CHAPTER II
MATERIALS & METHODS

The detailed description of materials, experimental methods and instruments for different characteristic techniques are presented in this chapter.

2.1 Materials

All chemical substances (indicated in table 2.1) were purchased and used without further purification. Commercially available CT DNA was used for DNA binding studies. The structures of different reactants are tabulated as below:

Table 2.1 List of structure of reactants

<table>
<thead>
<tr>
<th>Name of the reactants</th>
<th>Structure of the reactants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylhydrazine</td>
<td><img src="image" alt="Phenylhydrazine" /></td>
</tr>
<tr>
<td>2,3-Dimethylphenylhydrazine</td>
<td><img src="image" alt="2,3-Dimethylphenylhydrazine" /></td>
</tr>
<tr>
<td>3-Nitrophenylhydrazine</td>
<td><img src="image" alt="3-Nitrophenylhydrazine" /></td>
</tr>
<tr>
<td>Compound</td>
<td>Chemical Structure</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>2-Chlorophenyl hydrazine</td>
<td><img src="image" alt="2-Chlorophenyl hydrazine" /></td>
</tr>
<tr>
<td>2-Fluorophenyl hydrazine</td>
<td><img src="image" alt="2-Fluorophenyl hydrazine" /></td>
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<tr>
<td>2-Bromophenyl hydrazine</td>
<td><img src="image" alt="2-Bromophenyl hydrazine" /></td>
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<tr>
<td>2-Nitrophenyl hydrazine</td>
<td><img src="image" alt="2-Nitrophenyl hydrazine" /></td>
</tr>
<tr>
<td>4-Chlorophenyl hydrazine</td>
<td><img src="image" alt="4-Chlorophenyl hydrazine" /></td>
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<tr>
<td>4-Methoxyphenyl hydrazine</td>
<td><img src="image" alt="4-Methoxyphenyl hydrazine" /></td>
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<tr>
<td>4-Cyanophenyl hydrazine</td>
<td><img src="image" alt="4-Cyanophenyl hydrazine" /></td>
</tr>
<tr>
<td>Compound</td>
<td>Structure</td>
</tr>
<tr>
<td>-------------------------------</td>
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</tr>
<tr>
<td>4-Nitrophenyl hydrazine</td>
<td><img src="image" alt="4-Nitrophenyl hydrazine" /></td>
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<tr>
<td>4-Bromophenyl hydrazine</td>
<td><img src="image" alt="4-Bromophenyl hydrazine" /></td>
</tr>
<tr>
<td>4-Fluorophenyl hydrazine</td>
<td><img src="image" alt="4-Fluorophenyl hydrazine" /></td>
</tr>
<tr>
<td>Hydrazine</td>
<td><img src="image" alt="Hydrazine" /></td>
</tr>
<tr>
<td>3-Chlorophenyl hydrazine</td>
<td><img src="image" alt="3-Chlorophenyl hydrazine" /></td>
</tr>
<tr>
<td>3-Fluorophenyl hydrazine</td>
<td><img src="image" alt="3-Fluorophenyl hydrazine" /></td>
</tr>
<tr>
<td>3-Bromophenyl hydrazine</td>
<td><img src="image" alt="3-Bromophenyl hydrazine" /></td>
</tr>
<tr>
<td>3-Cyanophenyl hydrazine</td>
<td><img src="image" alt="3-Cyanophenyl hydrazine" /></td>
</tr>
</tbody>
</table>
2.2 Purification of solvents

The solvents were purchased from Merck and purified according to the methodology prescribed in Weissenburg series [139, 140]. The dimethyl sulphoxide and acetonitrile were purified and stored under inert atmosphere.

2.3 Preparation of supporting electrolyte

Tetrabutylammonium perchlorate (Me)$_4$NCIO$_4$ prepared from its bromide derivative as follows:

A clear solution of tetrabutylammonium bromide (25 mM) in 20 mL water was mixed with 2 mL of 50 % aqueous perchloric acid. The product (Tetrabutylammonium perchlorate) was formed, filtered, washed and dried. It was recrystallised using perchloric acid.

2.4 Preparation of Tris-HCl buffer

Accurately, 5 mM Tris-Hydrochloride and 50 mM sodium chloride were weighed and dissolved in double distilled water. The solution pH was corrected to 7.1 with sodium hydroxide and made up to 500 mL solution in a standard measuring
flask using double distilled water. This buffer was used to perform DNA binding studies.

2.5 DNA purity

The electronic absorbance of CT DNA in Tris-HCl buffer was measured. The absorption was measured at 260 and 280 nm. The ratio of absorption (A_{260}/A_{280}) gave information about the purity of DNA. The observed value is 1.8-1.9 suggested that the CT DNA is pure without any protein contamination [141]. The fresh stock solutions were prepared for the studies.

2.6 Analytical methods of characterization

2.6.1 Elemental analysis

CHN are most vital elements for structural characterization. It was done by using Elementar Vario ELIII Carlo Erba 1108 at CDRI Lucknow and EDTA as calibrant.

2.6.2 Determination of metal content

The amount of metal content was estimated using ammonium oxalate method. Exactly, 0.2g of metal complex was weighed and taken in a previously weighed silica crucible [142]. 0.6g of ammonium oxalate was added to metal complex. It was heated slowly and then strongly for 2 hrs using burner. It was cooled and weighed. The metal content in complex was calculated on the basis of metal oxide formation.

2.6.3 Molar conductance measurements

Conductivity measurements were carried out to find out the stoichiometry and the selectivity of complexes formed between ligands and cations in DMSO as solvent [143].

\[ \Lambda_m = \frac{(1000 \times \text{cell constant} \times \text{observed conductance})}{C} \quad \text{--------- (2.1)} \]

Where

\[ \Lambda_m = \text{Molar conductance} \]

\[ C = \text{Molar concentration} \]
Table 2.2  Molar conductance values for different metal complexes at 1mM solution in different solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Molar conductance (S cm$^2$ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Electrolyte Type</td>
</tr>
<tr>
<td>Nitromethane</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
</tr>
</tbody>
</table>

2.6.4 Magnetic susceptibility and Magnetic moment measurements

This technique is necessary for structural characterization of metal complexes and provides unique information about the nature of magnetic properties of metal complexes either paramagnetic or diamagnetic character [144-146]. The magnetic susceptibility of complexes was performed by taking the molar susceptibility using Guoy's magnetic balance. The Pascal's constants are essential for the calculation of diamagnetic corrections. The effective magnetic moment, $\mu_{eff}$ was calculated after making appropriate diamagnetic corrections by Pascal’s constants (2.2).

$$\mu_{eff} = 2.83 (\chi_M T)^{1/2} \text{BM} \quad \text{---------(2.2)}$$

Where

- $\mu_{eff}$ = effective magnetic moment
- $\chi_M$ = Molar susceptibility
- $T$ = Absolute temperature
- BM = Bohrmagnetron

Nickel(II) complex

Generally, the square planar nickel(II) complexes contain no unpaired electrons and therefore diamagnetic. The calculated magnetic moment value is 2.83 BM for the nickel(II) with two unpaired electrons while in octahedral nickel(II) complexes, the magnetic moment values in between 2.9 and 3.4 BM which indicates
that the sum of orbital and spin contribution. Tetrahedral complexes, magnetic moment values range from 3.0 to 4.0 BM is due to the larger distortion from a regular geometry.

**Cobalt (II) complex**

Generally, the calculated spin only value for the cobalt(II) with three unpaired electrons is 3.87 BM. In the Octahedral complexes, the magnetic moment values are in between 4.7 and 5.2 BM. On the basis of nature of ligand strength, the magnetic moment is below 4.7 BM for weak field ligand which corresponds to tetrahedral geometry whereas the value may be higher for stronger field ligand. Therefore, the magnetic moment values gave information about the magnetic properties and geometry of metal complexes.

**Copper (II) complex**

The electronic configuration of copper is [Ar]4s²4p⁶3d⁹ indicates copper in zero oxidation state. There is no Jahn-Teller distortion was observed for Cu(I) complex with d¹⁰ electrons and diamagnetic in nature. Some instance, Cu(I) complex was observed as colored substance may be due to its charge transfer transition in ligand. The Jahn-Teller distortion was observed for the copper(II) complex with d⁹ electrons and observed a splitting of e_g and t₂g orbitals. It is exhibited d-d transition ∼ 500 nm which corresponds to ²B₁g→²A₁g transition. It indicates that copper(II) complex exist in square-planar geometry. The absorption above 900 nm indicates the possibility of tetrahedral-planar geometry. The magnetic moment value for Cu(II) complex is 2.02 BM and black in colour expected for tetrahedral geometry.

**2.6.5 IR Spectra**

The IR spectra of the ligands and their metal complexes were recorded using Shimadzu FTIR IR Affinity-1 using KBr disc with the range 4000-390 cm⁻¹. In the graph, the percentage transmittance (%T) was plotted against wave number (cm⁻¹). The observed stretching and bending vibrations indicate the nature of group present in the metal complexes [147].
2.6.6 Nuclear magnetic resonance spectra

The $^1$H and $^{13}$C-NMR spectra of the ligands and their Ni(II) & Zn(II) complexes were recorded using Bruker Avance II 400 MHz spectrometer at SAIF, IIT Madras and TMS as standard. The chemical shift values are discussed in ppm with respect to TMS. The nature of protons is described in terms of singlet (s), doublet (d), triplet (t) and multiplet (m). The structural elucidation of the ligands as well as in precisely the donor sites of a ligand from the NMR spectra.

2.6.7 Mass spectra

The mass spectra of the ligands and their copper complexes were performed at CDRI, Lucknow on a JEOL SX 102/DA-6000 mass spectrometer/Data system using Argon/Xenon (6Kv, 10 Ma) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature using $m$-nitrobenzylalcohol (NBA) as the matrix.

2.6.8 Electronic spectra

Electronic spectra were recorded in a Systronics 2201 Double beam UV-Vis., spectrophotometer within the range of 200-1100 nm regions. In the graph, absorbance values are plotted against wave length (nm). The spectra of complexes were measured using DMSO (spectral grade) solvent. Based on the d-d transitions and the position of absorption, the geometry of the synthesized complex was assigned by the electronic spectral measurements.

2.6.9 Electron paramagnetic resonance (EPR)

EPR spectra of the paramagnetic complexes were recorded at liquid nitrogen temperature in the X-band region on a JES-X3 SERIES equipped with 100 KHz field modulation at IIT Chennai. Diphenylpicrylhydrazyl (DPPH)/ Tetracyanoethylene (TCNE) radical were used as field marker.

2.6.10 Thermal analyses

The thermal analyses (TGA/DTA) of metal complexes were measured by using a Shimadzu TG-50 Thermobalance, in the temperature range of 0ºC–1000ºC, under a dynamic atmosphere of nitrogen. The samples with the appropriate mass
were put into platinum crucibles with a raise of 10°C min\(^{-1}\). The complex was decomposed into metal oxide residue with respect to the metal complexes. The weight loss of thermal analyses (TGA/DTA) of metal complexes were calculated and compared with those of theoretical values for the suggested formula [148].

2.6.11 Powder X-ray diffraction

The powder sample was subjected to powder X-ray diffraction using analytical X’Pert Powder X’ Celerator Diffractometer. The K\(_\alpha\) radiations (\(\lambda = 1.54060\ \text{Å}\)) from a copper target were used. The specimen in the form of a thin film was scanned in the reflection mode in the 2\(\theta\) range 10-80° with four decimal accuracy [149].

2.6.12 SEM

The surface morphology of ligands and their metal complexes was studied by scanning electron microscope.

2.7 Biological Studies

The systematic procedure for the biological activities such as antibacterial, DNA binding, lipophilicity and antioxidant activities of synthesized ligand and their complexes are presented.

2.7.1 Antibacterial activity

The antibacterial activity of samples was determined using a well diffusion method [150]. The antibacterial activities were performed by using *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Pseudomonas aeruginosa*, respectively.

The nutrient agar medium was boiled to dissolve completely and sterilized at 15 lbs pressure (120°C). After sterilization, 20 mL of media was poured into the sterilized petri plates. These plates were kept at room temperature and the medium got solidified in the plates. Then, it was inoculated with microorganisms using sterile swabs. The stock solutions were prepared by dissolving the compounds in appropriate solvents. The sample solutions were filled in the incubated plates using a micropipette and incubated for 24 hrs at 37 °C. During incubation period, the sample
solution was diffused into the gel and inhibited the growth of the microorganism. The zone of inhibition was developed on the plate and measured.

2.7.2 DNA Binding Studies

2.7.2.1 Absorption titration experiment

UV–Vis., spectrophotometric titration is the most commonly used method for determination of DNA binding constants of ligands and their metal complexes. Absorption titration experiment was performed by maintaining a constant concentration of the complex and varying the DNA concentration. Then, dissolving an appropriate amount of the metal complex stock solution and mixing various amounts of DNA stock solutions while maintaining the total volume constant [151]. The absorbance (A) was recorded after each successive additions of CT DNA. The intrinsic binding constant, $K_b$, was determined from the plot of $\frac{[DNA]}{[\epsilon_a - \epsilon_f]}$ vs $[DNA]$, where $[DNA]$ is the concentration of DNA in base pairs, $\epsilon_a$, the apparent extinction coefficient which is obtained by calculating $A_{obs}/[\text{complex}]$ and $\epsilon_f$ corresponds to the extinction coefficient of the complex in its free form. The data were fitted to the following equation where $\epsilon_b$ refers to the extinction coefficient in the fully bound form of complex [152].

$$\frac{[DNA]}{[\epsilon_a - \epsilon_f]} = \frac{[DNA]}{[\epsilon_b - \epsilon_f]} + \frac{1}{K_b[\epsilon_b - \epsilon_f]}$$

$$\text{(2.3)}$$

2.7.2.2 Cyclic Voltammetry

The cyclic voltammetry is an electrochemical technique which measures the current that develops in electrochemical cell. The cyclic voltammogram of the metal complexes were recorded using CHI 604D electrochemical analyser. The cell contains three electrodes which are immersed in the solution to be analysed. The working electrode is the electrode where the reactions of the interest occur. The three electrodes are glassy carbon as working electrode, silver electrode (reference electrode) and platinum wire (auxillary electrode) used. TBAP is used as the supporting electrolyte. Cyclic voltammetry is an essential tool in the study of DNA
interaction with redox active molecules on the basis of change in electrode potential and current with addition of DNA solution.

2.7.3 Lipophilicity measurements

The lipophilic characteristics of synthesized substances were determined by measuring the distribution of the substance between water and n-octanol. The n-octanol/water partition coefficient (P_{ow}) is the ratio between the analyte concentration in the water layer (C_w) and n-octanol layer (C_O). The P_{ow} is calculated using the following equation.

\[
(P_{ow}) = \frac{(C_O)}{(C_W)} \quad \text{--------- (2.4)}
\]

Where

- \(P_{ow}\) = partition coefficient between n-octanol and water
- \(C_O\) = concentrations of the test substance in n-octanol phase
- \(C_W\) = concentrations of the test substance in water phase

2.7.4 Antioxidant Assay

2.7.4.1 Superoxide dismutase activity (SOD)

The superoxide dismutase activity (SOD) of the metal complexes was measured using alkaline DMSO as source of superoxide radicals (O_2•-) with nitrobluetetrazolium chloride (NBT) as a scavenger of superoxide [153]. 2.1 mL of 0.2 M potassium phosphate buffer (7.2 pH) and 1 mL of 56 µL of NBT solutions were added to the different concentration of metal complex solution. The reaction mixture was kept in ice for 15 min and then 1.5 mL of alkaline DMSO solution was added. The absorbance was monitored at 540 nm against a sample prepared under similar condition except alkaline DMSO [227].

\[
\text{Scavenging effect (\%)} = \frac{(A_0-A_1)}{A_0} \times 100 \quad \text{--------- (2.5)}
\]

Where “\(A_0\)” is the absorbance of the control reaction and “\(A_1\)” is the absorbance in the presence of the samples or standards.
2.7.4.2 Hydrogen peroxide Assay

10 mM solution of hydrogen peroxide was prepared in phosphate buffer and its concentration was determined at 230 nm using UV-Vis., Spectrophotometer. The complexes of different concentration of sample and standard, Vitamin C (100 µg/mL) were added to 2 mL of hydrogen peroxide solution. Under identical reaction mixture, without the sample was taken as control. The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against the blank [154].

2.7.5 Anti-inflammatory Activity

The anti-inflammatory activity of metal complexes was evaluated by using carrageenan induced paw edema method [154]. The rats were divided into four groups of six rats each. Group I received normal saline and behaved as control. Group II and III received the doses of 100 and 200 mg/kg bw of metal complexes, respectively. Group IV received the standard drug, Indomethacin 10 mg/kg bw. Edema was induced by injecting 100 µL of carrageenan (1% w/v; Sigma) in normal saline into the subplantar region of the left hind paw after 1 h of drug administration. The paw volume was measured with the help of mercury replacement plethysmometer at 0 h and then at 1, 2, 3 and 4 h after administration of drugs. The percentage inhibition of edema compared with that of the control was taken as anti-inflammatory activity. The percentage inhibition of edema was calculated by the formula (2.6),

\[
\text{Percentage inhibition of edema} = \frac{(A-B)}{A} \times 100 \quad \text{---------- (2.6)}
\]

Where,

- A represents the paw volume of the control at 3 h and
- B represents the paw volume of the test drug treated at 3 h.

2.7.6 Anti tuberculosis activity

The anti-mycobacterial activity of metal complexes was screened against *M. tuberculosis* using Micro Plate Alamar Blue assay method (MABA) [155]. This methodology is non-toxic, thermally-stable reagent and showed good correlation with proportional and BACTEC radiometric methods [156, 157]. This method is described as follows: 200 ml of sterile deionized water was added to 96 sterile well
plates to minimize evaporation of the medium in the tested wells during incubation period. The 96 wells received 100 mL of the Middlebrook broth and subsequent dilution of compounds was made directly on the plate. The final sample concentrations tested were 0.02-10.0 mg/mL. The plates were covered & sealed with parafilm and incubated at 37°C for five days. 25 mL of a freshly prepared 1:1 mixture of Alamar Blue reagent and 10% Tween 80 was added to the plate and incubated for 24 hrs [158, 159]. A blue color in the well was identified as no bacterial growth whereas a pink color was confirmed the growth of species. The minimal inhibition concentration (MIC) was defined as the lowest drug concentration, which prevented a colour change from blue to pink.

2.8 Catalytic study: Oxidation of benzyl alcohol to benzaldehyde

The catalytic reaction was performed in the substrate of benzyl alcohol into benzaldehyde as product. The substrate was taken in a two necked RB flask fitted with a water condenser. The oxidation reaction was carried out by varying the reaction conditions like solvent, temperature (30-90°C), time and the amount of catalyst. In the first step, the catalyst was allowed to mix well in the solvent for 10 min in RB flask followed by 1atm O₂ added. Then, the benzyl alcohol (substrate) was added. After the end of specific reaction time, the content from the reaction mixture was examined by GC. The position of peak was matched with the retention time of authentic sample to give a information about the product. A control experiment was also conducted in the absence of catalyst.

2.9 Conclusion

The detailed description about the materials, instruments, experimental methodologies used for the various studies are presented in this chapter.