3. EXPERIMENTAL METHODS AND TECHNIQUES

In this chapter a detailed description of the materials employed, general experimental techniques adopted to synthesize the compounds and purify them, analytical procedures followed to characterize them and techniques employed to assess their antibacterial, antifungal, antioxidant and anticancer activities.

3.1 MATERIALS EMPLOYED

High purity chemicals were used for all the syntheses. The following chemicals were used at various stages of this work:

2,6-dimethyl-5-heptenaldehyde, complying to Food and Drug Administration (Title 21, Volume 3 Sec. 172.515(21 CFR 172.515) was used for the schiff base ligand reaction. High purity Analytical reagent grade (AR) 4-chloroaniline, 4-fluoroaniline, copper(II)nitrate, nickel(II)nitrate, cobalt(II)chloride, chromium(II)chloride and samarium(III)nitrate sigma Aldrich and used without further purification. Hydrochloric acid and sodium sulphate were received from Merck chemicals. Double distilled water collected from the glass equipment was normally used in all preparations.

Gentamycin, peptone, amphotericin and sucrose were received from Merck Millipore. Other chemicals like sodium chloride, sodium nitrate, Potassium hydrogen phosphate (K₂HPO₄), Magnesium sulfate heptahydrate (MgSO₄.7H₂O), Potassium chloride (KCl), Ferrousulfate (FeSO₄), DPPH(2,2-diphenyl-1-picrylhydrazyl) were received from Hi media laboratories Pvt Ltd., Mumbai. Czapek-Dox yeast Agar from Sigma-Aldrich was used.

3.2 SOLVENTS

Analytical grade ethyl alcohol was received from Hayman Ltd., U.K and methyl alcohol was purchased from Hi media laboratories Pvt Ltd., Mumbai.
Other common solvents like acetone, Propan-2-ol, chloroform, dichloromethane, hexane, Dimethyl sulfoxide (DMSO), Dimethyl formamide (DMF)(HPLC Grade), and Deuterochloroform (CDCl₃) used at various stages of this work were of analytical grade (AR grade).

3.3 EXPERIMENTAL METHODS

3.3.1 Synthesis of Schiff Base Ligand [L1]

The Schiff base ligand (L1) was prepared by the condensation of 2, 6-dimethyl-5-heptenaldehyde and aniline in 1:1 molar ratio, by refluxing in propan-2-ol, for 3 h [62]. The solution was then left to stand at 298 K. The solid product formed was filtered, purified by crystallization from ethanol, washed with acetone and then dried in vacuum over anhydrous calcium chloride (Scheme 3.1). Beige colored crystalline product of Schiff base ligand, obtained was recrystallized (yield 80% and m. Pt. 180-182°C). The ligand was characterized by GC–MS, IR spectra, ¹H NMR, UV–Vis spectra and TGA. Its molecular formula was deduced as C₁₅H₂₁N.

![Scheme 3.1 Synthesis of Schiff base ligand [L1]](image)

Yield 80%, m. pt. 180-182 °C

3.3.2 Synthesis of Metal Complexes using L1

Schiff base - metal complexes using transition metals, viz., copper, nickel, cobalt and chromium and inner transition metal samarium were prepared as described by Nejati et al[69]. A 20ml of ethanolic solution of 0.004 mol of metal salt [Cu(NO₃)₂.3H₂O/ Ni (NO₃)₂.6H₂O/CoCl₂.6 H₂O/ CrCl₃.6 H₂O/ Sm(NO₃)₃.6H₂O] was added to 20ml 0.004 mol solution of the ligand
(L1) in ethanol. The resulting mixture was refluxed for 4 h. The solution obtained was then left to stand at 298 K. The molecular formula, molecular weight, colour of the compounds, their melting points, and yields are provided in the Table 3.1

### Table 3.1  Physical properties and the percentage yield of the Schiff base ligand [L1] and its metal complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Formula/wt.</th>
<th>Colour</th>
<th>Melting point(ºC)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand (L1)</td>
<td>C_{15}H_{21}N (215)</td>
<td>Beige</td>
<td>180-182</td>
<td>80</td>
</tr>
<tr>
<td>Cu (II)−L1 Complex</td>
<td>[Cu(L1)_2(H_2O)_2].2NO_3(654)</td>
<td>Brown</td>
<td>248-252</td>
<td>85</td>
</tr>
<tr>
<td>Ni(II)−L1 Complex</td>
<td>[Ni(L1)_2(H_2O)_2].2NO_3(649)</td>
<td>Green</td>
<td>228-232</td>
<td>80</td>
</tr>
<tr>
<td>Co(II)−L1 Complex</td>
<td>[Co(L1)_2(H_2O)_2].Cl_2 (667)</td>
<td>Reddish brown</td>
<td>229-233</td>
<td>86</td>
</tr>
<tr>
<td>Cr(III)−L1 Complex</td>
<td>[Cr(L1)_2(H_2O)_2].Cl_3 (625)</td>
<td>Reddish brown</td>
<td>220-224</td>
<td>81</td>
</tr>
<tr>
<td>Sm(III)−L1 Complex</td>
<td>[Sm(L1)_2NO_3H_2O]H_2O (678)</td>
<td>Grey</td>
<td>&gt;300</td>
<td>84</td>
</tr>
</tbody>
</table>

The microcrystals were filtered, washed with absolute ethanol, recrystallized from ethanol/chloroform (1:3, v/v) and dried in a vacuum desiccator over anhydrous calcium chloride. The metal complexes were characterized by IR, UV–Vis, Maldi–TOF, TGA, and EDAX.

### 3.3.3 Synthesis of Schiff Base Ligand [L2]

The schiff base ligand (L2) was prepared by the condensation of 2,6–dimethyl–5–heptenaldehyde and 4-chloro aniline in 1:1 molar ratio by refluxing in propan–2–ol for 3 h [62]. The solution obtained was then left to stand at 298 K. The solid product formed was separated by filtration, purified by crystallization frommethanol, washed with acetone and then dried in vacuum over anhydrous calcium chloride (Scheme 3.2). A beige coloured crystalline product of schiff base 4–chloro-N-(2,6–dimethyl–5–heptenylidene)
benzenamine obtained was recrystallized (yield and 82% m. Pt. 198-200°C). Its molecular formula was deduced as C\textsubscript{15}H\textsubscript{20}NCl.

![Chemical structure of benzenamine recrystallization](image)

Yield 82%, m. pt. 198-200°C

**Scheme 3.2 Synthesis of schiff base ligand [L2]**

### 3.3.4 Synthesis of Metal Complexes using L2

A 20ml of ethanolic solution of 0.004 mol of metal salt [Cu(NO\textsubscript{3})\textsubscript{2}.3H\textsubscript{2}O/Ni(NO\textsubscript{3})\textsubscript{2}.6H\textsubscript{2}O/CoCl\textsubscript{2}.6H\textsubscript{2}O/CrCl\textsubscript{3}.6H\textsubscript{2}O/Sm(NO\textsubscript{3})\textsubscript{3}.6H\textsubscript{2}O] was added to 20ml 0.004 mol solution of the ligand (L2) in ethanol. The resulting mixtures were refluxed for 4 h\textsuperscript{[63]}. The solution obtained was then left to stand at 298 K. The molecular formula, molecular weight and the colour of the compounds, their melting points and yields are provided in the Table 3.2.

**Table 3.2 Physical properties and the percentage yield of the schiff base ligand [L2] and its metal complexes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Formula/wt.</th>
<th>Colour</th>
<th>Melting point (°C)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand (L2)</td>
<td>C\textsubscript{15}H\textsubscript{20}NCl (250)</td>
<td>Beige</td>
<td>198-200</td>
<td>82</td>
</tr>
<tr>
<td>Cu(II)–L2 Complex</td>
<td>[Cu(L2)\textsubscript{2} (H\textsubscript{2}O)\textsubscript{2}].NO\textsubscript{3} (661)</td>
<td>Brown</td>
<td>252-256</td>
<td>86</td>
</tr>
<tr>
<td>Ni(II)–L2 Complex</td>
<td>[Ni(L2)\textsubscript{2} (H\textsubscript{2}O)\textsubscript{2}].NO\textsubscript{3} (656)</td>
<td>Green</td>
<td>230-234</td>
<td>85</td>
</tr>
<tr>
<td>Co(II)–L2 Complex</td>
<td>[Co(L2)\textsubscript{2} (H\textsubscript{2}O)\textsubscript{2}]. Cl\textsubscript{2} (665)</td>
<td>Reddish brown</td>
<td>232-236</td>
<td>84</td>
</tr>
<tr>
<td>Cr(III)–L2 Complex</td>
<td>Cr(L2)\textsubscript{2} (H\textsubscript{2}O)\textsubscript{2}. Cl\textsubscript{3} (694)</td>
<td>Brown</td>
<td>225-229</td>
<td>81</td>
</tr>
<tr>
<td>Sm(III)–L2 Complex</td>
<td>[Sm(L2)NO\textsubscript{3}(H\textsubscript{2}O)\textsubscript{2} (498)</td>
<td>Grey</td>
<td>&gt;300</td>
<td>80</td>
</tr>
</tbody>
</table>
3.3.5 Synthesis of Schiff Base Ligand [L3]

The microcrystals collected were filtered, washed with absolute ethanol, recrystallized from ethanol/chloroform (1:3, v/v) and dried in a vacuum desiccator over calcium chloride. The metal complexes were characterized by IR, UV–Vis, Maldi–TOF, TGA and EDAX.

The Schiff base ligand (L3) was prepared by the condensation of 2, 6–dimethyl–5–heptenaldehyde and 4–fluoro aniline in 1:1 molar ratio by refluxing in propan–2–ol for 3 h [69]. The solution was then left at 298K. The solid product formed was filtered, recrystallized from ethanol, washed with acetone and then dried in a vacuum over anhydrous calcium chloride (Scheme 3.3). A beige colored crystalline product of Schiff base was obtained and recrystallized (yield 84% and m. Pt. 190-192°C). Its molecular formula is C_{15}H_{20}NF.

![Scheme 3.3 Synthesis of Schiff base ligand [L3]](image)

Yield 84%, m. pt. 190-192°C

3.3.6 Synthesis of Metal Complexes using L3

A 20ml of ethanolic solution of 0.004 mol of metal salt [Cu(NO_3)_2·3H_2O/Ni(NO_3)_2·6H_2O/CoCl_2·6H_2O/CrCl_3·6H_2O/Sm(NO_3)_3·6H_2O] was added to 20ml 0.004 mol solution of the ligand (L3) in ethanol. The resulting mixtures were refluxed for 4 h. The solution obtained was then left to stand at 298 K [69]. The molecular formula, molecular weight and colour of the compounds, their melting points, and yields are provided in the Table 3.3.
The microcrystals were collected by filtration, washed with absolute ethanol, recrystallized from ethanol/chloroform (1:3, v/v) and dried in a vacuum desiccator over calcium chloride. The metal complexes were characterized by IR, UV–Vis, TGA, Maldi–TOF, and EDAX.

Table 3.3 Physical properties and the percentage yield of the Schiff base ligand [L3] and its metal complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Formula/wt.</th>
<th>Colour</th>
<th>Melting point(ºC)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand (L3)</td>
<td>C_{15}H_{20}NF (234)</td>
<td>Beige</td>
<td>190-192</td>
<td>84</td>
</tr>
<tr>
<td>Cu(II)–L3 Complex</td>
<td>[Cu(L3)_2(NO_3)_2H_2O] (610)</td>
<td>Brown</td>
<td>242-246</td>
<td>84</td>
</tr>
<tr>
<td>Ni(II)–L3 Complex</td>
<td>[Ni(L3)_2(NO_3)_2(H_2O)_2].2H_2O(721)</td>
<td>Green</td>
<td>220-224</td>
<td>81</td>
</tr>
<tr>
<td>Co(II)–L3 Complex</td>
<td>[Co(L3)_2(Cl)_2(H_2O)_2].2H_2O (1265)</td>
<td>Reddish brown</td>
<td>&gt;300</td>
<td>86</td>
</tr>
<tr>
<td>Cr(III)–L3 Complex</td>
<td>[Cr(L3)_2(Cl)_3].3H_2O (1304)</td>
<td>Brown</td>
<td>&gt;300</td>
<td>82</td>
</tr>
<tr>
<td>Sm(III)–L3 Complex</td>
<td>[Sm(L3)(NO_3)2H_2O] (526)</td>
<td>Grey</td>
<td>&gt;300</td>
<td>84</td>
</tr>
</tbody>
</table>

3.3.7 In vitro Antibacterial Study

The synthesized schiff base ligands L1, L2 and L3 and their respective metal complexes were screened for antibacterial activity. The studies were conducted against gram–negative bacterial strains, viz., *E. coli* (ETEC), *S. typhi*, and *P. aeruginosa* and gram–positive bacterial strains, viz., *S. aureus*, *B. subtilis*, and *B. megaterium* bacterial strains. The minimum inhibition concentration (MIC) obtained were compared with that of standard drug gentamycin for bacteria [70].

Agar diffusion method was used to screen for antibacterial activity[71]. The media was prepared using 10g of peptone, 10g of NaCl, 5g of yeast extract, 20g agar in1000 ml of distilled water. Initially, the stock
cultures of bacteria were revived by inoculating in broth media and grown at 310 K for 18 h. The agar plates of the above media were prepared and wells were made in the plate. Each plate was inoculated with 18 h old cultures (100 μl, 10^{-4} cfu) and spread evenly on the plate. After 20 min, the wells were filled with compound at different concentrations. The control wells with gentamycin were also prepared. All the plates were incubated at 310 K for 24 h and the diameter of inhibition zone was noted [72].

3.3.8 In vitro Antifungal Study

The schiff base ligands L1, L2 and L3 and their corresponding metal complexes were screened for antifungal activity. The studies were conducted against fungi species, viz., C. albicans, P. chrysogenum, A.niger, A.flavus, A.fumigatus and C. oxysproum. The minimum inhibition concentration (MIC) [73] obtained were compared with that of standard drug amphotericin for fungi.

Czapek-Dox agar was used for the activity study against fungi which has the composition of 30g of sucrose, 2g of sodium nitrate, 1g of K₂HPO₄, 0.5 g of MgSO₄.7H₂O, 0.5 g of KCl, 0.01g of FeSO₄, and 20g of agar per 1000ml

Initially, the stock cultures were revived by inoculating in broth media and grown at 310 K for 48 h. The agar plates of the above media were prepared and wells were made in the plate. Each plate was inoculated with 48 h old cultures (100 μl 10^{-4} cfu) and spread evenly on the plate. After 20 min, the wells were filled with compound at different concentrations. The control plates with antibiotic were also prepared. All the plates were incubated at 310 K for 96 h and the diameter of inhibition zone were noted.

The results were recorded by measuring the zones of growth inhibition surrounding the discs.
3.3.9 *In vitro* Anticancer Study

The efficiency of the compounds on cell cytotoxicity was analyzed in A549 cells (Mossman *et al.* 1983)[74]. Briefly the cells were seeded in 96 well plate in the log phase and allowed to reach 80% confluence. The cells were treated with 100 µg/ml of the compounds and without the compounds for 24 h. The medium was removed, 5mg/ml of MTT was added and incubated for 2h. The formation of formazan crystal was observed which were dissolved by addition of isopropanol (100µl). The purple colour developed was read at 490nm in Perkin Elmer multimode plate reader.

3.3.10 *In vitro* Antioxidant Study

The ability of the compounds to scavenge free radicals was analyzed by DPPH method (Blois *et al.* 1958). Briefly the methanolic solution of compounds was used at 100µg/ml concentration. To this 100µl of DPPH (4mg/100ml) was added and incubated for 30 minutes. The colour developed was read at 490nm in Perkin Elmer Multimode plate reader. Ascorbic acid was used as standard. The efficiency of the compounds to remove free radicals was compared with that of ascorbic acid.

3.4 **ANALYTICAL INSTRUMENTAL METHODS**

3.4.1 MALDI-TOF

Electron impact ionization (EI) technique was used with mass spectrometry for the characterization of schiff base ligand. However, for the metal complexes with higher molecular weights more accurate results are possible only with soft ionization technique viz., Matrix-assisted laser desorption/ionization (MALDI). While Maldi helps in ionization, it requires a detector to detect the fragmented ions. The type of a mass spectrometer most widely used with MALDI is the TOF (time-of-flight mass spectrometer).

Maldi–TOF Bruker Ultra flextreme model is used for deriving the empirical formulation of the metal complexes. 2 KHz laser and Energy of
30KJ is used for this purpose. α–cyano–4–hydroxycinnamic acid (4–HCCA) matrix used for Maldi–TOF investigation. This novel method helps to ascertain the higher molecular weights in binuclear complexes [75].

### 3.4.2 Other Analytical Instruments

GC-MS analysis was carried out to confirm the mass of schiff base ligand using Agilent 6890N GC fitted with 5973MSD. The injections were carried out using 7683 series auto-injector.

$^{1} \text{H NMR}$ is employed to further confirm the structure of schiff base ligand using Bruker Avance III, 400MHz and 9.4 Tesla super-conducting Magnet.

FT-Infrared spectra were recorded in the range of 400-4000cm$^{-1}$ with Thermo Nicolet, Avatar 370.

Absorption spectra were recorded in the range of 175 to 700nm using Varian, Cary 5000 UV-Visible spectrophotometer.

For studying the thermal decomposition Thermo gravimetric analysis (TGA) was carried out on a Perkin Elmer, Diamond TGA apparatus from 40°C to 740°C at a heating rate of 10°C/min under inert atmosphere.

EDAX was carried out to confirm the complex formation and the library matching to arrive at the constituents of the complex using ED – 2300 EDAX.

Molar conductivity measurements were recorded on systronic conductivity meter type 304.

Magnetic measurements were carried out using X-Band Electron paramagnetic resonance(EPR) using Bruker EMX Plus with the microwave frequency of 9.862953 GHz.
Magnetic susceptibility measurements were made using the vibrating sample magnetometer (VSM), LakeShore, 7407 model at room temperature with maximum 1 Tesla (FC/ZFC).

Fluorescence studies were conducted using Perkin Elmer LS45 Spectro fluorometer.

Single crystal X-Ray Diffraction (SXRD) studies were conducted using following materials:

- Data collection: CrysAlis PRO (Oxford Diffraction, 2010)
- Cell refinement: CrysAlis PRO
- Data reduction: CrysAlis PRO
- Program used to solve structure: SHELXS7
- Program used to refine structure: SHELXL2014/7
- Molecular graphics: ORTEP-3 for Windows and Mercury
- Software used to prepare material for publication: SHELXL97

### 3.5 COMPUTATIONAL TOOLS

- Molecular modeling
- Molecular docking studies
- Visualization

### 3.5.1 Materials Employed

- Molecular modelling : Structural studies
  - ab initio DFT studies using Gaussian 03
  - Conformal Optimization using MOE
- Molecular docking studies
  - Binding affinity
• Autodock-Vina
  - Binding Scores
• HEX 8.0.0

• Visualization
  - PyMol
  - MOE

3.5.2 Experimental Methods

**ab initio** DFT studies using MOE and Gaussian 03: The Gaussian 03 studies were undertaken to model the probable the structural implications. The molecules were built in chem sketch, and minimized by the MMFF94x force fields in MOE before being saved as .Pd files that were subsequently studied in G03.

To optimize the computational cost versus the accuracy, all the molecules were optimized under ONIOM bi-layer model with the high layer consisting of the inner core metals and the complexing atoms, while the rest of the complex was treated as a low layer. The high layer was optimized on HF/LANL2DZ while the low layer was optimized on B3PW91/STO-3G. Alternatively, where higher accuracy was demanded, the mono-layer DFT studies was used with higher basis set of 6-31G* and LANL2DZP to incorporate the diffused polarization effects.

The optimized structures were evaluated for their energetics and spatial conformations.

**Binding affinity using Autodock-Vina, GOLD and HEX 8.0.0:** The protein (PDB IDs) responsible for the antifungal activity in *P. chrysogenum*, and antibacterial mechanism in *P. aeruginosa* were obtained from the online protein data bank (PDB). These were accordingly modified, removing the undesired solvents and existent ligands and heteroatoms.
Docking studies were consequently carried out using Autodock-Vina for plain organic moieties and Hex 8.0 for the metal complexes. In Hex, by default, the parameters used for docking calculations were correlation type shape only, FFT mode at the 3D level, and grid dimension 6 with receptor range 180 and ligand range 180 with twist range 360 and distance range 40.

**Visualization of the binding interactions:** The binding interactions to note the active amino acid residues were pictured using MOE and PyMol.