/restraints/parameters	9670 / 0 / 442
β (°)	99.374(4)	Goodness-of-fit (GOF) on F^2	1.089
γ(°)	90	Final R indices [I > 2σ(I)]	R1 = 0.0772 wR2 = 0.2263
V ($Å^3$)	8045.2(5)	R indices (all data)	R1 = 0.1276 wR2 = 0.2554
λ (Å)	0.71073	Largest peak and hole(e $Å^{-3}$)	1.456 and -0.426
ρ_{calcd} (mg m^{-3})	1.276		

4.1.5. Crystal structure of Complex 1

Complex **1** is binuclear with the monoclinic crystal system and C2/c space group. Copper atom is located in a five-coordinated environment completed by two N atoms and one O atom from HL ligand and the remaining sites are occupied by oxygen atom from carboxylate group of aspirin. The distance between metal and coordination atoms were: Cu(1)-O(1) = 1.894 (3) Å, Cu(1)-N(1) = 1.941(4) Å, Cu(1)-O(3) = 1.976(3) Å, Cu(1)-N(2) = 2.065(4) Å, Cu(1)-O(2) = 1.901(3) Å, Cu(2)-O(4) = 1.947(3) Å, Cu(2)-N(4) = 2.062(4) Å . The selected angles between metal and coordination atoms are found to be to the tune of previously reported structures[70]. The space configuration of five-coordinated complexes could be well defined by the τ value [τ = (β – α)/60 where α and β being the two largest coordination angles], τ = 0 for an ideal square pyramid as well as τ = 1 for an ideal triangular bipyramid. For complex **1** the value of τ = 0.058, thus it forms slightly distorted square pyramidal geometry. Figure 4.13 shows the ORTEP diagram of complex **1**.

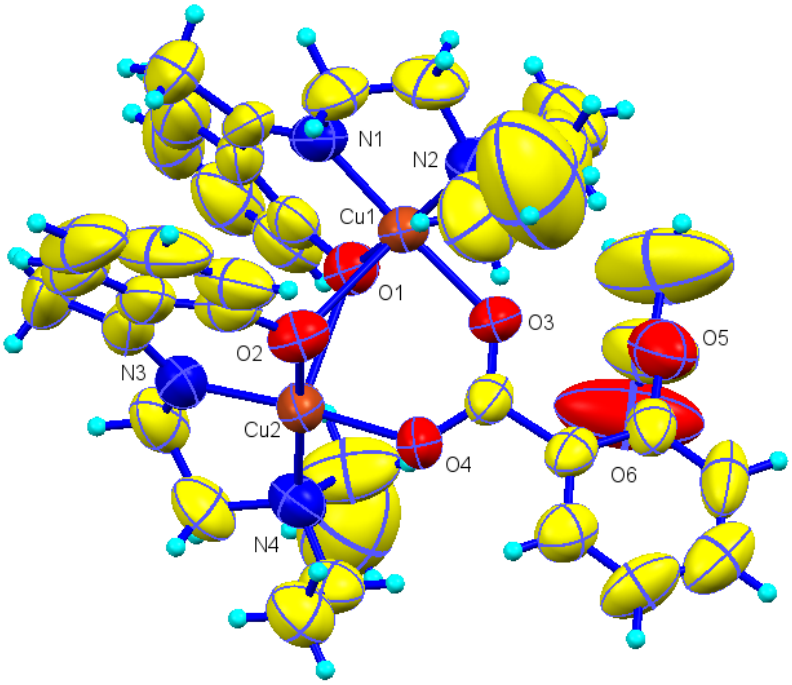


Figure 4.13: ORTEP diagram of complex **1**

4.2 HSA binding study:

To understand the interaction of synthesized complexes with proteins, their effect on the intrinsic fluorescence emission band of human serum albumin has been analyzed. For this purpose separate solution of HSA was titrated against addition of complexes **1-4**. It was observed that significant decrease of initial fluorescence intensity (figure **4.14**) is caused by the addition of complexes to the respective protein solution.

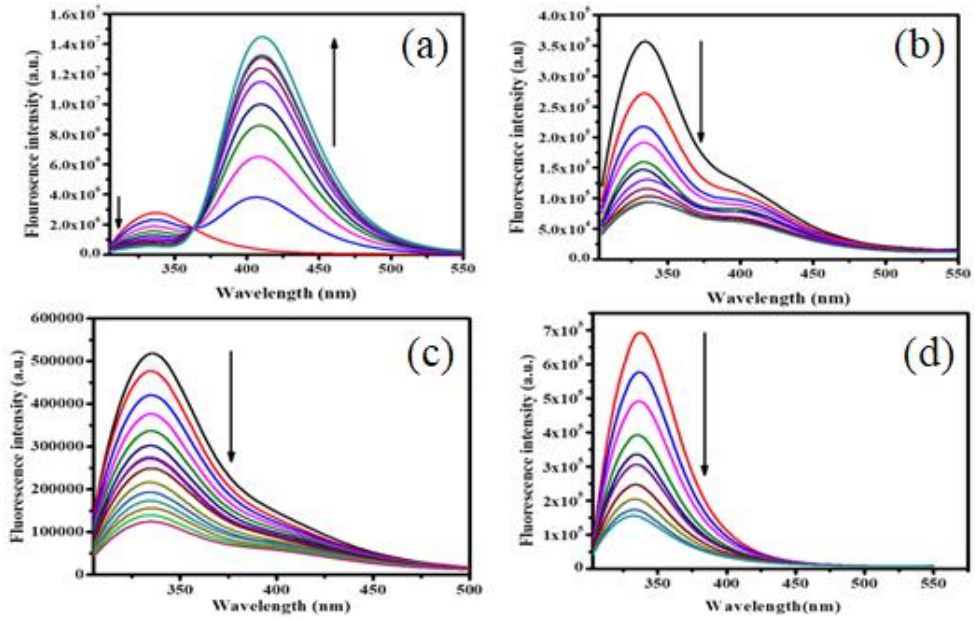


Figure 4.14: Fluorescence quenching of HSA by (a) complex **1,** (b) complex **2**, (c) complex **3** and (d) complex **4**.

To get further insight in the quenching process, the fluorescence quenching data were analyzed with Stern-Volmer equation, (graphs shown in figure **4.15**) according to which:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q]$$

where F_0 and F are the fluorescence intensities in the absence and the presence of a quencher, k_q is the bimolecular quenching rate constant, τ_0 is the average lifetime of fluorophore in the absence of a quencher and [Q] is the concentration of a quencher (metal complexes). K_{SV} is the Stern–Volmer quenching constant in M^{-1}.The K_b value obtained for complexes **1-4** are of order 10^4-10^5 (M^{-1}) with complex **1** showing maximum affinity for protein binding with $K_b = 4.5795 \times 10^5$ (M^{-1})

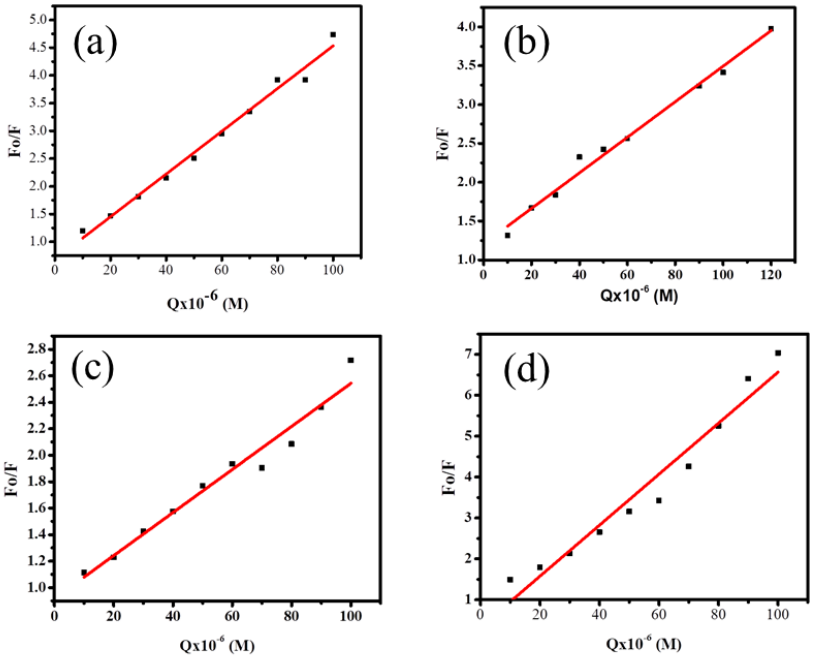


Figure 4.15: Stern volmer plot for HSA binding (a) complex **1,** (b) complex **2**, (c) complex **3** and (d) complex **4**.

The binding constant (K_b) and number of binding sites (n) have been determined by Scatchard plot (figure **4.16**), equation of which given by

$$\log \left[\frac{F_0 - F}{F} \right] = \log K_b + n \log [Q]$$

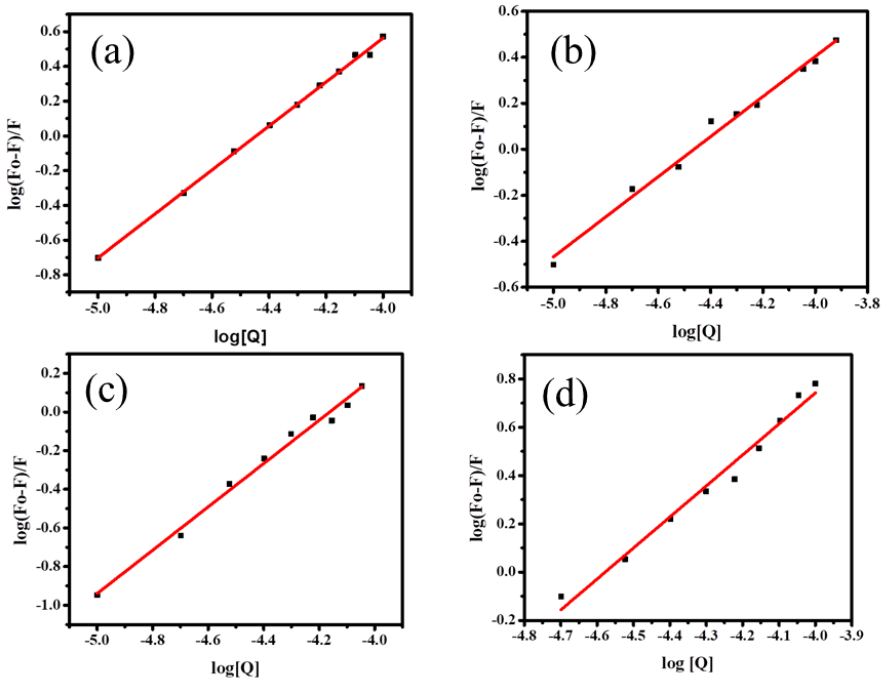


Figure 4.16: Scatchard plot for HSA binding (a) complex **1,** (b) complex **2**, (c) complex **3** and (d) complex **4**.

The calculated values of Ksv, n and kq for the interaction of the complexes with the HSA are given in Table 4.3 indicating substantial HSA binding affinity of the complexes.

Table 4.3: Table for stern-volmer constant, quenching constant, binding constant and number of binding sites for HSA binding complex **1– 4**.

Complex	K_{SV} (M^{-1})	k_q (M^{-1} s^{-1})	K_b (M^{-1})	n
Complex 1	3.8531×10^4	2.3108×10^{12}	4.1654×10^5	1.12
Complex 2	2.2895×10^4	1.3818×10^{12}	0.0239×10^5	0.87
Complex 3	0.9897×10^4	8.5557×10^{12}	4.5795×10^5	1.12
Complex 4	3.7672×10^4	7.2945×10^{12}	1.9105×10^4	1.90

4.3 BSA binding study:

Separate solutions of BSA were also titrated against addition of complexes **1-4** at excitation wavelength at 296, excitation spectra was studied at 340 nm. It was observed that significant decrease of initial fluorescence intensity (figure **4.17**) is caused by the addition of complexes to the respective protein solution. Fluorescence quenching data were analyzed with Stern-Volmer equation and Scatchard equation.

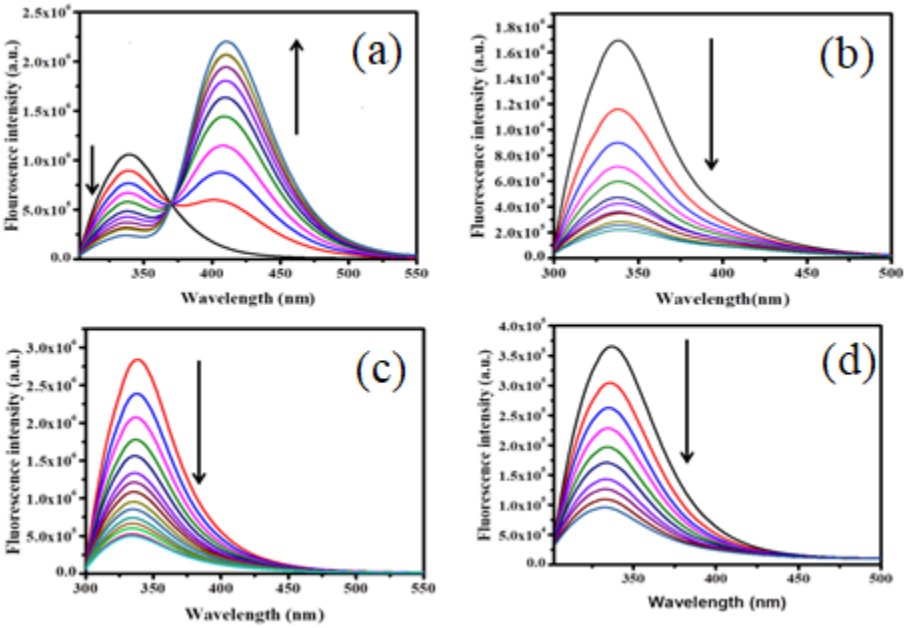


Figure 4.17: Fluorescence quenching of BSA by (a) complex **1,** (b) complex **2**, (c) complex **3** and (d) complex **4**.

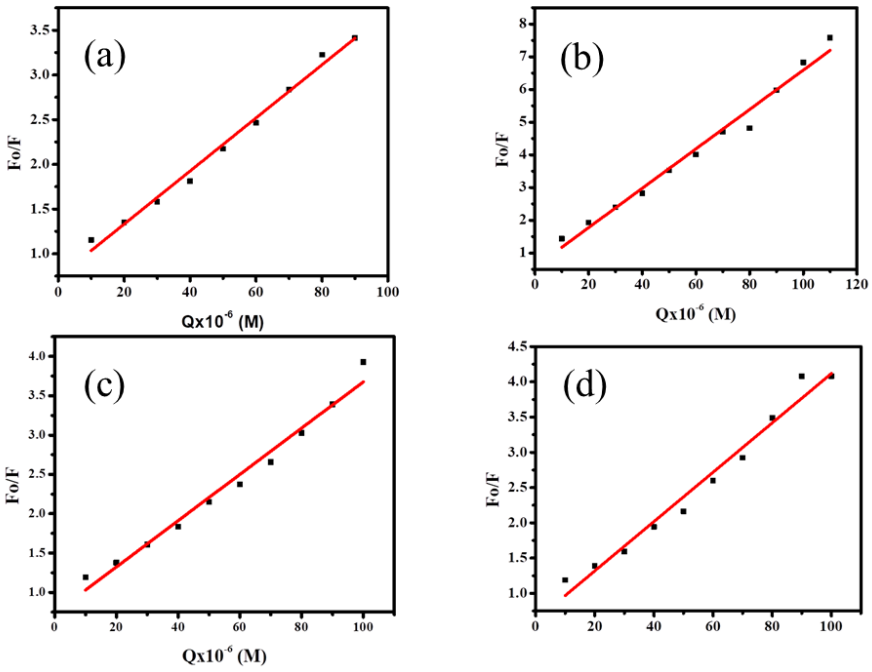


Figure 4.18: Stern volmer plot for BSA binding (a) complex **1**, (b) complex **2**, (c) complex **3** and (d) complex **4**.

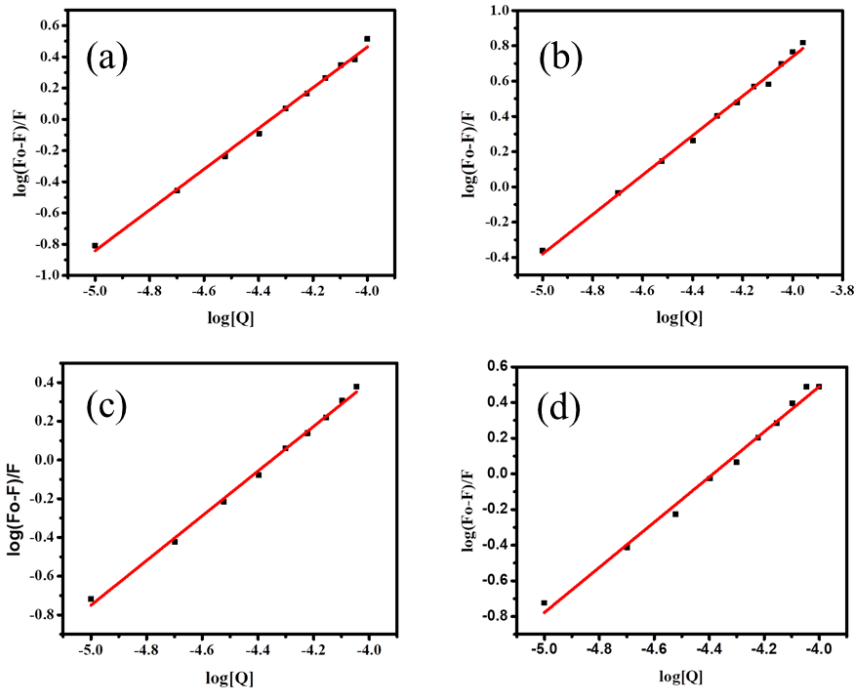


Figure 4.19: Scatchard plot for BSA binding (a) complex **1,** (b) complex **2**, (c) complex **3** and (d) complex **4**.

Table 4.4: Table for stern-volmer constant, quenching constant, binding constant and number of binding sites for BSA binding complex **1– 4**.

Complex	K_{SV} (M^{-1})	k_q (M^{-1} s^{-1})	K_b (M^{-1})	n
Complex 1	2.9422×10^4	1.7685×10^{12}	1.0963×10^3	1.34
Complex 2	6.0295×10^4	3.6158×10^{12}	7.4123×10^2	1.18
Complex 3	2.9434×10^4	1.7657×10^{12}	2.3481×10^2	1.15
Complex 4	3.4276×10^4	2.0595×10^{12}	2.3941×10^4	1.26

4.4 DNA binding studies

DAPI is a fluorogenic probe that binds to minor groove of DNA, if there is other molecular species present in solution that binds with minor groove of DNA, there will be competition between DAPI and molecular specie to bind with DNA. In all complexes flouroscence quenching was observed that is due to displacement of DAPI from ct-DNA. Fluorescence quenching data were analyzed with Stern-Volmer equation and Scatchard equation.

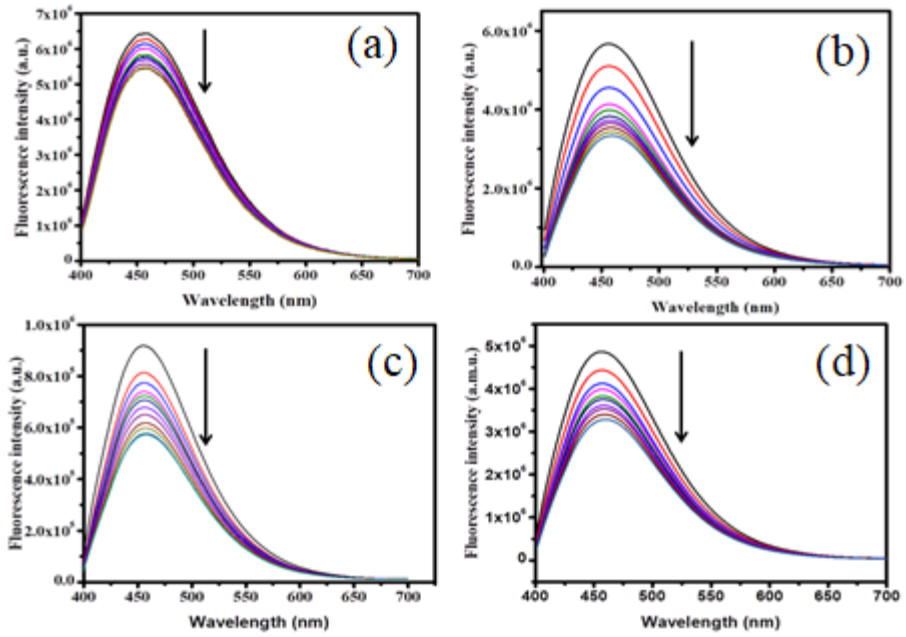


Figure 4.20: Fluorescence quenching of ct-DNA- DAPI by (a) complex **1,** (b) complex **2**, (c) complex **3** and (d) complex **4**.

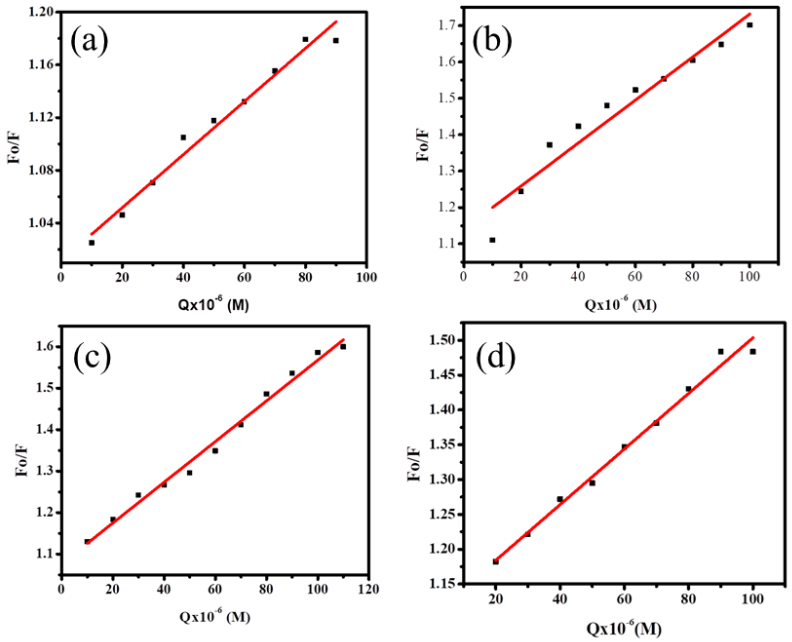


Figure 4.21: Stern volmer plot for DNA binding complex **1(**a**),** complex **2**(b), complex **3**(c) and complex **4**(d).

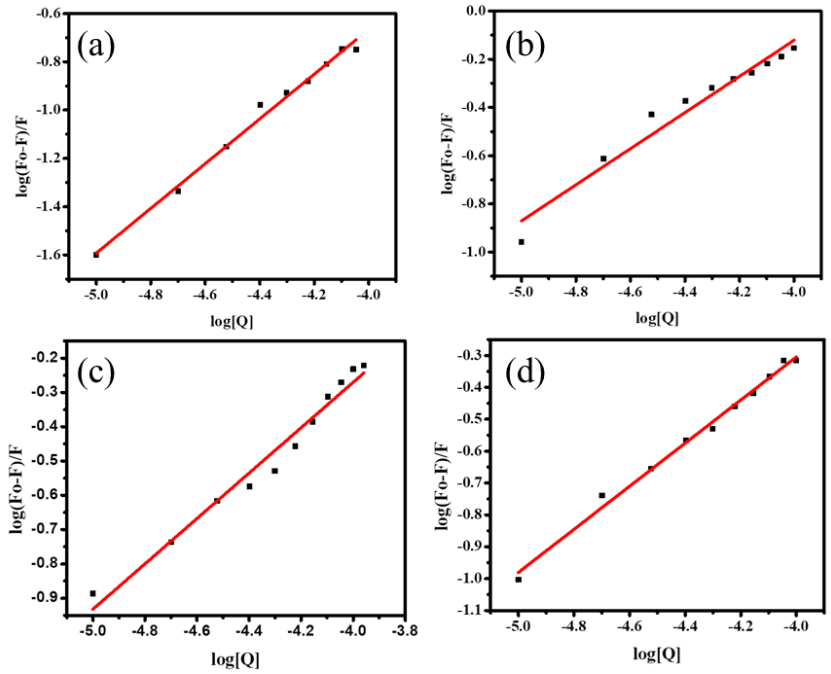


Figure 4.22: Stern volmer plot for DNA binding (a) complex **1,** (b) complex **2**, (c) complex **3** and (d) complex **4**.

Table 4.5: Table for stern-volmer constant, quenching constant, binding constant and number of binding sites for DNA binding complex **1– 4**.

Complex	K_{SV} (M^{-1})	K_b (M^{-1})	N
Complex 1	2.9422×10^4	1.0963×10^3	0.92
Complex 2	6.0295×10^4	7.4123×10^2	0.74
Complex 3	2.9434×10^4	2.1881×10^2	0.66
Complex 4	3.4276×10^4	2.4541×10^4	0.67

4.5. Cytotoxicity by MTT assay

The potential anti proliferative impacts of the compounds on cancer cell line MCF7 and A549 were tested by the regular MTT assay. NSAID does not show cytotoxicity activities on MCF7 and A549, but metal complex **1- 4** shows cytotoxic activities against MCF7 and A549, Complex **1- 4** shows IC_{50} in between 60 – 80 µM against MCF7 cell line having highest cytotoxicity for complex **1**, IC_{50} at 61.23 µM in 24 hour time period and

lowest for complex **4** at 76.03 µM. complex **1** and **3** shows cytotoxic activity against A549 cell line shows IC_{50} values at 16.23 and 120.26 µM respectively in 24 hour time period.

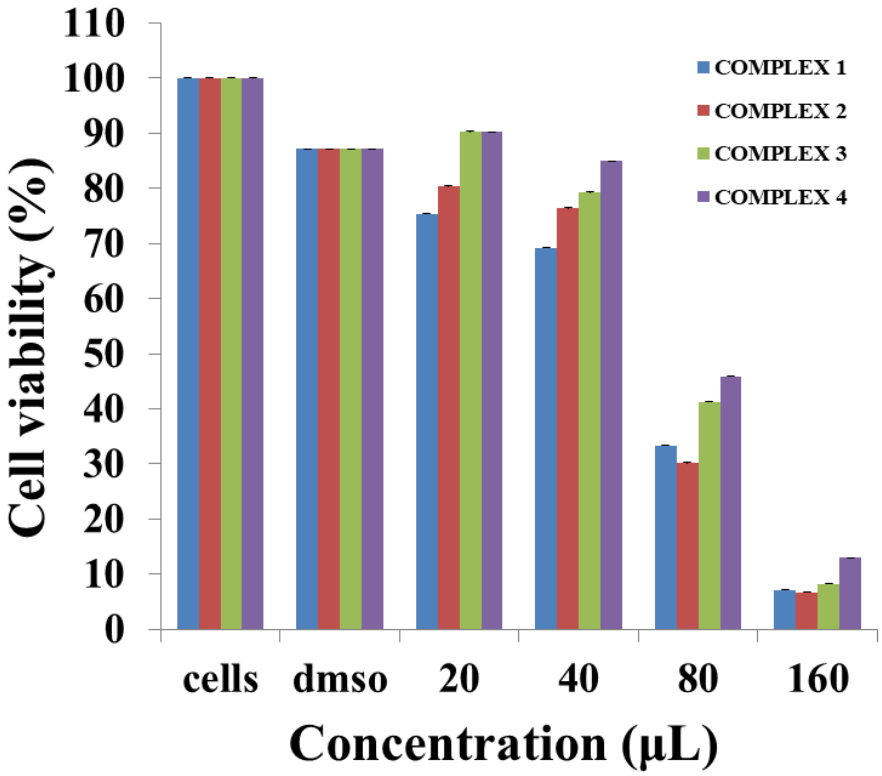


Figure 4.23: Cell viability of MCF7 cells after treatment with complexes **1-4** for 24 hours.

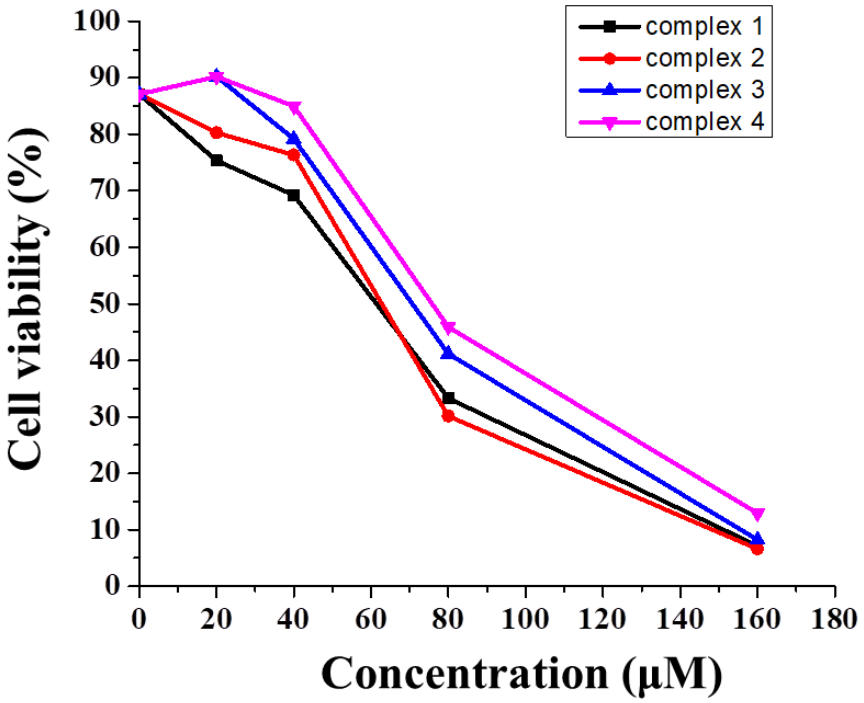


Figure 4.24: Cell viability of MCF7 cells after treatment with complexes **1-4** for 24 hours.

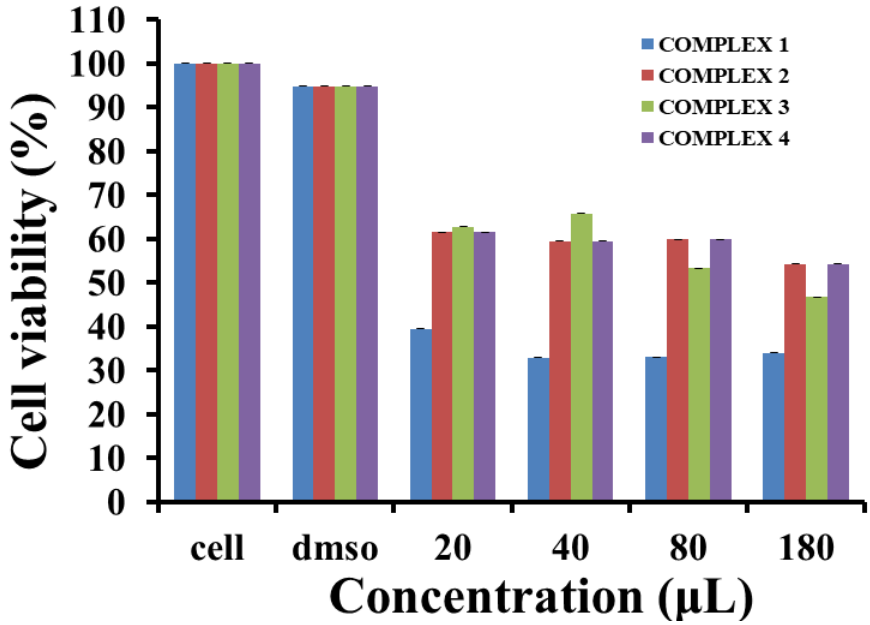


Figure 4.25: Cell viability of A549 cells after treatment with complexes **1-4** for 24 hours.

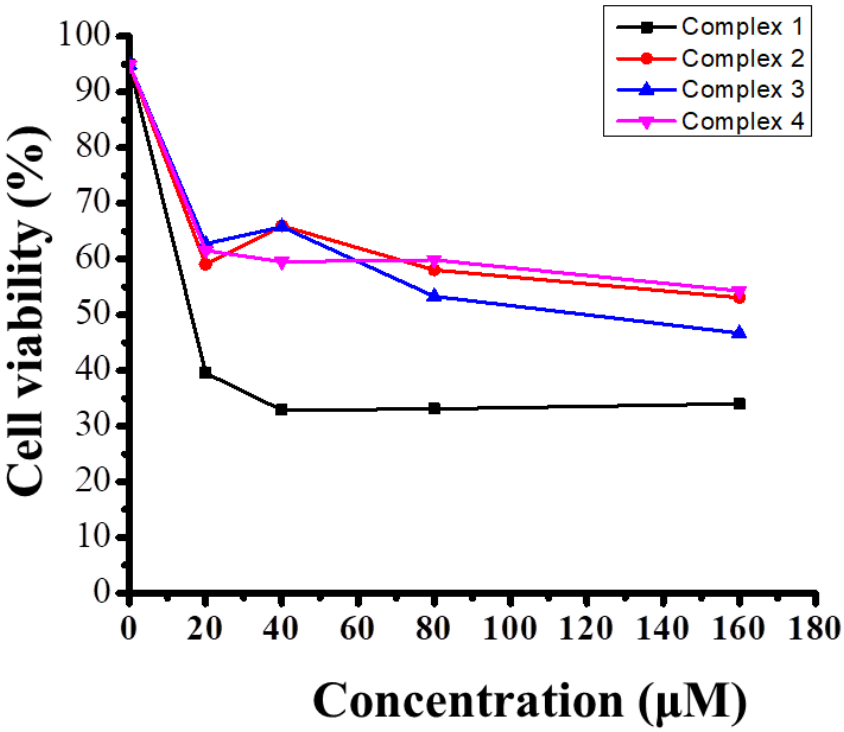


Figure 4.26: Cell viability of A549 cells after treatment with complexes **1**-**4** for 24 hours

Chapter 5

Conclusion and Future Scope

Four new binuclear complexes based on Schiff-base ligand and NSAID have been synthesised and characterized successfully. All the four complexes showed BSA, HSA binding, DNA binding activity. Complex **1-4** has shown cell cytotoxic activity against breast cancer cell line MCF7, also complexes **1** and **3** have shown cell cytotoxic activity against lung cancer cell line A549 in 24 hour time period.

Although free NSAID does not shows cytotoxic activity against A549 cell line, but with complexation to copper, they shows cytotoxic activity, the results obtained indicate that copper complex with Schiif Base ligand with "ONO" donor set and NSAID can form interesting copper based complexes, which can show better cytotoxic activities. In future more compounds can be formed with different NSAIDs and different set of schiff bases and can be tested for biological activities.

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