Chapter 2

Materials, Methods and Instruments
Chapter 2

This chapter deals with a brief description of materials used, methods adopted and instruments employed in the present research project. The analytical methods, physicochemical measurements and biochemical techniques employed for the characterization and exploration of biological applications of the free ligands and their transition metal complexes are also compiled.

2.1. CHEMICALS

Ethyl pyruvate (Spectrochem, India), pyruvic acid (Spectrochem, India), hydrazine hydrate (Spectrochem, India), carbondisulphide (Spectrochem, India), sodium acetate anhydrous (Himedia, India), Aniline (s.d. fine chemicals India), 4 bromo aniline (s.d. fine chemicals India), 4 chloro aniline (Spectrochem, India), 4 fluoro aniline (s.d. fine chemicals India), ethylchlorocetate (s.d. fine chemicals India), o-phenylene diammine (Himedia, India), Isatin (s.d. fine chemicals India) were of analytical grade and used as supplied without further purification. Spectral grade deuterated DMSO-\textsubscript{d6} and CDCI\textsubscript{3} were used to record the NMR spectra and HPLC grade methanol, acetonitrile were used to record GCMS spectra of ligands.

Solvents were purified using standard procedures documented in Vogel’s text book of pracitcle organic chemistry [1, 2]. The drying reagents employed at various stages viz., anhydrous so-lium sulphate, anhydrous magnesium sulphate, calcium chloride, phosphorus pentachloride and mineral acids such as hydrochloric acid, sulphuric acid, nitric acid and bases like sodium hydroxide pellets and ammonia were of analytical grade obtained from s.d. fine chemicals India. Distilled water was used throughout the experimental processes.

2.2. ANALYSES OF COMPLEXES

Metal content and chloride contents of the transition metal complexes were analyzed following standard methods [3]. Cobalt, nickel and copper were determined gravimetrically as cobalt oxinate, nickel-dimethylglyoximate and copper salicylaldoximate respectively, while zinc was determined volumetrically using EDTA as the titrant. Chloride was determined gravimetrically as AgCl.
2.3. PHYSICAL MEASUREMENTS

2.3.1. Thin Layer Chromatography (TLC)

Progress of the reaction and purity of the compounds prepared in each step of the reactions was checked by TLC on pre-coated silica gel plates using different eluent systems. The aluminum pre-coated silica gel plates were obtained from Merck Chemicals India.

2.3.2. Melting points

Melting points were determined by using melting point apparatus supplied by Sheetal Scientific Instruments and are uncorrected.

2.3.3. Elemental analysis

All the compounds were analyzed for carbon, hydrogen and nitrogen on a TruSpec CHN/CHNS analyzer at University Scientific Instrumentation Centre, Karnataka University, Dharwad.

2.3.4. Gas chromatography and mass spectrometry

The mass spectra of the ligands which have boiling points below 280 °C were obtained on SHIMADZU GCMS-QP2010S at University Scientific Instrumentation Centre, Karnataka University Dharwad.

2.3.5. Conductance measurements

The molar conductance measurements were made on ELICO conductivity bridge type CM-82 provided with a dip type conductivity cell fitted with platinum electrodes. The cell constant was determined by measuring the conductance of aqueous KCl solution of known specific conductance. The value of cell constant was found to be 0.51. The conductance values of the complexes were determined by using 10^{-3} M solution in DMF. The molar conductance is calculated as follows,

\[ \Lambda_M = 1000 \times k \times \text{observed conductance (in mhos)/C} \]

Where \( \Lambda_M \) = Molar conductance; \( k \) = cell constant; \( C \) = Molar concentration (10^{-3})
2.3.6. Magnetic susceptibility measurements

Magnetic susceptibility measurements were carried out at room temperature on a Johnson Matthey magnetic susceptibility balance using Hg[Co(NCS)4] as the calibrant whose magnetic susceptibility is $16.44 \times 10^{-6}$. Effective magnetic moment at room temperature was calculated using following expression. \( \chi_m \) is molecular susceptibility. Diamagnetic corrections were made using Pascal’s constants.

\[
\mu_{\text{eff}} = 2.83(\chi_m T)^{1/2}
\]

The magnetic moment measurements were carried out at London Metropolitan University London, U. K.

2.3.7. Fourier Transform Infrared spectra

Infrared spectra of complexes and parent ligands were recorded in KBr discs in the region 400-4000 cm\(^{-1}\) on a Nicolet 170SX FT-IR spectrometer. IR spectra were recorded at University Scientific Instrumentation Centre, Karnataka University Dharwad.

2.3.8. Nuclear Magnetic Resonance spectra (NMR)

1D spectra of ligand and Zn(II) complexes and 2D HETCOR spectra of ligands were recorded in DMSO-\(d_6\) on Bruker AMX-300 MHz and Bruker AMX-500 MHz operating at 500.23 MHz for \( ^1\text{H} \) NMR and 100.63 for \( ^{13}\text{C} \) using \( ^1\text{H}/^{13}\text{C} \) dual probe using tetramethysilane (TMS) as internal standard. The number of scans set to record \( ^1\text{H}, ^{13}\text{C} \) and 2D HETCOR NMR were 16, 621 and 8 respectively, from University Scientific Instrumentation Centre, Karnataka University, Dharwad and London Metropolitan University London, U. K.

2.3.9. Electronic spectra

The UV-visible spectra of all the compounds in DMF were recorded on Varian Cary Bio UV-Visible spectrophotometer using \( 10^{-3} \text{ M} \) solution in the range of 200-1000 nm.
2.3.10. Electron paramagnetic resonance spectra (EPR)

The EPR spectra of a polycrystalline Cu(II) complexes were recorded at room temperature and liquid nitrogen temperature on a Varian E-4 X-band spectrometer using TCNE (tetracyanoethylene) as 'g' marker (g = 2.00277) at a frequency of 9.1 GHz under the magnetic strength of 3000 G. EPR spectral results were obtained from Sophisticated Analytical Instrument Facility, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India.

2.3.11. Thermal studies

Thermogravimetric studies were carried out in the 25–1000 °C temperature range using a TGA7 ANALYSER, Perkin-Elmer, US, with a heating rate of 10 °C per min in a N₂ atmosphere. The data were collected at University Scientific Instrumentation Centre, Karnataka University, Dharwad.

2.3.12. Cyclic voltammetric measurements

The cyclic voltammetric experiments were carried out with a three electrode apparatus using a CHI1110A electrochemical analyzer (USA). Cyclic voltammetric data were recorded using a glassy carbon working electrode (0.082 cm²), a platinum counter electrode, and an Ag/Ag⁺ reference electrode. Glassy carbon electrode surfaces were polished with 0.05-mm alumina, rinsed in water, and air-dried immediately before use. The electrochemical experiments were carried out and the positions of the waves were compared to the potential of the ferrocene/ferrocenium couple. The DMSO/DMF solution (containing 0.1 M Tetramethylammoniumbromide, as supporting electrolyte, 10⁻³ molar concentration of the ligand and each of the complexes) was placed in a single-compartment electrochemical cell and degassed by bubbling with N₂(g) saturated with DMSO. A nitrogen atmosphere was continuously maintained in the solution while the experiments were in progress.

2.3.13. Crystallographic studies

Single crystal X-ray diffraction studies were analyzed at 273 K using BRUKER SMART APEX-CCD diffractometer with MoKα radiation (λ = 0.71073).
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All the non-hydrogen atoms were refined with anisotropic temperature factors. The structures were solved using SHELXL-97 and refined using Full-matrix least squares F^2 [4]. All the calculations were performed using WinGX [5]. Molecular geometry calculations were performed using PARST and the structure drawing was obtained with ORTEP-3 for windows program [6]. This work was carried out at I.H.R.P. London Metropolitan University London, U. K.

2.4. BIOLOGICAL ACTIVITY

2.4.1. In Vitro Antiproliferative Activity

Under a sterile condition, human liver carcinoma cell line (HepG2) and human cervix carcinoma cell line (HeLa), Human acute lymphoid leukaemia (REH), Human Caucasian colon adenocarcinoma (COLO-205), Human promyelocytic leukemia cells (HL-60), Human acute lymphoblastic leukemia (MOLT-4) and Human erythroleukemia type cancer cell line (K562) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Antiproliferative activity of the synthesized ligands and their Co(II), Ni(II) and Cu(II) complexes at 100 μM concentration was tested against various cell lines using MTT assay [7,8]. Each compound was initially solubilized in dimethyl sulfoxide (DMSO), however, each final dilution contained less than 1% DMSO.

The cells at approximately 80% confluence were selected for trypsinization. The cells were harvested by removing the medium and then 1 mL of trypsin-EDTA (200 mg/L for EDTA, 500 mg/L for trypsin in a ratio (1:250) is added and incubated at 37°C for about 5 minutes. The cells were detached from the plate and collected in a centrifuge tube and centrifuged at 1000 rpm for 5 minutes. Supernatant solution was removed and the cells were resuspended in 10 mL RPMI-1640 culture medium. Cell number was determined using hemocytometer. The cell suspension was diluted to the required concentration of 5x10^4 cells/mL. 24-well microplates were seeded with 500 μL of cell suspension and incubated at 37 °C and 5% CO₂ for 24 h.

After 24 h incubation, the cells were treated with the newly synthesized compounds and then incubated for further 24 h. 100 mL of PBS solution of MTT (3-
(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/mL) was added to each well and incubation was continued for another 4 h. The medium was discarded and the cells were washed with sterile PBS and then the resultant formazan blue crystals were dissolved in 500 μL DMSO and the optical density (OD) was measured at 570 nm (reference filter 690 nm) using an automatic microplate reader. Triplicate readings were obtained for each sample and optical density values of the three wells were averaged for one sample. The results were recorded and expressed as % inhibition in cell growth of cell lines compared with the control.

2.4.2. DNA interaction studies

2.4.2a. Methodology for DNA cleavage analysis using gel-electrophoresis method

Culture media

Potato dextrose broth (Peptone 10, NaCl 10 and yeast extract 5 g/L) was used for the growth of the *E. coli*. The 50 mL media was prepared, autoclaved for 15 min at 121 °C and 15 lb pressure. The autoclaved media were inoculated with the seed culture and incubated at 37 °C for 24 h.

Isolation of DNA

The fresh bacterial culture (1.5 mL) was centrifuged to obtain the pellet and made to dissolve in 0.5 mL of lysis buffer (100 mM tris pH 8.0, 50 mM EDTA, 50 mM lysozyme) and to which added 0.5 mL of saturated phenol followed by the incubation at 55 °C for 10 min. The resultant mixture was centrifuged at 10,000 rpm for 10 mins and to the supernatant, equal volume of chloroform and isoamyl alcohol (24:1) and 1/20th volume of 3M sodium acetate (pH 4.8) were added and further, centrifuged at the same conditions. Soon after, 3 volumes of chilled absolute alcohol were added and so obtained DNA was separated by centrifugation. Finally the pellet was dried and dissolved in TE buffer (10 mM tris pH 8.0, 1 mM EDTA) and stored in cool.

Sample preparation

The samples (10 mg/mL) were prepared in DMSO.

Treatment of DNA with the samples

The synthetic compounds (100 μg) were added separately to the DNA sample of *E. coli*. The samples mixtures were incubated at 37 °C for 2 h.
2.4.2b. Agarose gel electrophoresis

To know the DNA cleavage action of compounds, 200 mg of agarose was dissolved in 25 mL of TAE buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/1L) by boiling and poured into the gel cassette fitted with a comb. When the gel attained ~55 °C, allowed to solidify and then, comb was removed carefully. So obtained solid gel was placed in the electrophoresis chamber flooded with TAE buffer. Subsequently 20 µL of DNA sample (mixed with bromophenol blue dye at 1:1 ratio) was loaded carefully into the wells, along with standard DNA marker and the constant 50 V of electricity was made to pass for around 30 mins. Finally, the gel was removed carefully and stained with ETBR (Ethidium bromide) solution (10 µg/mL) for 10-15 min and the bands were observed under UV transilluminator. The illuminated gel was photographed by using a polaroid camera (a red filter and polaroid film were used) [9].

2.4.2c. DNA binding analysis using electronic spectral method

The concentration of E. coli DNA was determined spectrophotometrically by assuming ε 260 = 6600 M⁻¹ cm⁻¹. All the experiments involving the interaction of the complex with DNA were carried out in deionized water buffered with tris(hydroxymethyl) aminomethane (Tris, 5 mM) and sodium chloride (50 mM), and adjusted to pH 7.1 with hydrochloric acid. A solution of DNA gave a ratio of UV absorbance at 260 and 280 nm of ca. 1.3 : 1, indicating that the DNA was sufficiently free of protein. In constructing the UV–Vis spectrophotometric DNA titration curves, the DNA absorption was subtracted. In order to evaluate quantitatively the DNA-binding strength, the intrinsic DNA-binding constant K₅ was derived by nonlinear regression analysis using equation [10, 11]

\[
\frac{[\text{DNA}]}{(e_a - e_f)} = \frac{[\text{DNA}]}{(e_a - e_b)} + \frac{1}{K_b (e_a - e_b)}
\]

where [DNA] is the concentration of DNA per base pairs, εₐ is the apparent absorption coefficient, which was obtained by calculating Aₐₐₐ/[complex] and εₕ and εₖ are the extinction coefficients for the free complex and complex in the fully bound form, respectively.
2.4.2d. DNA binding analysis using viscosity measurement

Viscosity measurements were carried out on an Ostwald micro-viscometer, immersed in a water bath maintained at a temperature of 26.0 ± 0.01 °C. The sample flow times were measured three times and the mean value was used. Data are presented as (\(\frac{\eta}{\eta^0}\))^{1/3} versus the ratio [complex] / [DNA], where \(\eta\) and \(\eta^0\) are the specific viscosity of DNA in presence and in the absence of complex respectively. The values of \(\eta\) and \(\eta^0\) were calculated by using equation,

\[
\eta = (t-t^b)/t^b
\]

Where \(t\) is the observed flow time of DNA containing solution and \(t^b\) is the flow time of buffer alone. Relative viscosities for DNA were calculated from the relation \(\frac{\eta}{\eta^0}\) [12, 13].

2.4.2e. Cyclic voltammetric studies

The cyclic voltammetric experiments were carried out with a three electrode apparatus using a CHI1110A electrochemical analyzer (USA). The complexes were dissolved in DMSO to the desired concentrations. The cyclic voltammogram of fixed concentrations of complexes in the absence and presence of DNA were performed. The shifts in the values of \(E_p\) (separation of anodic and cathodic peak potentials), \(i_{pc}/i_{pa}\) (the ratio of cathodic to anodic peak currents), and the formal potential \(E_{1/2}\) explains the binding ability of complexes with the DNA [14].

2.4.2f. DNA thermal denaturation studies

Thermal denaturation studies were carried out on a UV spectrometer equipped with temperature controlling thermostat. The melting curves (\(T_m\)-curve) of both free \(E.\ coli\) DNA and DNA-bound complexes were obtained by measuring the hyperchromicity of DNA at 260 nm as a function of temperature. The melting temperatures were measured with 60 mM DNA in phosphate buffer at pH 6.8 (\(\mu = 0.2\) mol L\(^{-1}\)M NaCl). The temperature was varied from 25 °C to 85 °C at a rate of 5 °C min\(^{-1}\). The melting temperature (\(T_m\)) was taken as the mid-point of the hyperchromic transition [10, 11].
2.4.3. Antimicrobial Activity

The prepared compounds were evaluated for their antimicrobial strategy against bacteria *Staphylococcus aureus, Bacillus subtilis & Escherichia coli* and fungi *Aspergillus niger, Candida albicans & Cladosporium*. The methodology used for the investigation is described below. *Gentamycin* and *Amphotericin* were used as standards for bacteria and fungi respectively, in the present task.

2.4.3a. Antibiogram analysis of compounds against bacteria

Media used in the analysis was prepared by adding 10 g of peptone and 10 g of sodium chloride to the homogeneous mixture of 5 g of Yeast extract and 20 g of nutrient agar taken in 1000 mL of distilled water. Initially, the stock cultures of *Staphylococcus aureus, Bacillus subtilis* and *E. coli* were revived by inoculating in broth media and grown at 37 °C for 18 h. The agar plates of the above media were prepared and wells were made in the plate. Each plate was inoculated with 150 µL of 18 h old cultures and spread evenly on the plate. After 20 min, the wells were filled with 500 µL of each compound (10 mg/mL in DMF). The control plates with *Gentamycin* (10 mg/mL) and DMF were also prepared. All the plates were incubated at 37 °C for 24 h and the diameter of inhibition zone were noted in centimeters. The values were compared with that of *Gentamycin* and the samples showing significant inhibition were selected for the further calculation of minimum inhibition concentration (MIC). The MICs of compounds were determined by assaying at 400 and 800 µg concentrations along with standard *Gentamycin* at the same concentrations. The cultures were grown for 24 h and the zones were compared with that of *Gentamycin* and percentage of inhibition were calculated.

2.4.3b. Antibiogram analysis of compounds against fungi

Potato dextrose agar (PDA) medium used in the analysis was prepared by mixing 20 g of dextrose to the filtrate obtained by the 250 g of peeled potato boiled for 20 min squeezed and filtered; the volume was made up to 1000 mL by adding distilled water. Initially, the stock cultures of *Aspergillus niger* and *Candida albicans, Cladosporium* were revived by inoculating in broth media and grown at 37 °C for 48
h. The agar plates of the above media were prepared and wells were made in the plate. Each plate was inoculated with 150 μL of 48 h old cultures and spread evenly on the plate. After 20 min, the wells were filled with 500 μL of each compound (10 mg/mL in DMF). The control plates with *Amphotericin* (10 mg/mL) and DMF were also prepared. All the plates were incubated at 37 °C for 48 h and the diameter of inhibition zone were noted in centimeters. The values were compared with that of *Amphotericin* and the samples showing significant inhibition were selected for the further calculation of minimum inhibition concentration (MIC). The MICs of compounds were determined by assaying at 400 and 800 μg concentrations along with standard *Amphotericin* at the same concentrations. The cultures were grown for 48 h and the zones were compared with that of *Amphotericin* and percentage of inhibition were calculated.
References


