Chapter 2

Material and Methods

List of Chemicals & Reagents

All reagents, chemicals and solvents used were of analytical grade. $CuCl_2.2\ H_2O$, $VOSO_4.5H_2O$, $NiCl_2.6H2O$, $MnCl_2.4H_2O$, $FeCl_3.3H_2O$, $Cu(acetate)_2.2H_2O$, $ZnCl_2$ (anhydrous) (Rankem), $RuCl_3.\ H_2O$ (Hi-media), Moxifloxacin (MFL), Gatifloxacin (GFL), Levofloxacin (LFL) (Gift from Alembic Pharmaceuticals), Curcumin, 2,2–bipyridine, 1,10-phenanthroline, Leucine, Phenylalanine, Tryptophan, Tyrosine, Cytosine, Adenosine triphosphate (ATP) Benzaldehyde, Isatin, Isoniazid Ferrocenecarboxyaldehyde (SRL), Bovine serum albumin (BSA), glycyl-glycine, glycyl-leucine, glycyl-alanine(Sigma), Tris(hydroxymethyl) aminomethane(Hi-media), Disodium salt of calf thymus DNA (CT-DNA) (SRL), Agar-agar, glycerol, Bromophenol blue & xylene cyanol FF (Hi-media), 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide ; Ethidium Bromide (EtBr), Nicotine amide adeninde dinucleotide (NADH), Nitro blue tetrazolium(NBT). Phenazine methosulphate (PMS), Dimethyl sulphoxide (DMSO) (Merck), 4',6-diamidino-2-phenylindol(DAPI), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT), 2,7-Dichlorodihydrofluorescein diacetate (DCFDA), AnnexinA5, Propidiumiodide, *N,N,N′,N′*-Tetramethylacridine-3,6-diamine (Acridine orange), , mercaptopropionic acid (MPA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich) and Super coiled pBR322 DNA (Genei) were utilized as received. All the solvents were purified by standard procedures, whenever required.

2.0 Characterization techniques

Carbon, hydrogen and nitrogen contents were determined using Carlo Erba analyzer model 1108. Melting points were determined by electrothermal apparatus. TLC was performed on commercial aluminium sheets precoated with 0.20 mm layers of silica gel. The visualization of spots on TLC plates was effected by exposure to iodine.

2.1 Infrared spectroscopy

The infrared spectra of all the complexes/ligands in solid state were recorded on a Perkin Elmer RX-1 Fourier Transform Infrared spectrometer in the range of 4000-400cm^{-1} (used resolution 4 cm^{-1}; minimum resolution 0.125 cm^{-1}) as KBr pellets. About 5 mg of finely powdered sample, thoroughly mixed with uniformly dried spectral grade KBr and pressed into a thin pellet under anhydrous conditions was applied for recording IR spectrum.

2.2 Ultraviolet and visible spectroscopy

The electronic spectra of the complexes in solution state were recorded on UV lamda 50 (Perkin Elmer) double beam spectrophotometer in the range 200-900 nm.

2.3 Nuclear Magnetic resonance spectroscopy

NMR samples were prepared by dissolving the compounds in a deuterated lock solvent. Samples for ^{1}H NMR were dissolved in DMSO or $CDCl_3$ and the ^{1}H peak of tetramethylsilane (TMS) is used as the internal reference . ^{1}H NMR spectra were recorded at 400.00 MHz on an Avance-400 Bruker, Switchzerland NMR spectrometer.

2.4 Electronic spin resonance spectroscopy

In the present study the x-band (9.1 GHz) ESR spectra of the complexes were recorded in DMSO at 298K and at liquid nitrogen temperature (L.N.T) on Perkin-Elmer 450X spectrometer with 100 KHz field modulation at IIT Bombay. The ESR spectrum obtained from the instrument is a plot of the first derivation of the absorption curve as function of magnetic field. The magnetic field was calibrated with an NMR gauss meter and the exact frequency was determined by using TCNE radical as a field marker.

2.5 Mass spectrometry

Mass spectra of the synthesized ligands and complexes were recorded on JEOL GC MATE II GC-Mass spectrometer in ESI ionization mode at Oxygen healthcare, Ahmedabad.

2.6 Thermal Analysis

The thermal behaviour of the synthesised complexes was determined by using EXSTAR 6300, TA instruments (USA), operating at heating rate of 10˚C per minute in the range of 30-800˚C in N_2.

2.7 Cyclic voltammetry

Cyclic voltammetry was performed with CH Instruments 660C Electrochemical Analyzer, using a three-electrode configuration comprised of a Pt wire as the auxiliary electrode, a platinum sheet as the working electrode and Ag/AgCl as the reference electrode. Electrochemical measurements were made under nitrogen atmosphere and at 298 K. All solutions were prepared in DMSO containing 0.1M tetraethyl ammonium phosphate (TEAP).

2.8. DNA binding studies

The mode and DNA propensity of the synthesized complexes were analysed with different experimental techniques.

All the experiments involving interaction of complexes with CT-DNA were conducted in Tris buffer containing 150 mM NaCl and 15 mM trisodium citrate adjusted to pH 7.2 with HCl. DNA stock solution was prepared by dilution of CT DNA with buffer (followed by exhaustive stirring at 4 °C for three days, and kept at 4 °C for no longer than a week. The stock solution of CT DNA gave a ratio of UV absorbance at 260 and 280 nm (A_{260}/A_{280}) ≤ 1.90, indicating that DNA was sufficiently free of protein contamination [1].DNA concentration was determined by absorption spectroscopy using the molar absorption coefficient 6600 $M^{-1}cm^{-1}$ [2] at 260 nm.

2.8.1 Absorption spectral studies

In UV titration experiments, the binding constant K_b of complexes with CT-DNA has been determined through the UV-spectra of complexes recorded for constant complex concentration in the absence and presence of CT-DNA for diverse [complex]/CT-DNA mixing ratios(r). The magnitude of binding strength of compounds with CT-DNA was estimated through the binding constant K_b which can be obtained by monitoring the changes in the absorbance at corresponding λ_{max} with increasing concentration of CT-DNA and is given by the ratio of slope to the *y*-intercept in the plot **[DNA]/(ε_A-ε_f) vs [DNA] ,** according to the equation [3]:

$$[DNA]/(\varepsilon_A-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f)+1/K_b(\varepsilon_b-\varepsilon_f) \quad \ldots\ldots\ldots\ldots\ldots\ldots(2.1)$$

Where [DNA] is the concentration of DNA in base pairs $\varepsilon_A = A_{obsd}$ / [complex]; ε_f= extinction coefficient of the free complex and ε_b= extinction coefficient for the complexes in fully bound form. A plot of [DNA]/(εa-εf) versus [DNA] gave a slope of 1/(εb-εf) and Y- intercept equal to 1/ Kb(εb-εf); K_b is the ratio of the slope/intercept.

2.8.2 Fluorescence spectral studies

The competitive studies of each complex with DNA have been investigated with fluorescence spectroscopy in order to examine if the complex can displace EB from its CT DNA-EB complex. The CT DNA-EB complex was prepared by adding 20μM EB and 26 μM CT DNA in buffer (150 mM NaCl and 15 mM trisodium citrate at pH 7.0). The relative binding of the complex to DNA was studied by addition of the complex at different concentrations to a DNA-EB solution. The influence of the addition of each complex to the DNA-EB complex solution was obtained by recording the variation of the fluorescence emission spectrum. Fluorescence intensities between 550 and 800 nm (λ_{ex}= 440 nm) were measured at each step of the titration The Stern–Volmer constant K_{SV} is used to evaluate the quenching efficiency for each complex according to stern volmer the equation (Eq.):

$$\frac{Io}{I} = 1 + K_{SV}[Q] \qquad \text{..... (2.2)}$$

Where Io andI are the emission intensities in the absence and the presence of the quencher, respectively, [Q] is the concentration of the quencher (complexes) and K_{SV} is the Stern–Volmer constant and which was obtained from the slope of plot Io/I vs [Q].

2.8.3 Cyclic voltammetry

The interaction of the complexes with CT-DNA was investigated by recording the cyclic voltammograms of 0.4 mM dmso/buffer (1:1 v/v) solution of the complexes upon addition of CT-DNA. Buffer was used as a supporting electrolyte and cyclic voltammogram were recorded at 100 mV s^{-1}.

2.8.4 Viscometry studies

Viscosity measurement is sensitive to length change, least ambiguous and the most critical method for determining the binding mode of compounds to DNA in solution in the absence of crystallographic data. Interaction of foreign moiety to DNA which alters the length of DNA duplex is directly reflected on the intrinsic viscosity of DNA depending on the mode of interaction.

In the present study, viscometric experiments were performed using Schott Gerate AVS 310 Automated Viscometer that was thermostated at 27 (±0.1) ºC in a constant temperature bath. The concentration of CT DNA was 100 μM. The flow times were measured with an automated timer. Each sample was measured 3 times and an average flow time was calculated. The viscosity values were calculated using equation [4].

$$\eta = (t - t_0) \quad \text{..... (2.3)}$$

where t_0 is the flow time of buffer alone and t is the flow time for buffer containing DNA. Data are represented in terms of $(\eta/\eta_0)^{1/3}$ versus concentration ratio ([Complex]/[DNA]), where η is the viscosity of DNA solution in the presence of complex and η_0 is the viscosity of solution of DNA alone. Viscosity values were determined from the observed flow time of DNA-complex solutions (t) corrected for that of the buffer alone (t_0).

2.8.5 CD Spectral studies

Circular dichroism (CD) spectra were recorded on a Jasco J -815 spectrometer at a scanning speed of 50 nm/min at room temperature using fixed concentration of complexes (50 μM) in DMSO in the absence and presence of increasing amount of DNA (0 -60 μM).

2.8.6 Gel electrophoresis

(i) **Preparation of the sample solution**:

About 5 mg of the sample was accurately weighed using digital electronic balance and made the solution with H_2O/DMSO. The solution was diluted suitably to 10 μM concentration.

(ii) Buffer solutions

Tris-borate ethylenediaminetetraacetate (TBE) electrolyte buffer (5X stock) 54 g of tris base (MW 121.14) was dissolved in 100 ml of water. 27.5 g of boric acid was and 20 ml of 0.5 M EDTA (pH 8.0) solution was added and diluted to 1 litre with water. The resulting buffer was stored at 4°C.

(iii) Loading buffer (6X)

0.25% bromophenol blue in 40% sucrose/water mixture was used as the loading buffer. This buffer was prepared by first dissolving 2.0 g of sucrose in 3 ml of water and adding a 12.5 mg of bromophenol blue to this solution. The volume was made upto 5.0 ml. The resulting buffer was stored at 4°C

(iv) Ethidium bromide stock solution (1 mg/ml)

1 mg of ethidium bromide was dissolved in 1 ml of water by stirring in dark for several hours. The resulting solution was stored in an aluminum foiled bottle at ambient temperature. A working concentration of 0.5 μg/ml was used for staining the gels after electrophoresis.

(v) 0.8% agarose gel casting:

0.8 g of accurately weighed (molecular biology grade, Gelrose) agarose was added to 100 ml of TBE buffer. The slurry was then heated on boiling -water bath until the agarose dissolved completely. The resulting clear solution was poured onto the gel mould and immediately the comb was clamped into position near one end of the gel. The teeth of the comb formed the sample wells. Care was taken to see that at least 0.5–1.0 mm of agarose was left between the bottom of the teeth and base of the gel, so that the sample wells are completely sealed. After 30 – 45 min. the comb and auto clamped tape were removed carefully and the gel was mounted in a tank. 1.5 litre of working buffer (TBE) was transferred into gel until the gel was covered to a depth of about 1.5 mm.

(vi) Assay for nuclease activity

The solution containing metal complex was taken in a clean eppendroff tube and 1µg of plasmid pBR322/PUC19 DNA was added. The contents were incubated for 30 minutes at 37^0C and loaded on 0.8% agarose gel after mixing 3µl of loading buffer and 0.25% bromophenol blue + 0.25% Xylene cynaol + 30% glycerol. Electrophoresis was performed at constant voltage till the bromophenol blue reached to the 3/4 of the gel. The gel was stained for 10 min by immersing it in ethidium bromide solution (5 µg/ml) and then de-stained for 10 min by keeping it in sterile distilled water. The plasmid bands were visualized by photographing the gel under a UV Transilluminator.

The efficiency of DNA cleavage was measured by determining the ability of the complex to form open circular (OC) or nicked circular (NC) DNA from its super coiled (SC) form.

2.9 Protein Binding studies

2.9.1 Fluorescence spectral studies

The binding of the complexes with BSA has been estimated from the change in the fluorescence intensity of the protein after the addition of complexes.

The interaction between the metal complex and BSA (6.6 μM) was studied from tryptophan fluorescence quenching experiments in phosphate buffer (pH 6.8). Quenching of the emission intensity of tryptophan residues of BSA at 344 nm (excitation wavelength at 295 nm) was monitored in the presence of increasing complex concentrations. The values of the Stern-Volmer quenching constants and the quenching rate constants for the complexes interacting with BSA are calculated by Stern–Volmer quenching equation

$$Io/I = 1 + K_{SV}[Q] \quad \text{..... (2.4)}$$

and the Stern-Volmer plots Io/I versus [Q] where Io is the initial tryptophan fluorescence intensity of BSA, I the fluorescence intensity after addition of the complexes, K_{SV} (M^{-1}) the quenching constant, [Q] is the concentration of the quencher.

2.9.2 Isothermal Calorimetric studies

Isothermal Titration calorimetry is a thermodynamic technique for monitoring any chemical reaction initiated by the addition of a binding substrate. When substances bind, heat is either generated or absorbed. Measurement of this heat allows accurate determination of binding constants, reaction stoichiometry, enthalpy and entropy of binding, thereby providing a complete thermodynamic profile of the molecular interaction in a single experiment. The BSA solution was located inside the sample cell and the complex solution in the injector syringe (Fig 2.1). A feedback control system

supplies thermal power continuously to maintain the same temperature in both reference and sample cells. Any event taking place in the sample cell, usually accompanied by heat change, will change the temperature in that cell and the feedback control system will modulate the power supplied in order to minimize such temperature imbalance.

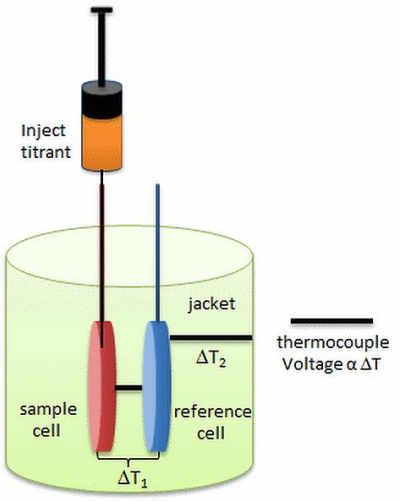

Fig 2.1: Isothermal Titration Calorimeter Cells

A sequence of injections is programmed & the complex solution was injected periodically into the sample cell. The value of the association constant (or binding affinity), Ka, governs the equilibrium, i.e. the partition between the different species (free & bound). The change in composition inside the sample cell after each injection triggers the binding reaction & the rearrangement of populations leading to the formation of complex. The heat changeassociated with each injection is proportional to the increase in complex concentration (advance of the reaction) and it was calculated by integrating the area under the deflection of the signal measured (amount of heat per unit of time provided to maintain both cells, sample & reference, at the same temperature). At the end of the experiment, saturation of the macromolecule was reached with the added complex. Applying non-linear regression using the appropriate models in the data

analysis, binding constant, Ka, binding enthalpy, ΔH, entropy change, ΔS and the stoichiometry of binding (n), in a single experiment was determined.

The energetics of the binding of complexes to BSA was studied by using an isothermal titration calorimeter ITC measurements were performed at 298 k with Nano ITC. All the solutions were thoroughly degassed before loading. Titrations were carried out using 0.25 cm^3 syringe filled with complex solution stirring at the speed of 300 rpm during the run. The reference cell was filled with buffer. A titration experiment consisted of 25 consecutive injections of 10 μl each, of the complex solution into the protein (BSA) solution for durations of 20 s with a 4 min interval between consecutive injections. To correct the heat effects of dilution and mixing, control experiments were performed at the same concentrations of the protein and the complex and the data subtracted from the respective complex protein titrations data. The heat released or absorbed upon each injection was measured, and the data were plotted as integrated quantities. The data fitted well to a two sets of binding sites model.

2.9.3 Protein cleavage studies

Protein cleavage experiments were carried out following literature procedures [5, 6]. Freshly prepared solution of BSA in 50 mM Tris-HCl buffer (pH 7.2) was used for the protein cleavage studies. The protein solutions in Tris-HCl buffer (Eppendorf vials in) medium containing complexes (with varied concentrations of 50, 100, 150 and 200 μM) were incubated at 37 °C for 1.0 h The incubated samples (50 μL) were dried in an EYELA Centrifugal Vaporizer (Model CVE-200D) and the samples were dissolved in the loading buffer (24 μL) containing SDS (7% w/v), glycerol (4% w/v), Tris-HCl buffer (50 mM, pH 6.8), mercaptoethanol (2% v/v) and bromophenol blue (0.01% w/v). The protein solutions were boiled for 3 min for denaturation. The samples were loaded on a 3% polyacrylamide stacking gel. The gel electrophoresis was done at 60 V until the dye

passed into the separating gel from the stacking (3%) gel, and then the voltage was increased to 120 V. The gels were run for 1.5 h, stained with Coomassie Brilliant Blue R-250 solution (acetic acid/methanol/water = 1:2:7 v/v) and destained with water/methanol/acetic acid mixture (5:4:1 v/v) for 4 h. The gels, after destaining, were scanned with a HP Scanjet G3010 scanner and the images were further processed using Adobe Photoshop 7.0.

2.10 Biological studies

2.10.1Antimicrobial activity

The antibacterial activity of complexes strains of bacteria to complexes were evaluated by measuring the size of the bacteriostatic diameter (Inhibition zone).

Antibacterial activities were investigated using agar well diffusion method [7]. All the synthesized complexes were screened in vitro for their antibacterial activity against two Gram-negative (*Escherichia coli, Staphylococcus Ptyphi.*) and two Gram-positive (*Bacillus subtulis, Staphylococcus aureus*) bacterial strains. Ciprofloxacin and Moxifloxacin were used as standards for antibacterial activity. Each compound was dissolved in water at different concentrations (5, 10 and 20 mg/ ml). 1 $cm^3$of a 24 h broth culture containing 106 CFU/cm^3 was placed in sterile petri-dishes. Molten nutrient agar (15 cm^3) maintained at 45°C was then poured into the Petri-dishes and allowed to solidify. Holes of 6 mm diameter were formed in the agar using a sterile cork borer and these holes were completely filled with the test solutions. The plates were incubated for 24 h at 37°C and the zones of inhibition based upon zone size around the discs were measured three times by caliper to get an average value. The minimum inhibitory concentration (MIC) was determined by serial dilution technique and the activities of

the prepared complexes were confirmed by calculating the activity index according to the following relation:

$$\text{Activity index}(A) = \frac{\text{Inhibition zone of compound (mm)}}{\text{Inhibition zone of standard drug (mm)}} \text{ X 100} \quad \text{..... (2.5)}$$

2.10.2 Anticancer Activity: MTT assay

Traditionally, the determination of cell growth is done by counting viable cells after staining with a dye, Yellow MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) a tetrazole, which is reduced to purple formazan in the mitochondria of living cells and absorbance of this coloured solution can be quantified by measuring between 500-600 nm by a spectrophotometer. The absorption maximum is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversions can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an cytotoxic agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the cytotoxic agent in causing death of cells can be deduced, through the production of a dose-response curve. Solutions of MTT solubilized in tissue culture media or phosphate buffer, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals, which are dissolved in acidified isopropanol and the resulting purple solution is spectrophotometrically measured.

The MTT assay was done to evaluate the cytotoxic activity of the compounds on A549 human lung carcinoma cells. 5.0×10^3 cells $well^{-1}$ were plated in triplets in 96 well cell culture plates (Tarson India Pvt. Ltd.). The cell were treated with various concentration

of complexes (5.0 - 94.5µg/ml) and incubated for 16-18 hrs at 37°C. At the end of the incubation period, 10 µl of MTT (5 mg ml^{-1}) was added to each well, and further incubated for 2-4 h at 37 °C. The culture media was discarded and the wells were washed with Phosphate Buffer Saline (Hi-Media, India Pvt. Ltd.), followed by addition of 150µl DMSO and subsequent incubation for 30 min. The absorbance was measured at 540nm using an ELX800 Universal Microplate Reader. To ensure that all experiments were performed in the linear range of the assay, initial standardization assays were carried out. The IC_{50} values were determined by non-linear regression analysis using GraphPad Prism (5.0)

2.10.3 Staining Techniques

2.10.3.1 Fluorescent staining studies: AO/EB staining

Apoptosis i.e programmed cell death is normal in the development and maintenance of the health of multicellular organisms. Cells die in response to a variety of external and physiological stimuli, and during apoptosis, they do so in a controlled and regulated fashion. This makes apoptosis distinct from the other form of cell death, namely, necrosis in which uncontrolled (accidental) cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Necrosis involves karyolysis, cytoplasmic vacuolation culminating in loss of integrity of cell membrane followed by cell lysis. Apoptosis, by contrast, is a process in which cells play an active role in their own death (cell suicide). Most tumor cells retain their sensitivity to some apoptotic stimuli which seems to be a primary factor in determining the efficacy of chemotherapeutic agents, and in this context, the apoptosis-inducing ability of drugs.

Acridine orange is a vital dye that is taken up only by viable cells (Fig 2.2). After its uptake, it gets intercalated into the double stranded DNA of a live cell and emits green fluorescence. Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA (Fig 2.3). To investigate the mode of cell death (viz. apoptosis or necrosis) in A549 cells caused by the complexes, cells were stained with AO/EB dual dye and observed under the fluorescence microscope.

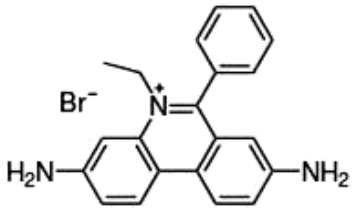

Fig 2.2: Ethidium bromide
3,8-Diamino-5-ethyl-6 phenylphenanthridinium bromide. Ex: 285 Em: 605

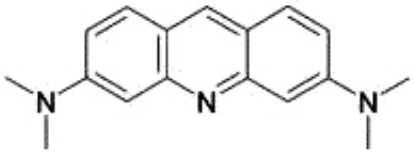

Fig 2.3: Acridine orange
N,*N*,*N*′,*N*′-Tetramethylacridine-3,6-diamine
Ex: 502 Em: 525

The cell suspension of sample containing $5x10^5$ cells treated with complexes, were examined under a fluorescent microscope (Carl Zeiss, Germany) using an UV filter (450-490 nm) after treatment with 25 µL of AO/ EB solution (1 part of 100 µg ml^{-1}AO and 1 part of 100 µg ml^{-1} EB in PBS). Three hundred cells per sample were counted in triplicate for each dose point. The cells were scored as viable, apoptotic or necrotic as judged by the staining, nuclear morphology and membrane integrity, and the percentages of apoptotic and necrotic cells were then calculated. Morphological changes were also observed and photographed.

2.10.3.2 Nuclear condensation: Dapi Staining

DAPI staining reveals the changes in the gross cytology of the cell, with special reference to cytoplasm and nucleus. DAPI (4',6-diamidino-2-phenylindole, Fig.2.4) is a fluorescent dye that binds strongly to A–T rich regions in DNA and produces blue florescence at excitation wavelength 358 nm and emission wavelength 461 nm. Nuclear staining was performed by reported procedures. Briefly, the cells after exposure with complexes for 12-16 h were washed with PBS and fixed in 3.7% paraformaldehyde for 10 min. The fixed cells were then permeabilized with TBST [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100] for 5 min. Cells were washed with PBS and then DAPI solution (10 μgmL^{-1} in PBS) was added and kept for 5 min. After several washings with PBS, the cells were observed under a fluorescence microscope (Leica DM IL microscope with integrated Leica DFC 320 R2 camera and IL50 image software)

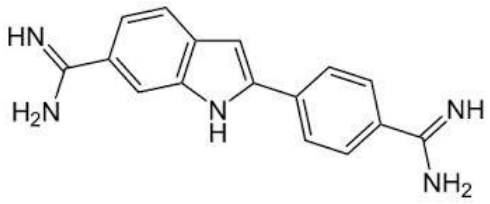

Fig 2.4: DAPI (4',6-diamidino-2-phenylindole)
Ex: 358nm Em :461nm

2.10.3.3 Determination of intracellular ROS generation

Free radicals or reactive oxygen species (ROS) are molecules generated by biochemical redox reactions during normal cell metabolism. The deleterious effects of these ROS are kept under check by a delicate balance between the rate of their production and the rate of their elimination by endogenous antioxidant defence systems. But under conditions of toxic insult, overproduction of ROS burdens the cellular antioxidant defence

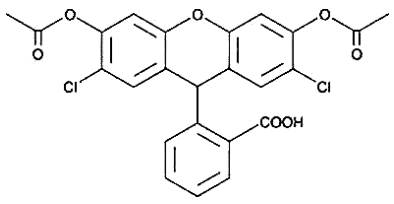

Fig 2.5: DCFDA
2,7-Dichlorodihydrofluorescein diacetate
Ex:498 Em:523

system that results in oxidative status eventually culminating in cellular oxidative damage. Cellular oxidative stress is studied using DCFDA (2,7-Dichlorodihydrofluorescein, Fig. 2.5) stain that gets converted to DCF and emits green fluorescence by reacting with hydrogen peroxide and peroxyl radicals.

DCFDA stain was used to decipher metal complexes induced intracellular oxidative stress. A549 cells (1.0×10^5 cells well^{-1}) were grown on cover slips using 12 well cell culture plates for 12 h. After a 12 h treatment with complexes **1-5** (25 μg/ml), the cells were incubated with 7.5×10^{-6} M DCFDA at 37 °C for 30 min and observed with a Leica DMRB florescence microscope. The intracellular mean fluorescence intensity was quantified using Image J 1.40 g software.

2.10.3.4 DNA fragmentation assay by agarose gel electrophoresis

The formation of oligo-nucleosomal DNA fragments were assessed using an electrophoretic technique called "DNA ladder" that reveals a visual profile of DNA damage**.** A549 cells were cultured in the presence of 25μg/ml of complexes for 12 h, harvested by trypsinisation, washed and suspended in PBS. Cells ($1x10^6$) were resuspended in lysis buffer [20 mM EDTA, 0.8% (w/v) sodium lauryl sarcosiate, 100 mM Tris (pH 8.0)] and 10 mg/ml RNase (Boehringer Manneheim, Sussex, UK) and incubated at 37 °C for 24h. Proteinase K (1 mg/ml) was subsequently added and the samples were incubated for a further 2h at 50°C. DNA was then extracted with phenol/ chloroform/isoamyl alcohol (25:24:1) mixture and ethanol from the cell lysate and the extracted DNA (10 μg) was subjected to 1.5% agarose gel electrophoresis [8].

2.10.3.5 Flow cytometry: Annexin V-FITC/PI staining

Flow cytometry (FACS analysis) is a method to evaluate cell membrane proteins and intracellular proteins as well as peptides and DNA. The principle behind FACS is an antigen-antibody reaction, with the antibodies being fluorescently labelled. Flow cytometry quantification is carried out with intercalating color labels (without the antibody).

The basis of a FACS analysis is a labelled (colored) suspension of individual cells which passes a focused laser beam. Capillary forces cause the cells to pass the flow cell, where the labels are stimulated by the laser light. The emitted florescent light from the fluorophores (coupled to the antibodies) and the scattered-light are detected separately. The cells scatter a fraction of the light which is then detected by photomultipliers (light detectors). The amount of light measured correlates with the size of the cells and their complexity. A measurement for the diffraction of light in a flat angle is the forward scatter (FSC), which depends on the volume of the cell. A measurement for the diffraction of light in a right angle is the so called side wards scatter (SSC). It depends on the granularity, the size of the cells, the structure of its nucleus, and the amount of vesicles inside the cells.

Annexin-V FITC/PI double-staining assay was used to quantify apoptosis, according to the manufacturer's protocol (Sigma Aldrich, Ltd.USA). A549 cells were incubated in presence of complexes of desired concentration for 12-18 hrs. The cells from each well were then centrifuged, washed with PBS and suspended in 100 µL buffer. 5µl of Annexin V FITC Conjugate and 10 µl of Propidium Iodide solution were added to each cell suspension and incubated for 10 min at room temperature in the dark. The samples were analysed on flow cytometer (MoFlo™ Cytomation, Modular Flow Cytometer) using Cell Quest software. Double staining of cells with FITC-Annexin V and PIpermits

the discrimination of live cells ($FITC^{-}P^{-}$), early apoptotic ($FITC^{+}$ PI^{-}), late apoptotic ($FITC^{+}$ PI^{+}) or necrotic cells ($FITC^{-}$ PI^{+}).

2.10.3.6 Gene expression assay

Expression of apoptosis related genes, BCL-2 and BAX were studied using reverse transcriptase PCR (RT-PCR) wherein GAPDH was used as a control. Total RNA was isolated using trizol reagent (Invitrogen, California, USA). cDNA was synthesized by reverse transcription of 1 µg of total RNA using I Script cDNA Synthesis kit (BIORAD, California, USA). PCR was carried out using PCR Master Mix (Thermo Scientific, California, USA).Total volume was 25 µl: 2 µl c-DNA, 12.5 µl Dream Taq™, 1 µl of each primer and 8.5µl ultrapure water. Cycler conditions were as follows: Initial denaturation at 95°C for 3 min, further it was followed by PCR cycle of denaturation at 95°C for 30 sec, annealing at 55.2°C (GAPDH), 55.0°C (BAX) and 55.5°C (BCL-2) for 30 s and strand extension at 72 °C for 30 s and ends with final extension at 72°C for 8-10 mins. PCR cycles were 35 for GAPDH and 37 for BAX and BCL-2. The PCR products were separated on 2% agarose gel which was stained with ethidium bromide and quantified by ImageJ. Statistical analysis was done by using ANOVA. Primers used for this study are listed in Table-1.

Table-1 Primers used for gene expression study

LIST OF GENES	DETAILS	LENGTH OF AMPLICON
GAPDH	Accession number (NM_002046) Forward primer (Template: 113-132) 5′-GCTCTCTGCTCCTCCTGTTC-3′ Reverse primer (Template: 366-385) 5′-CAGTTCCGACTCTTGCCCT -3′	273bp
Bcl-2	Accession number (NM_000633) Forward primer (Template: 959-978) 5′-GTCATGTGTGTGGAGAGCGT-3′ Reverse primer (Template: 1083-1102) 5′-GGAAACACCTTGACATGCCG -3′	144bp
BAX	Accession number (NM_001291428) Forward primer (Template: 138-157) 5′-GGCCCTTTTGCTTCAGGGTT -3′ Reverse primer (Template: 341-360) 5′-AGAAAAAGGCTCACCGTCGA-3′	223bp

2.10.3.7 Protein Extraction and Western Blot Analysis.

Western Blotting (also called immunoblotting) is a technique used for analysis of individual proteins in a protein mixture (e.g. a cell lysate). In Western blotting the protein mixture is applied to a gel electrophoresis in a carrier matrix (SDS-PAGE, native PAGE, isoelectric focusing, 2D gel electrophoresis) to sort the proteins by size, charge, or other differences in individual protein bands (Fig. 2.6). The separated protein bands are then transferred to a carrier membrane (e.g. nitrocellulose, nylon or PVDF). This process is called blotting. The proteins adhere to the membrane in the same pattern as they have been separated due to interactions of charges and are then accessible for antibody binding for detection.

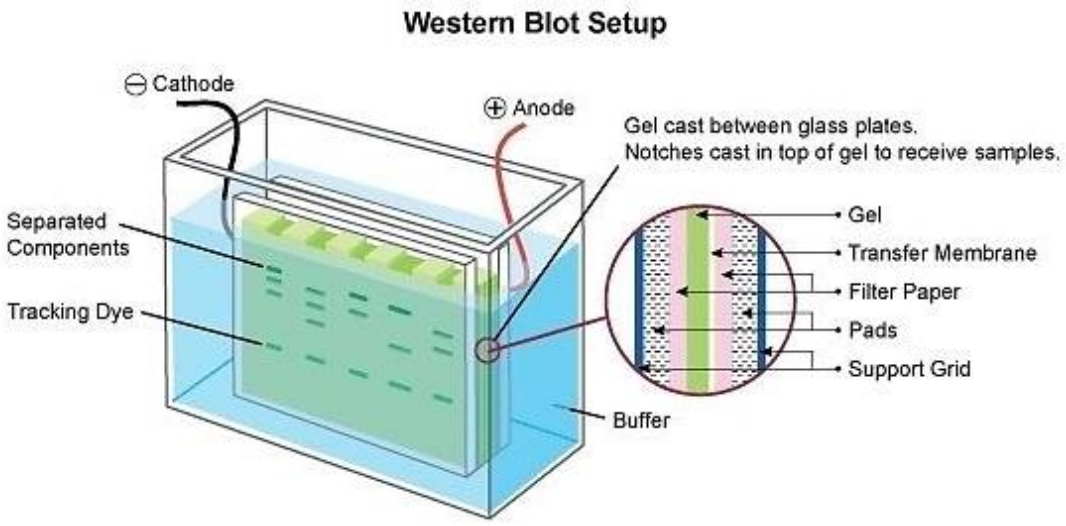

Fig 2.6: Illustration of western blot.

Antibodies used to detect target proteins on the western blot are conjugated with fluorescent or radioactive labels or enzymes that give a subsequent reaction with an applied reagent, leading to a coloring or emission of light, enabling detection.

A549 cells were plated and cultured followed by the addition of 200 mg/ml of complexes The incubation was continued for 24 h. Cells were harvested by scraping the cells from culture dishes with a scraper and collected by centrifugation. Cells were resuspended in 125 mM Tris-HCl buffer, sonicated and lysed with 4% SDS. Cell extracts were boiled for 10 min, chilled on ice, and centrifuged at 2000 rpm for 5 min before collecting the supernatant. The protein content of the samples was quantified using the BCA protein assay kit (Pierce, Rockford, IL). 50 µg of proteins were subjected to 14% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Each membrane was blocked with 10% milk prior to incubation with antibodies i.e Bax (1:7500 dilution; Trevigen, Gaithersburg, MD), Bcl2 (1:1000 dilution; Dako, Carpinteria, CA), washed with TBST (Tris buffered saline), and incubated with secondary antibodies conjugated with peroxidase. The signal was detected using the chemiluminescent detection system.

2.11 Antiglycation activity

This test was used to evaluate the ability of the complexes to inhibit the methyl glyoxal mediated development of fluorescence of BSA. Activity was evaluated by using the reported method [9,10] with the following modifications:

Triplicate samples of BSA 100 mg/ml, 14 mM MGO, 0.1 M phosphate buffer (pH 7.4) containing NaN_3(30 mM) was incubated under aseptic conditions at 37°C for 9 days in the presence or absence of various concentrations of the test compounds. After 9 days of incubation, each sample was examined for the development of specific fluorescence (excitation, 330 nm; emission, 440 nm) on a microtitre plate spectrophotometer. The reaction was stopped by adding 10µl tricholoroacetic acid and after ten minutes the mixtures were centrifuged. The precipitate was re-dissolved in alkaline phosphate buffered saline (PBS) and quantified for the relative amount of fluorescent Advance glycation end products (AGEs).

Any sample giving fluorescence equal to the fluorescence of BSA/glucose implied that there was no inhibition of glycation; whereas, any sample giving fluorescence lower than that of BSA/glucose indicated that there was inhibition of glycation by the complexes. Rutin(standard) was used as a positive control (IC_{50}= 25.46 ± 1.50 SEM). Results were expressed as percentages compared to negative control and the corresponding IC_{50} were calculated. The percent inhibition of AGE formation in the test sample versus control was calculated for each compound by using the following formula:

$$\% \text{ inhibition} = (1 - \text{fluorescence of test sample/fluorescence of the control group}) \times 100 \quad \text{..... (2.6)}$$

2.11.1 Antioxidant activity

2.11.1.1 SOD mimic activity

SOD activity is frequently assayed with indirect assay that involves the addition of superoxide generator and a scavenger whose reaction with superoxide is spectrophotometrically monitored. The presences of SOD in a sample inhibit the reaction between superoxide and the scavenger by competing for the superoxide supplied by generator. The degree of inhibition is proportional to the activity of SOD in the sample. Several super oxide generator and detector have been used in combination to assess in vitro SOD activity. In the present study ,the superoxide radical was generated in the test system by using NBT and determined spectrometrically by the Nitroblue tetrazolium photoreduction method with a minor modification [11].

Nonenzymatic system made up of 30 μM PMS, 79 μM NADH, 75 μM NBT andphosphate buffer (pH = 7.8) was used to produce superoxide anion (O_2•–). The scavenging rate of O_2^{-} under the influence of complexes (0.25 to 5.0μM) was determined by monitoring reduction in rate of transformation of NBT to monoformazan dye. The reactions were monitored at 560 nm with a UV–Vis spectrophotometer and the rate of absorption change was determined.

The % inhibition of NBT reduction was calculated using equation

$$\%\ inhibition\ of\ NBT\ reduction\ (\eta) = \left(1 - \frac{k'}{k}\right) \times 100 \qquad \text{..... (2.7)}$$

where, *k'*and *k* represent the slopes of the straight line of absorbance values as a function of time in presence and absence of SOD mimic compound, respectively. The IC_{50} values for the complexes were determined by plotting the graph of percentage inhibition of NBT reduction (η) against an increase in the complex concentration. The concentration

of the complex which causes 50% inhibition in the reduction rate of NBT to monoformazan is reported as IC_{50} value for the tested complex.

2.11.1.2 DPPH activity:

DPPH (1,1-diphenyl-2-picryl hydrazyl) is characterized as a stable free radical by virtue of the delocalization of the unpaired electron over the molecule as whole, so that the molecules do not dimerise, as would be the case with most other free radical. The delocalization also gives rise the deep violet colour, characterized by an absorption band in ethanol solution, centered at about 520nm.When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (**2**) Shown in (Fig.2.7) with the loss of this violet colour.

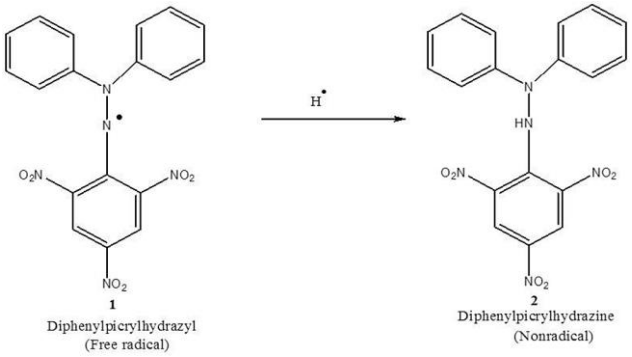

Figure 2.7: Reaction mechanism of DPPH free radical scavenging assay

The determination of the DPPH scavenging activity was carried out by using a methanolic solution of DPPH (25 mg L^{-1}) mixed with different concentrations (0.65-20 μM) of complexes in methanol. The above mixture was stirred vigorously for 5 min and was allowed to stand for 1 h at room temperature before its absorbance was measured at 517 nm [12]. The percentage of scavenging activity was calculated by using following equation.

$$\text{DPPH scavenging activity (\%)} = (A_0 - A_i)/A_0 \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (2.8)$$

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