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SYNTHESIS, CHARACTERIZATION, DNA-BINDING, PHOTOCLEAVAGE AND ANTI-MICROBIAL STUDIES OF COMPLEX $[Ru(4\text{-ETHYL- PYRIDINE})_4(DPPZ)]^{2+}$

Padmaja Naishadham and S. Satyanarayana*

Department of Chemistry, Osmania University, Hyderabad, Andhra Pradesh, India

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Correspondence to Author:

Dr. N. Padmaja

Department of Chemistry, Osmania University, Hyderabad, Andhra Pradesh, India

E-mail: tapadmaja@gmail.com

ABSTRACT: The new Ru (II) complex $[Ru(4\text{-Et-py})_4(dppz)]^{2+}$ (dppz = dipyrido phenazine) have been synthesized and characterized by different analytical techniques like elemental analysis, LC-MS, ^1H NMR , ^{13}C NMR. The interaction of this complex with calf thymus DNA has been explored by U.V. absorption spectroscopy, Fluorescence measurements, and viscosity measurements. Their photocleavage behavior towards pBR 322 and anti-bacterial properties of this complex was also investigated. The experimental results show that the complex binds with DNA by intercalation mode.

INTRODUCTION: Transition metal complexes with anticancer activity gained place in the chemotherapy of some cancers [1-3]. While Platinum based drugs have been in clinical use for the past 30 years [4], their inherent toxicity often limit their use as the last option. Cisplatin, Carboplatin, Oxaliplatin etc. owe their activity to their capacity to intercalate nuclear DNA and prevent transcription.

The square planar, inert Pt(II) drugs are activated by slow hydrolysis of the anionic ligands and the antitumour properties of the metabolites are associated to their capacity to bind genomic DNA in the cell nucleus forming stable intrastand crosslinks which then block replication or prevent transcription. Metal complexes carrying charge and containing planar aromatic ligands such as phenanthroline are used as probes [5-9].

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Some other transitional metals also have the potential for intercalation of DNA and prevent growth in cancer cells. Chief among them is ruthenium. Ruthenium complexes have been widely investigated and two such complexes NAMI – A and KP – 1019 are under clinical trials for metastatic and colorectal cancers. Ru (II) polypyridyl complexes have the possibility to become anticancer agents [10-19] because they are effective against primary tumours and easily absorbed, their cytotoxicity is relatively low (compared to Pt(II)). The ruthenium complexes exhibit Photocleavage as evidenced by spectroscopic methods and viscosity measurements [20-27]. This has prompted us to synthesize some novel asymmetric bidentate derivatives of ruthenium with dipyridophenazine which shows quenching of luminescence in aqueous media due to the interaction of the phenazine nitrogens with water via - hydrogen bonding or excited state proton transfer [28, 29] and their ability to bind with DNA using physicochemical and spectroscopic methods has been investigated.

Now, we are reporting the synthesis, characterization of $[Ru(4\text{-Et-py})_4(dppz)]$ $(Clo_4)_2.2H_2O$ using different techniques.

The binding of this complex with CT DNA was explored using electronic absorption measurements, fluorescence measurements, viscosity measurements. Their photo cleavage behavior towards pBR 322 was studied. The antimicrobial property towards *E. coli* bacteria was also studied.

EXPERIMENTAL:

Materials: All the common chemicals used in this study were obtained from Sigma Aldrich. The solvents were purchased from Merck. DNA was purchased from Sigma Chemicals was sonicated and purified according to Chaires *et al* [30].

a. **Physical measurements:** Elemental analysis (C, H and N) was performed on Elemental analyser Flash EA 1112 Thermofinnigon, Molecular weight determinations were performed on LC-MS 2010A Shimadzu with Column- C-18, Detector-UV (254) with MS probe of ESI, ^{1}H and ^{13}C NMR Spectra were recorded on a Bruker ARX-300 NMR Spectrometer with DMSO as Solvent at COSIST, University of Hyderabad, Hyderabad. Electronic absorption spectra were recorded on a *Elico BL 198* Model spectrophotometer with temperature control.

b. **Spectroscopic characterization:** $[Ru(4\text{-}Et\text{-}py)_4(dppz)](ClO_4)_2.2H_2O$ Complex was characterized by elemental analysis (Anal. Calc. for $C_{38}H_{34}N_8Cl_2O_{10}Ru$: C, 55.2; H, 4.6; N, 11.2; found C, 55.2; H, 4.6; N, 11.2%) given in **fig. 1**, ESI-MS m/z = 1009 ($-2H_2O$) displays intense molecular peaks at 807, 739, 609 and 595 given in **fig. 2**. In 4- Et-pyridine scrambling of hydrogen occurs before fragmentation [31]. ^{1}H NMR (DMSO) $H_2(H_6)$ 7.81 ppm $H_3(H_5)$ 7.6 ppm and H_4 at 7.91 ppm for pyridine and dipyridophenazine 8.54(1H), 8.57(2H), 8.61 (3H), 8.53(4H), 8.64(5H) given in **fig. 3**, ^{13}C NMR spectrum dppz signals at C_1-154, C_2-127, C_3-130, C_4-152, C_5-134, C_6-139, C_7- 150, C_8- 132, C_9 - 133 and 4- Ethyl Pyridine signals at 150 ppm(C_2, C_6) 124.2 ppm(C_3,C_5) 139 ppm (C_4) given in **fig. 4**.

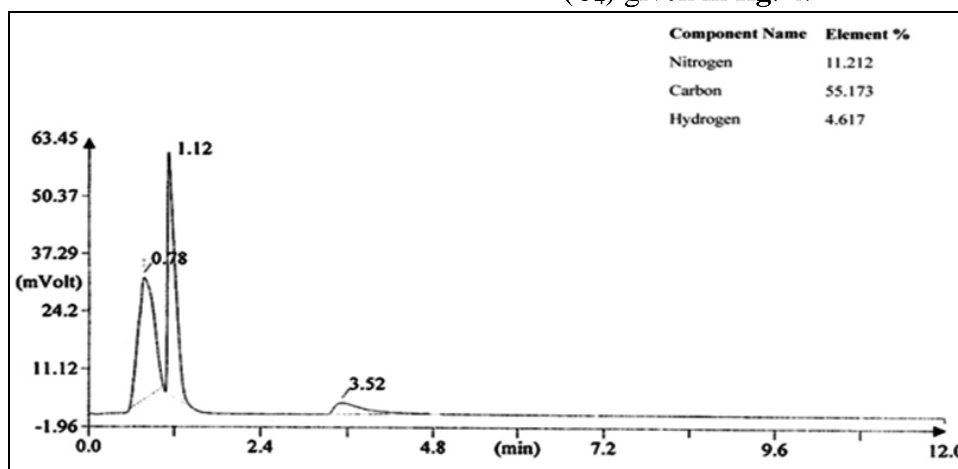

FIGURE 1: CHNS REPORT OF $[Ru(4\text{-}Et\text{-}Py)_4(dppz)](ClO_4)_2$

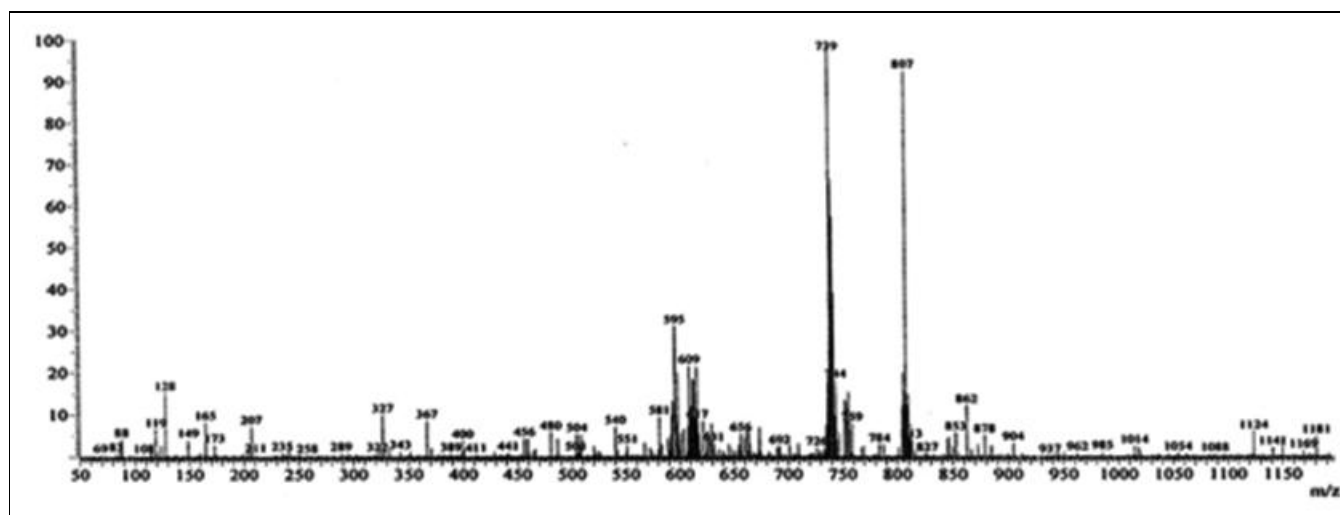

FIGURE 2: LC-MS SPECTRUM OF $[Ru(4\text{-}Et\text{-}Py)_4(dppz)](ClO_4)_2$

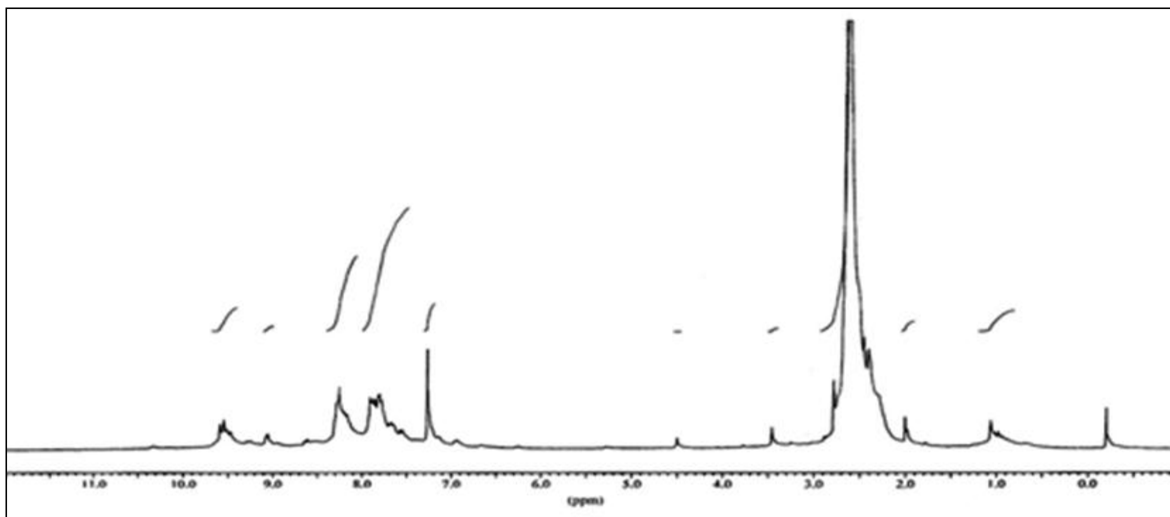

FIGURE 3: ^{1}H NMR SPECTRUM OF [Ru(4-Et-Py)$_4$(dppz)](ClO_4)$_2$

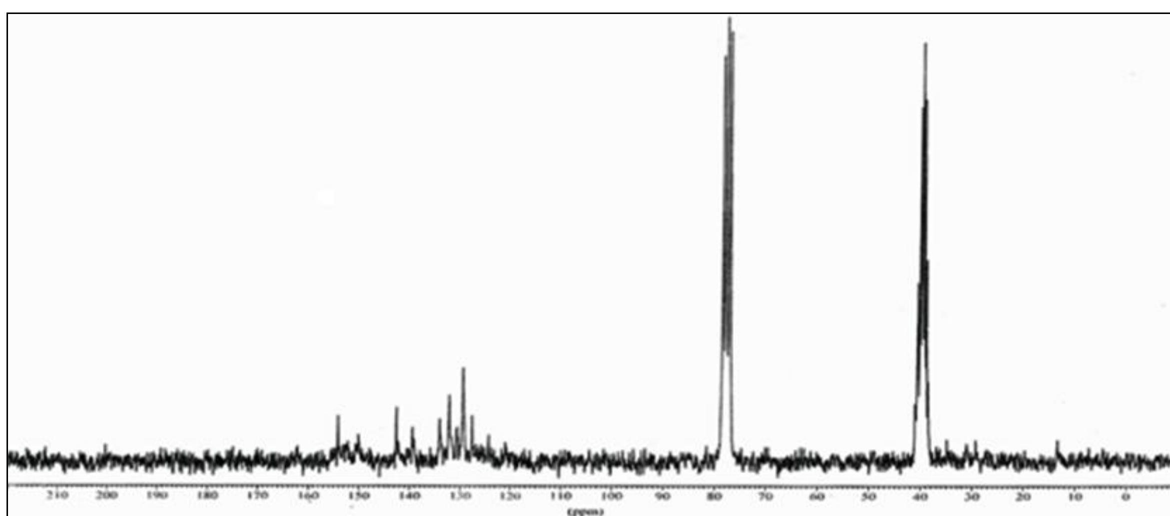

FIGURE 4: ^{13}C NMR SPECTRUM OF [Ru(4-Et-Py)$_4$(dppz)](ClO_4)$_2$

Synthesis: ligands like phen-dione and dipyrido phenazine (dppz) were synthesized as per the reported procedures [32, 33].

a. **Synthesis of Phen-dione:** 1, 10 Phenanthroline hydrate (1gm), Potassium bromide or Sodium bromide (5.95gm) were taken in a flask. conc. H_2SO_4 (20ml) was added through the ice cooled walls of flask drop by drop followed by conc. HNO_3 (10 ml). Reflux for 2 hrs, the mixture was taken in a 400ml of H_2O. The solution was neutralized with $NaHCO_3$ and then extracted with CH_2Cl_2. Removal of CH_2Cl_2 give 0.91gm of Phen-dione as yellow needles followed by recrystallization with ethanol or toluene.

b. **Synthesis of Dipyrido phenazine (DPPZ)**: A solution of Phendione (0.210 gm) and ortho phenylenediamine (OPDA 0.124 gm) in ethanol (20ml) was heated and refluxed for 4hrs. After cooling, the yellow precipitate was collected by filtration, washed with cold ethanol and vacuum dried.

c. **Synthesis of [Ru(dppz)Cl_4]:** Catherine J. Murphy *et al*., [34] reported the synthesis of [Ru(dppz)Cl_4] via modification of the literature method for [Ru(bpy)Cl_4] [35]. A 0.5572 gm amount of $RuCl_3.xH_2O$ and a 0.7583 gm amount of dipyridophenazine were added to 50ml of 1M HCl and stirred for about 30 min under nitrogen atmosphere and then allowed to sit under nitrogen for 10 days. The insoluble product was filtered, washed with water and dried in air.

d. **Synthesis of [Ru(4-Et-py)$_4$(DPPZ)] (ClO_4)$_2$.2H_2O:** According to the reported procedure of Liang- Nian Ji et al[36] [Ru(L)$_4$ (dppz)]$^{2+}$ was synthesized. A mixture of [Ru(dppz)Cl_4] (0.131 gm) 4-Et-Pyridine (0.34gm) and DMF (N,N- dimethyl formamide) 20ml was refluxed under argon atmosphere for

8hrs to give a dark red solution. After being cooled to room temperature, the solution was filtered to remove a small amount of insoluble components. The filtrate was reduced to 5ml and then diluted with 15 ml water. Upon dropwise addition of saturated aqueous $NaClO_4$ solution, a lot of red solid was collected by filtration. It was purified by column chromatography on alumina using acetonotrile-toluene as eluent. The deep red product was further recrystallized with acetonitrile – ether and dried in vacuo.

DNA binding studies: Concentration of CT DNA was measured by using its known extinction coefficient at 260 nm [37]. Buffer A (5mM tris, pH 7.1, 50mM NaCl) was used for absorption titration experiments and luminescence measurements, buffer C (1.5 mM Na_2HPO_4, 0.5mM NaH_2PO_4, 0.25mM Na_2EDTA, pH =7.0) was used for the viscometric titrations.

a. **U.V. Absorption measurements:** The application of electronic absorption spectroscopy in DNA-binding studies is one of the most useful techniques [38]. Absorption titration experiment was performed by maintaining the metal complex concentration constant (10µM) and varying the concentration of DNA from 20- 200 µM. While measuring the absorption spectra, equal amount of DNA was added to both complex solution and the reference solution to eliminate the absorbance of DNA itself. From the absorption data, the intrinsic binding constant K_b was determined from the plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ v_s [DNA], where [DNA] is the concentration of DNA in base pairs, ε_a, the apparent extinction coefficient obtained by calculating A_{obsd}/ [complex] and ε_f corresponds to the extinction coefficient of the complex in its free form where ε_b refers to the extinction coefficient of the complex in the fully bound form.

$$[DNA]/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f) + 1/(K(\varepsilon_b-\varepsilon_f) \quad \ldots(1)$$

Each set of data, when fitted to the above equation, gave a straight line with a slope of $1/ K_b (\varepsilon_a - \varepsilon_f)$. K_b was determined from the ratio of the slope to intercept. The binding constants indicate that the complex binds more strongly.

b. **Fluorescence Measurements:** Fluorescence quenching experiments [39] were carried out at 20°C by addition of microlitre aliquots of 0.02M $K_4[Fe(CN)_6]$ to 2ml samples of the complex. For the quenching experiments, samples were excited at 559 nm and emission was monitored. In these experiments, ionic strength was maintained by addition of KCl along with $K_4[Fe(CN)_6]$ so that the final total concentration of K^+ was held constant. Emission was recorded in the absence and presence of 20- 200 µM CT-DNA.

$$F_o/F = 1 + K_{sv}[K_4Fe(CN)_6],$$

Where F_o is the intensity of fluorescence in the absence of quencher, F is the fluorescence intensity in the presence of quencher and K_{sv} is the Stern-Volmer quenching constant. Quenching curves were analyzed by linear and nonlinear least squares methods [40].

c. **Viscometry:** Viscosity experiments were carried out using an Ubblehode viscometer maintained at a constant temperature at 30.0 ± 0.1^0C in a thermostatic water bath. Each compound was introduced into the degassed DNA solution (Degassed by sonication). Mixing of the complex and DNA was done by purging with nitrogen. Flow time was measured with a digital stop watch and each sample was measured three times and the average flow time was calculated. Reduced specific viscosity was calculated according to Cohen and Eisenberg [41]. Plots of $[\eta/\eta_o]^{1/3}$ (η and η_o are the reduced specific viscosities of DNA in the presence and absence of the complex.) versus [complex]/[DNA] were constructed. Plot of $[\eta/\eta_o]^{1/3}$ versus [DNA] was found to be similar to that reported in the literature [42].

d. **DNA Photocleavage:** Irradiation of sample by U.V light at a wavelength of 365 nm containing pBR 322 DNA and the complex were carried out for 1hr. The cleavage reaction on plasmid DNA was monitored by agarose gel electophoresis. When circular plasmid DNA was subjected to gel electrophoresis, relatively fast migration is observed for the intact supercoiled form (Form 1). If scission occurs on one strand (nicking), the supercoils will relax to generate a slower- moving open circular form (Form II) [43].

If both strands are cleaved, a linear form (Form III) is generated that migrates between Form I and II.

e. **Anti-bacterial activity:** The presence of metal ions in biological matter created great interest and curiosity among chemists as well as biologists, due to their anti-bacterial activities. It may be anticipated that a stable metal chelate will produce its effect either by structural or functional activation or inactivation of a susceptible biological site. Attempts have been made to correlate the stability of the metal drug chelates with their anti-bacterial activity. The Complex shows inhibiting activity against *E. coli*.

RESULTS AND DISCUSSION:

The U.V. absorption measurement: The electronic absorption spectroscopy is the most common way to investigate the interactions of metal complexes with DNA [44-49]. A metal complex binding to DNA through intercalation usually results in hypochromism and bathochromism, due to the intercalation mode involving a strong stacking interaction between an aromatic chromophore and the base pairs of DNA. It seems to be generally accepted that the extent of the hypochromism in the UV- Visible band is consistent with the strength of intercalative interaction [50-53]. The absorption spectra of complex $[Ru(4\text{-}Et\text{-}py)_4(dppz)]$ $(ClO_4)_2$ in the absence and presence of CT-DNA (at a constant concentration of complexes, [Ru] = 20µM) are measured. The absorption spectra of complex show three bands with comparable intensity as in **fig. 5**, lying in the range of 200- 600nm. The first one is a strong and broad absorption band centered at 360 nm, which is generally assigned to a singlet, metal to ligand transfer (MLCT). There are two narrow separated bands within the range of 265 - 255 nm and one strong and narrow band centered at 220nm, both of them are characteristic of the intraligand (IL) transitions. These assignments of absorption bands can be confirmed and analyzed in detail by TDDFT calculations (Time-dependent density functional theory (TDDFT)) in aqueous solution. With the increase in concentration of DNA, the absorption band of the complex display clear hypochromism. The spectral characteristics indicate that the Ru(II) complex interact with DNA.

The intrinsic binding constants K_b of complexes were measured to be 1.4 x 10^5 M^{-1} respectively. The K_b values are in the same order of those bidentate $[Ru(L)_2(dppz)]^{2+}$ complexes. Where L= bpy, phen, dmb, dmp. This suggests that the complex most likely intercalatively bind to DNA, involving a strong stacking interaction between the aromatic chromophore (dppz) and the base pairs of the DNA. It indicates that the ancillary ligands 4- Ethyl Pyridine enhance the DNA – binding affinity. The DNA- binding constant of complex shows that it has larger hypochromism value but smaller K_b value implying stronger binding to DNA.

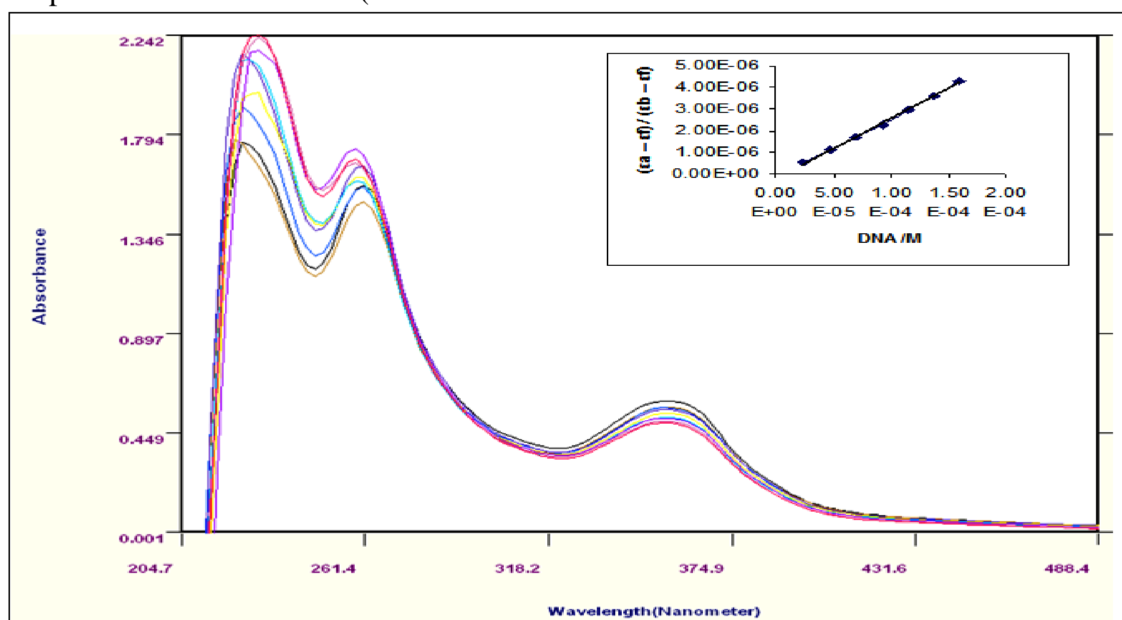

FIG. 5: UV-VISIBLE SPECTRA OF $[Ru(4\text{-}Et\text{-}py)_4(dppz)](ClO_4)_2$. Tris- HCl buffer upon addition of CT DNA in absence (top) and presence of CT DNA (bottom) the [Ru] = 10 µM; [DNA] = 0-126 µM. Insert: plots of [DNA] / (Σ_a - Σ_f) vs [DNA] for the titration of DNA with complex

a. **Fluorescence Emission studies:** In the absence of CT- DNA, complex [Ru(4-Et-py)$_4$(dppz)] (ClO$_4$)$_2$ emit luminescence in tris buffer at ambient temperature, with a maximum appearing at 558 nm. Upon addition of CT DNA the emission intensities of this complex increase and it implies that complex interacted strongly with DNA and was protected efficiently.

The hydrophobic environment inside the DNA helix restricts the mobility of the complex at the binding site. Emission quenching experiments using $[Fe(CN)_6]^{4-}$ as quencher may provide further information about the binding of complexes with DNA, the emission of complex was efficiently quenched by $[Fe(CN)_6]^{4-}$ as in **fig. 6 & 7** and the plot was not linear which imply that the quenching process can be both dynamic and static [54].

However, in the presence of DNA, the Stern – Volmer plots are changed drastically and the emission intensity was hardly affected by the addition of anionic quencher. This may be explained by repulsion of the highly negative $[Fe(CN)_6]^{4-}$ from the DNA polyanion backbone which hinders access of $[Fe(CN)_6]^{4-}$ to the DNA bound complexes [55].

The plot for the DNA bound complexes become linear, indicative of only dynamic quenching, which was consistent with the strong DNA – binding affinity of complex. The curvature reflects degrees of protection or relative accessibility of bound cations, a larger slope for the Stern – Volmer curve parallels poorer protection and low binding.

From the results, it was suggested that the complex has stronger DNA- binding affinity.

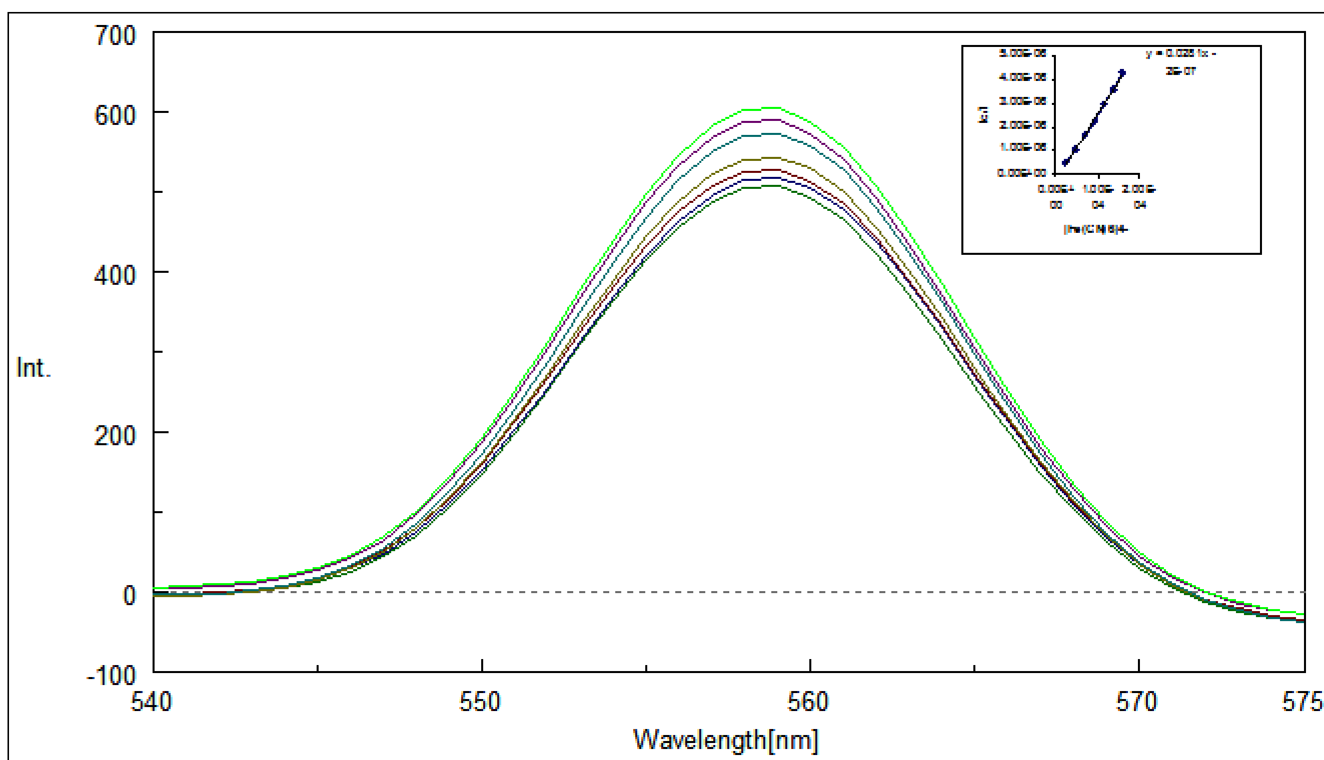

FIGURE 6: FLUORESCENTE EMISIÓN SPECTRA OF COMPLEX [Ru(4-Et-py)$_4$(dppz)]ClO$_4$.2H$_2$O IN AQUEOUS BUFFER. Tris 5mM, NaCl 50mM, pH 7.0) in the presence of CT DNA, [Ru] = 20µM, [DNA] / [Ru] 0, 5, 10, 15, 20 (The arrow shows the intensity changes upon increasing concentration). Insert: Plots of relative integrated emission intensity vs [DNA]/[Ru].

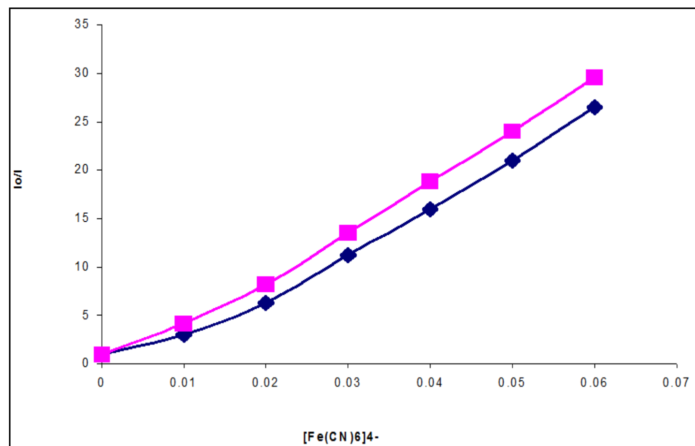

FIGURE 7: EMISSION QUENCHING OF [Ru(4-Et-Py)$_4$(DPPZ)] $(ClO_4)_2$ WITH INCREASING $[Fe(CN)_6]^{4-}$ IN THE PRESENCE AND ABSENCE OF DNA

Viscosity studies: Viscosity measurements were carried out to elucidate the binding mode of the complex, by keeping [DNA] = 0.4mM and varying the concentration of the complex. The changes in relative viscosity of rod- like CT-DNA in the presence of [Ru(4-Et-py)$_4$(dppz)]$^{2+}$ were observed. From the figure it was observed that with the addition of increasing amounts of complex to the DNA the relative viscosity of DNA increases steadily. The increased degree of viscosity, depend on the binding affinity to DNA. These results offer further proof that the complex effectively intercalates between the base pairs of DNA with high affinity which is inserted in **fig. 8**.

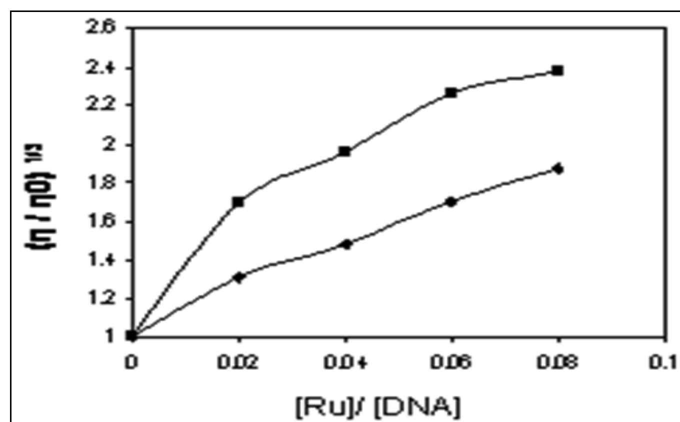

FIGURE 8: EFFECT OF INCREASING AMOUNT OF COMPLEXES ([Ru(py)$_4$(dppz)]$ClO_4.2H_2O$ & [Ru(4-Et-py)$_4$(dppz)]$^{2+}$) ON THE RELATIVE VISCOSITY OF CT DNA AT 25± 0.1°C.

Photoactivated cleavage of pBR 322 DNA: There was substantial and continuing interest in DNA endonucleolytic cleavage reactions that were activated by metal ions [56, 57]. The order of migration of plasmid DNA during agarose gel electrophoresis, the three forms of plasmid DNA can be used to study cleavage induced by radiation at 365 nm. When circular plasmid DNA was subjected to electrophoresis, relatively fast migration was observed for the intact supercoil form (Form I).

If scission occurs on one strand (nicking), the supercoil will relax to generate a slower moving open circular form (Form II). If both strands were cleaved, a linear form (Form III) that migrates between Form I and II will be generated [58]. **Fig. 9** shows gel electrophoresis separation of pBR 322 DNA after incubation with the complex and irradiation at 365nm.

No DNA cleavage was observed for controls in which complex was absent (lane 0), or incubation of the plasmid with the Ru (II) complex in dark. During irradiation experiments, the increasing concentration of Ruthenium complex result in gradual decrease in Form I with corresponding increase in Form II and formation of some Form III indicating cleavage.

FIGURE 9: PHOTO CLEAVAGE STUDIES OF [Ru(4-Et-py)$_4$(DPPZ)]$^{2+}$

Anti-bacterial activity: The antibacterial screening effects of these complexes were tested against *Escherichia coli* bacteria by the well diffusion method [59], using agar nutrient medium inoculated with bacteria. The well was filled with the test solution using a micropipette and the plate was incubated at 35°C. The test solution was diffused and the growth of the inoculated bacteria was affected. The diameter of the inhibition zone against each concentration was noted. The increase in the delocalization of π- electrons over the whole chelate ring enhances the penetration of the complexes blocking of the metal binding sites in the enzymes of bacteria [60].

This complex also disturbs the respiration process of the cell and thus blocks the further growth of the organisms [61]. [Ru(4-Et-py)$_4$(dppz)] $(ClO_4)_2$ shows inhibiting activity against *E. coli* as shown in **fig. 10**.

FIGURE 10: ANTI-BACTERIAL ACTIVITY OF [Ru (4-Et-Py)$_4$ (DPPZ)] $(ClO_4)_2$

CONCLUSIONS: The study shows Ru(4- Et-py)$_4$(dppz)]$^{2+}$ compounds bind with DNA inter calatively resulting in antibacterial activity possibly due to DNA binding. Since, Ruthenium complex with Ethylene diamine was shown to exhibit anti tumour and anticancer activity. It is likely the novel ruthenium complexes have the potential for antitumour and anticancer properties [62-64].

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