CHAPTER II
EXPERIMENTAL SECTION

2.1. Chemicals and Reagents

All the analytical grade (A.R.) chemicals were used without further purification for the syntheses of various compounds and metal complexes. However, for spectroscopic techniques and conductivity measurements only proper spectroscopic grade solvent were used. The list of various chemicals and reagents are given in Table 2.1.

Table.2.1. List of chemicals and reagents.

Chemicals	Molecular Weight (g/mol)	Source	CAS No.
3, 3' Methylenedianiline	198.26	Sigma-Aldrich, Germany	19471-12-6
2-hydroxy-5-nitro benzaldehyde	167.12	Sigma-Aldrich, Germany	97-51-8
5- bromo salicylaldehyde	201.02	Sigma-Aldrich, Germany	1761-61-1
1-methyl imidazole	82.10	Sigma-Aldrich, Germany	616-47-7
2-bromoethylamine hydrobromide	204.89	Sigma-Aldrich, Germany	2576-47-8
Potassium hexa fluorophosphate	184.06	Sigma-Aldrich, Germany	17084-13-8
Salicylaldehyde	122.12	Sigma-Aldrich, Germany	90-02-8
4-chloroaniline	127.57	Sigma-Aldrich, Germany	95-51-2

4-hydroxy benzoic acid	138.12	Sigma-Aldrich, Germany	99-96-7
Ethidium bromide	394.31	Sigma-Aldrich, Germany	1239-45-8
Deoxyribonucleic acid sodium salt from calf thymus		Sigma-Aldrich, Germany	73049-39-5
Trizma hydrochloride	157.60	Sigma-Aldrich, Germany	1185-53-1
Cobalt acetate	177.021	Sigma-Aldrich, Germany	71-48-7
Zinc acetate. $2H_2O$	219.50	Thomas Baker	5970-45-6
Copper(II) nitrate trihydrate	10031-43-3	Sigma-Aldrich, Germany	241.60
Acetone (AR)	58.08	S. D. Fine Chemicals, India	67-64-1
Methanol	32.04	Sigma-Aldrich, Germany	67-56-1
Ethanol	46.07	S. D. Fine Chemicals, India	64-17-5
Dimethylformamide	73.09	S. D. Fine Chemicals, India	68-12-2
Diethylether	74.12	S. D. Fine Chemicals, India	60-29-7
Sodium nitrite	68.99	Merck, India	7632-00-0
Sodium Hydroxide	39.99	Merck, India	1310-73-2
Sodium carbonate	105.98	Merck, India	497-19-8
Spectroscopic grade	18.02	S. D. Fine	7732-18-5

water (For Spectroscopy)		Chemicals, India	
Dimethylsulfoxide (For spectroscopy)	78.13	S. D. Fine Chemicals, India	67-68-5

2.2. Physico-chemical methods used to characterize synthesized compounds:

To characterize the structure of synthesized ligands and their metal complexes, Different Physico-chemical techniques have been used. The descriptions of these methods are given below:

2.2.1. Elemental analysis:

Euro VECTOR EA 3000 analyzer was used for the elemental micro-analyses (C, H and N) of all the synthesized compounds. The metal contents of the complexes were determined with Atomic Absorption Spectrophotometer (Varian SpectrAA 50B) by using suitable standard metal solutions procured from Sigma-Aldrich, Germany.

Fig.2.1. Atomic Absorption Spectrophotometer (Varian SpectrAA 50B).

2.2.2. Thin layer chromatography:

The thin layer chromatography (TLC) on silica gel plates were used to confirm the purity of the prepared ligands and the complexes. UV-light was used to view the TLC plate and iodine is used as and when required.

2.2.3. Magnetic susceptibility measurement:

Magnetic susceptibilities were measured at room temperature with a Sherwood Scientific Ltd magnetic susceptibility balance (Magway MSB Mk1). The MSB works on the basis of a stationary sample and moving magnets. The pairs of magnets are placed at opposite ends of a beam so placing the system in balance. Introduction of the sample between the poles of one pair of magnets produces a deflection of the beam that is registered by means of phototransistors. A current is made to pass through a coil mounted between the poles of the other pair of magnets, producing a force restoring the system to balance. At the position of equilibrium, the current through the coil is proportional to the force exerted by the sample and can be measured as a voltage drop.

The solid sample is tightly packed into weighed sample tube with a suitable length (*l*) and noted the sample weight (*m*). Then the packed sample tube was placed into tube guide of the balance and the reading (*R*) was noted. The mass susceptibility, χ_{g} is calculated using by equation 1:

$$\chi_{\mathrm{g}} = C_{\mathrm{Bal}} \times l \times \frac{(R - R_0)}{m \times 10^9} \quad \textbf{(1)}$$

where l = the sample length (in cm), m = the sample mass (in g), R = the reading for the tube plus sample, R_0 = the empty tube reading and C_{Bal} = the balance calibration constant. Thus molar susceptibility is $\chi_{\mathrm{M}} = \chi_g \times \mathrm{M.Wt\ of\ the\ sample}$. The molar susceptibility is the corrected with diamagnetic contribution. The effective magnetic moment μ_{eff} is calculated by using equation 2:

$$\mu_{eff} = 2.83\sqrt{T \times \chi_A} \quad \mathrm{B.M} \quad (2)$$

where χ_A is the corrected molar susceptibility. The MSB was calibrated with $Hg[Co(SCN)_4]$ before the actual measurements.

Fig 2.2. The magnetic susceptibility balance (Magway MSB Mk1).

2.2.4. Infrared spectroscopy:

FTIR spectra were recorded in KBr pellets with a Perkin-Elmer Spectrum FTIR spectrometer (RX-1) working in the range 4000-400 cm^{-1} at ambient temperature. KBr used for preparing the pellets was dryed in a hot air oven for 24 hours and then kept in vacuum desiccators over anhydrous $CaCl_2$ before use.

Fig 2.3. Perkin-Elmer Spectrum FT-IR spectrometer (RX-1).

2.2.5. Electronic spectroscopy

UV-Visible spectra of the ligands and their complexes were recorded in a quartz cell (1 cm path length) on a Jasco (V-530) double beam spectrophotometer equipped with a thermostated bath (maintained at 25±0.1 °C) using either water or DMSO as the reference solvent in accordance with the solubility of the compounds.

Fig 2.4. Jasco V-530 double beam UV-VIS Spectrophotometer.

2.2.6. Nuclear Magnetic Resonance (NMR) spectroscopy (^{1}H NMR and ^{13}C NMR):

^{1}H and ^{13}C NMR spectra were recorded on a FT-NMR (Bruker Avance-II 400 MHz) spectrometer using D_2O, $CDCl_3$ and DMSO-d_6 as the solvent. Chemical shifts **(*δ*)** are quoted in ppm downfield of internal standard tetramethylsilane (TMS).

Fig 2.5. FT-NMR (Bruker Avance-II 400 MHz).

2.2.7. ESI-MS:

Mass spectra were recorded on a Waters ZQ-4000 spectrometer using water and chloroform as the solvent. Chemical shifts **(δ)** are quoted in ppm downfield of internal standard tetramethylsilane (TMS).

Fig 2.6. ESI-MS (Waters ZQ-4000 spectrometer).

2.2.8. Conductivity

Specific conductances were measured at (298.15 ± 0.01) K with a Systronic conductivity TDS-308 meter. The conductance measurements were carried out by using a dip-type immersion conductivity cell, CD-10 with a cell constant of 1.0 ± 10% cm^{-1}. The instrument was standardized by using 0.1 (M) aqueous KCl solution. Measurements were made in a thermostatic water bath maintained at the experimental temperature with an accuracy of ± 0.01 K. The molar conductance of the complexes (Λ_m) was determined by using the relation $\Lambda_m = 1000 \times \kappa / c$, where c and κ stands for the molar concentration of the metal complexes and specific conductance, respectively. The complexes (1×10^{-3} M) were dissolved in respective solvent and their specific conductance was measured at (298.15±0.01) K.

Fig 2.8. Systronic conductivity TDS-308 meter.

2.2.9. Mass measurements:

Mass measurements were carried out on digital electronic analytical balance (Mettler Toledo, AG 285, Switzerland) as shown in Fig 2.9. This Digital balance can measure mass to a very high precision and accuracy. The mass measurements were accurate to ± 0.01 mg.

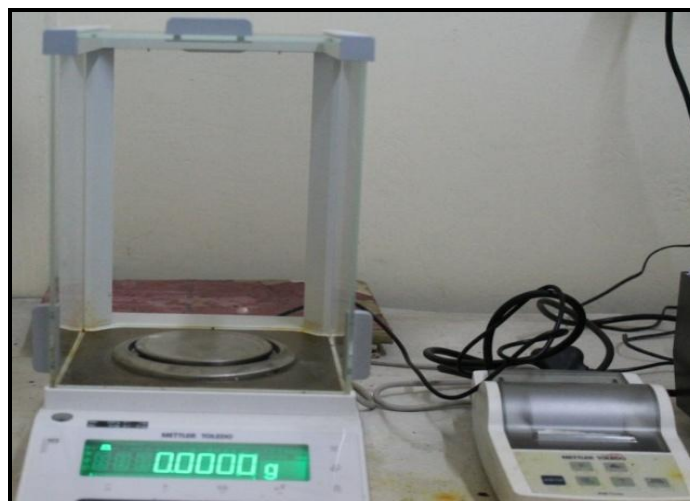

Fig 2.9. Digital electronic analytical balance (Mettler Toledo, AG 285).

2.3. DNA interaction study:

2.3.1 Absorption study:

The DNA binding experiment of synthesized metal complexes with CT-DNA were performed in Tris-HCl buffer (50 mM, pH 7.2). The ratio of UV absorbance at 260 and 280 nm was used to acutain protein contamination of DNA. If this ratio >1.86 for CT-DNA solution, DNA is considered to be was free from protein contamination [1]. The CT-DNA concentration is also determined by absorption spectroscopy at 260 nm (ε_{260}= 6600 mol^{-1} cm^{2}). The absorbances measurements were recorded for solution with a constant/preferred concentration of the synthesized complex (10 μM) but with varied concentration of the CT-DNA. The stock aqueous solutions of the complexes were prepared in the range of 1×10^{-4} M and such solution were then diluted with Tris-HCl buffer to get the desired experimental concentrations. During the titration equal amount of CT-DNA stock solutions were added to both the complex solutions and the reference solutions to eliminate the effect of CT-DNA absorbance. Using the absorption data, the intrinsic binding constant (K_b) can be obtained as usual [2] by plotting [DNA] / (ε_a - ε_f) *versus* [DNA] using Wolfe-Shimer equation [3] as shown below:[3]

$$\frac{[\mathrm{DNA}]}{\varepsilon_a - \varepsilon_f} = \frac{[\mathrm{DNA}]}{\varepsilon_b - \varepsilon_f} + \frac{1}{K_b(\varepsilon_a - \varepsilon_f)} \quad (3)$$

where, ε_a, ε_b and ε_f correspond to apparent extinction coefficient of the complex, A_{obs}/[complex], extinction coefficient for the complex in fully bound form, and in free form, respectively. The intrinsic binding constant (K_b) can be obtained from the ratio of slope to intercept.

2.3.2. Ethidium Bromide (EB) competitive study with fluorescence emission spectroscopy:

To investigate the effectiveness of binding modes of the complexes to DNA, a competitive binding study was performed by following EB displacement strategy through fluorescence emission spectroscopy. The competitive binding experiment was carried out

in Tris-HCl buffer by keeping a constant ratio of concentration of [DNA]/[EB] =1.13 constant and varying the complex concentrations from 0 to 60 μM. During this experiment the emission spectra were recorded in the wavelength range 550-700 nm by exciting the EB-DNA conjugate at the wavelength 540 nm (i.e., λ_{ex}=540 nm). The obtained spectra were analyzed by using the classical Stern–Volmer equation[4] which is given below as equation [4]:

$$\frac{I_0}{I} = 1 + K_{SV}[Q] \quad (4)$$

where I_0 and I are the fluorescence intensities in the absence and presence of the quencher, respectively, K_{sv} is the linear Stern–Volmer quenching constant, Q is the concentration of the quencher. The relative binding affinities of the complexes to CT-DNA was determined from the (K_{sv}) values.

2.3.3. Thermal denaturation study:

Thermal denaturation studies of DNA were carried out by Jasco UV-Visible V-530 model spectrophotometer equipped with a temperature controlling programme assembly that helps to raise the temperature of the experimental solutions by 1 $^{\circ}$C min $^{-1}$. The absorbance of the CT-DNA solutions (100 μM) both in absence and presence of soluted metal complexes (10 μM) were monitored continuously at 265 nm [4].

2.3.4. Viscosity measurements:

Viscosities of the experimental solutions determined through Ostwald capillary type viscometer thermostated at 25 ± 1 $^{\circ}$C. The concentration of CT-DNA was kept constant (100 μM) during the measurement and concentration of ligand/complex solution was gradually increased (10 -100 μM). With a digital stopwatch the flow times were measured with an accuracy of ± 0.20 s. The relation [5] used to calculate the viscosities:

$$\eta = (t - t^o)/ t^o \quad (5)$$

where, t is the flow time of CT-DNA containing ligand or complex solution and t^o is the flow time of buffer alone in seconds.[2] The obtained data were presented as $(\eta/\eta_o)^{1/3}$ versus 1/ R (R = [DNA]/[Complex]), where η_o and η is the viscosity of DNA in the absence and that in the presence of the ligand or complex in experimental solutions, respectively.

2.4. DNA cleavage study:

The DNA cleavage reaction was investigated by gel electrophoresis technique. The DNA cleavage activity was adjudged by examining the transformation of supercoiled form of pBR322 DNA (Form I) to circular (Form II) and linear (Form III) form. To perform cleavage reaction, solution of pBR322 DNA was prepared in Tris-HCl buffer and each reaction mixture contains 20µM DNA, 200µM H_2O_2 and 5-15µM synthesized complex. After incubation for 2 hours, 2 µL of the loading buffer and 1% agarose gel (containing 1 µg/cm^3 ethidium bromide) was mixed to each reaction mixture. The electrophoresis was performed in Tris-acetic acid-EDTA buffer for 2h at 50V. After the completion of electrophoresis, the gel was captured photographically under UV light.

2.5. Antioxidant and free radical scavenging activity study:

Several biochemical experiments were performed to determine the antioxidant or free radical scavenging activities of the synthesized complexes.

2.5.1. DPPH radical scavenging assay:

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity was monitored by using a literature method given by Saha *et al* [5]. Various concentrations of complexes were prepared and mixed with freshly prepared DPPH solution (1mM; diluted in 95% methanol) and kept in dark. After 30 minutes of reaction absorption was measured at 517 nm to determine the percent of inhibition and compared with the standard ascorbic acid. The percent of inhibition was calculated using the following relation [6]:

$$\text{Percentage of scavenging} = \frac{A_0 - A_1}{A_0} \times 100 \qquad (6)$$

where, A_0= absorbance of the control and A_1= absorbance in the presence of samples and standard.

2.5.2. Hydroxyl radical scavenging assay:

Hydroxyl radical scavenging activity was studied according to a standard protocol with slight modification.[6] Hydroxyl radical ($OH^•$) was generated through Fenton reaction and inhibition was measured at 532 nm. Mannitol was used as a standard for hydroxyl radical scavenging activity. Equation [7] was used for calculation of percent of inhibition.

2.5.3. Superoxide radical scavenging assay:

Superoxide radical scavenging assay was studied by following a literature method[7] with slight modifications. Non-enzymatic combination of phenazine methosulfate (PMS) and reduced nicotinamide adenine dinucleotide (NADH), superoxide radical ($O_2^{•-}$) was produced and measured by the reduction of nitro blue tetrazolium (NBT) to purple-colored formazan. Quercetin was used as standard for the scavenging of superoxide radical.

2.5.4. Nitric oxide (NO) radical scavenging assay:

Nitric oxide radical (NO) quenching activity was studied as per GriessI-llosvoy reaction [8]. NO was generated through the reaction between aqueous sodium nitroprusside (SNP) and oxygen. Diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-(1-naphthyl) ethylenediamine dihydrochloride (NED), pink azo dye was generated. Curcumin was used as standard for the above experiment. The percentage inhibition was calculated using equation [6].

2.5.5. Hydrogen peroxide scavenging assay:

Hydrogen peroxide (H_2O_2) scavenging activity was studied by following FOX reagent method [9]. Sodium pyruvate was used as standard.

2.5.6. Peroxynitrite scavenging activity:

Peroxynitrite ($ONOO^-$) scavenging activities of β-cyclodextrin derived Schiff base Zn(II) complexes were compared against the standard gallic acid following a previously standardized method [10].

2.5.7. Singlet oxygen scavenging assay:

Singlet oxygen ($_1O^2$) generated through the reaction of sodium hypochloride (NaOCl) with H_2O_2 and scavenging activity of singlet oxygen was determined by monitoring the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO) using the method of Pedraza-Chaverrí *et al*[11]. with minor modifications. Lipoic acid was used as standard for the above experiment.

2.5.8. Hypochlorous acid scavenging assay:

HOCl scavenging activity was studied by monitoring the decrease in absorbance of catalase following a standard method with minor modification [12]. Ascorbic acid was used as standard for this experiment.

2.5.9. Measurement of reducing power:

The ferric reducing power was determined by the method of Oyaizu *et al* [13]. and Butylated hydroxytoluene (BHT) was used as standard.

2.5.10. Iron chelation assay:

Iron chelation activity was studied by the method of Haro-Vicente *et al* [14]. by measuring the decrease of intensity of violet complex (generated on coupling of Fe^{2+} and ferrozine) on addition of various concentrations of compounds. The absorbance was measured at 562 nm. Etylenediaminetetraacetate (EDTA) was used as standard.

2.5.11. Total antioxidant activity (TAA):

Total antioxidant activity (TAA) was measured following the method of Prietto *et al* [15]. by the reduction of Mo^{6+} to Mo^{5+}. Various concentrations of complex solution

were mixed with 1 mL of reaction mixture containing sulfuric acid, sodium phosphate and ammonium molybdate. The absorbance was measured at 695 nm against a suitable blank. Ascorbic acid was used as standard for this experiment. The antioxidant activity was measured by the capacity of the complex to reduce molybdenum (VI) to molybdenum (V) using equation [7]:

$$\text{Total antioxidant activity} = \frac{A_0 - A_1}{A_0} \times 100 \quad (7)$$

where, A_0 = the absorbance of the blank and A_1 = absorbance in the presence of the samples and standard (ascorbic acid).

2.5.12. Lipid peroxidation inhibition assay:

Lipid peroxidation inhibitory activity was determined by studying the inhibition of OH^- catalyzed malondialdehyde (MDA) production from the polyunsaturated fatty acid (PUFA) as per the method of Kizil *et al* [16-18]. Mice brain homogenate was used for the lipid peroxidation inhibition assay. Brain homogenate was prepared by centrifuging of mice brain with phosphate buffer (and potassium chloride. Various concentrations of complexes were mixed with the homogenate (100 µl) followed by addition of ferrous sulfate and ascorbic acid and incubated for 1 h at 37°C. Following incubation, TCA (Trichloroacetic acid) and TBA (thiobarbituric acid) were added in the reaction mixture and then heated at 95°C in water bath for 30 min. Then the mixtures were cooled to room temperature and centrifuged for 2 min. The absorbance of the supernatant liquid was measured at 532 nm. The resultant data was compared with the standard Trolox.

2.6. Antibacterial activity of the synthetic compounds using well diffusion method:

2.6.1. Preparation of synthetic compounds for microbiological assay:

The test solution of synthetic compound was prepared in DMSO at a concentration of 5, 7.5 and 10 mg/ml.

2.6.2. Test Organisms:

Two pathogenic gram positive bacteria *Staphylococcus aureus*, *Bacillus subtilis* and two pathogenic gram negative bacteria *Escherichia coli*, *Klebsiella pneumonia* were used for assessing the antibacterial activities using well diffusion method. All bacterial strains were cultivated in nutrient broth medium (NB) and incubated at 37 °C for 24 hr. This was used for the antibacterial activity in the well diffusion assay.

2.6.3. Preparation of media:

The nutrient agar (NA) medium was obtained from Hi-media laboratory Pvt Ltd, Mumbai, India. NA (2.8g) was suspended in 100 ml of distilled water, boiled and dissolved completely. It was sterilized by autoclaving at 15 lbs. pressure (121 ºC) for 15 min and poured into sterile Petri dish and allowed to set. Sterility check was done after the agar was solidified [19].

2.6.4. Preparation of culture media and inoculation method:

The pure cultures of each bacterium were prepared in Nutrient broth and incubating over night at 37 ºC. After incubation, all the required plates were inoculated by dipping a non-toxic sterile cotton swab into the microbial growth. Excess of inoculate was removed by pressing and rotating the swab firmly against the side of the tube, above the level of the liquid. The medium was inoculated by evenly streaking the swab over the entire surface of the plate in three directions.

2.6.5. Agar well diffusion method:

The assay for antibacterial activities was conducted by agar well diffusion method [20]. Sterile nutrient agar (NA) was poured into sterile Petri dishes, which was inoculated with the test organisms. Sterile cork borer was used to prepare well (9 mm) on each Petri plates. About 200 µl of synthetic compound (10mg/mL) was poured into each well (each microorganism inoculated plates) using sterile pipette and incubated at 37 ºC for 24h. After incubation all the tested plates were examined and the diameters of the inhibition zones were measured. All the experiments were carried out in triplicates. DMSO was

used as a control. The zone of inhibitions is expressed as mean values with standard deviation of mean (SD).

2.7. Molecular modeling analysis:

2.7.1. Swiss target prediction:

The bioactive small molecules, drugs and metabolites have tendency to bind with proteins and other macromolecule targets to modulate their activities which has visible expression in organisms [2]. The synthesized complexes were checked for possible bioactivity with Swiss target prediction web server (http://www.swisstargetprediction.ch).

2.7.2. Molecular Docking:

The target proteins identified by Swiss target prediction were chosen as receptor protein. The X-ray crystallographic structures of these receptor proteins were obtained from RCSB PDB (*https://www.rcsb.org).* They were used for molecular docking analysis against the synthesized chemicals. Molecular docking was performed using Auto Dock Vina. The receptor structures were defined as rigid and the grid dimensions varied from being 80, 80 and 80 for X, Y and Z dimensions for proteins having PDB ID's 1D3G, 1JY1, 1R2D, 2NL9 and 4LXD while 100, 100 and 100 for 3D2Q and 4CIM. Whereas for the protein with PDB ID 2ON9 it is 60, 60 and 60 respectively. The Exhaustiveness of the simulation was set to 8. Every protein of interest was made ready by removing the water molecules and by adding polar hydrogen to them. Gasteiger charges were added to the proteins on the basis of electronegativity equilibration and all non-polar hydrogens were merged [2]. This was performed because Auto Dock uses the united atom model to represent molecules. Gasteiger charges were also calculated for the respective complexes and all the torsions were allowed to rotate.

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