Diketone Based Cu(II), Ni(II) and Zn(II) Complexes Containing N_2O_2 Donor Ligand: Synthesis, Characterization, DNA Binding and Cleavage Studies.

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Abstract - **A new series of Schiff base Cu(II), Ni(II) and Zn(II) complexes have been designed and synthesized using the ligand(1,2,8,9-Tetraphenyl-3,7-diazaduohepta-2,7-diene-1,9-dione) which is obtained from the condensation of benzil and 1,3-diaminopropane. The ligand and their complexes have been characterized with the aid of FT-IR, NMR, Mass, elemental analysis, EPR and absorption studies. The interactions of the synthesized complexes with calf thymus (CT) DNA have been investigated by absorption, emission, viscosity and circular dichoric techniques. It was found that all the complexes binds with CT-DNA presumably *via* an intercalation mechanism with an intrinsic binding constant (K_b) values of 8.6 x 10^5 M^{-1}, 8.3 x 10^5 M^{-1} and 7.8 x 10^5 M^{-1} respectively which are generated from absorption experiment. All the complexes shows efficient oxidative cleavage of SC-DNA involving singlet oxygen species as evidenced from the control data showing inhibition of DNA cleavage in the presence of sodium azide and L-histidine.**

Key words: Schiff base, Cu(II) complex, Ni(II) complex, DNA binding studies, DNA cleavage activities.

I. Introduction

Cancer is a syndrome that has tormented human throughout the history [1]. The phosphate-diester bonds are known to serve as nucleotide linkages in the genetic DNA and the half life time for the spontaneous hydrolysis of DNA under physiological conditions is reported to be 1,30,000 years [2]. This remarkable stability is one of the essential requirements for survival and maintenance of life. The design and synthesis of simple metal complexes for the cleavage of the phosphodiester of DNA is a very active area of bioinorganic research. Cisplatin was initially synthesized by Peyrone in 1844, and its biological activities were accidentally determined by B.Rosenberg and co-workers in 1965. And then many succedent experiments indicate that cisplatin and its derivatives such as oxaliplatin and carboplatin can be used as an efficient anticancer drugs through binding to DNA *via* an intercalation mode [3,4], however their clinical use is restricted by undesirable side effects, general toxicity and acquired drug resistance. These drawbacks drive inorganic chemists to develop novel strategies for the preparation of more effective, less toxic, target specific and preferably non covalently bound anti cancer drugs [5-9].

Numerous studies have demonstrated that DNA is the primary intracellular target of anticancer drugs due to the interaction between small molecules and DNA, which can cause DNA damage in tumour cells, resulting in apoptosis [10, 11]. In order to develop new antitumor drugs which specifically target DNA, it is necessary to understand that the metal complexes interact with the double helix DNA in either a non covalent or a covalent way. The former way includes three binding modes; intercalation, groove binding and external static electronic effects. Among these interactions, intercalation is of therapeutic importance, because small molecules binding to DNA via intercalation often exhibits potential anticancer activities. Intercalative mode usually presents in compounds of planar automatic ring systems that occupy the space between two adjacent base pairs in DNA [12, 13].

Survey of literature demonstrates that interest on the design of novel transition metal complexes capable of binding and cleaving duplex DNA with high sequence and structure selectivity increases continuously [14-16]. Transition metal macrocyclic complexes have received a great attention because of their biological activities, including antiviral, anticarcinogenic, antifertile, antibacterial, antifungal, antiarthritic [17-19] and antitumor [20].

Prompted by these and in continuation of our work, (21-24), we have synthesized macrocyclic complexes derived from benzil and 1,3-diamino butane and characterized the complexes by physiochemical techniques like IR, NMR, elemental analysis, absorption and mass spectral analysis. The DNA binding, cleavage activities have been examined against CT DNA and SC DNA for the Cu(II), Ni(II), and Zn(II) complexes.

II. Materials and instrumentation

All the reagents and chemicals were purchased from commercial sources (Sigma-Aldrich Co., Thomas Baker Co.) and used as received without further purification. The supercoiled pBR322 DNA was purchased from Bangalore Genei (India). Elemental analysis was recorded on a Carlo Erba model 1106 elemental analyzer. FT-IR spectra were obtained from Perkin Elmer FTIR spectrometer with the samples prepared as KBr pellets. NMR spectra were recorded in DMSO-d_6 by using TMS as an internal standard on a BRUKER 400 instrument. UV–Visible spectra were recorded using Perkin Elmer Lambda 35 spectrophotometer operating in the range of 200–1000 nm with quartz cells and ε values are expressed in M^{-1} cm^{-1}. The emission spectra were recorded on a Perkin Elmer LS-45 fluorescence spectrometer. Mass spectral analysis was performed in Q-TOF Mass Spectrometer. ESR spectra were recorded at room temperature Varian E-4 X-band spectrometer using DPPH as the g-marker. Viscosity measurements were recorded using a Brookfield Programmable LV DVII+ viscometer. Tris (hydroxymethyl) aminomethane–HCl (Tris–HCl) buffer solution was prepared by using deionized and sonicated triple distilled water. The supercoiled pBR322 DNA and Calf thymus (CT) DNA were procured from Bangalore Genie (India).

A. Synthesis Preparation of ligand (1,2,8,9-Tetraphenyl-3,7-diazaduohepta -2,7-diene-1,9-dione)

1,2,8,9-Tetraphenyl-3,7-diazaduohepta -2,7-diene-1,9-dione was synthesized according to the literature procedure [25].

B. Synthesis of Cu(II), Ni(II) and Zn(II) complexes

A methanolic solution of ligand (0.01 mol) and respective metal perchlorate salt (0.01 mol) was refluxed for 5 h. The mixture was concentrated to half of its original volume and kept in desiccators overnight. On overnight cooling dark coloured precipitates formed were filtered, washed with methanol, acetone, diethylether and dried in *vacuo.*

C. Cu(II) complex: Yield: 78%, Anal.(%) Calc. for $[C_{31}H_{26}ClCuN_2O_6^-]$ C, 59.95; H, 4.23; N, 4.53: Found: C, 59.91; H, 4.28; N, 4.51: ESI-MS: 620 $[L+Cu+ClO_4]^+$; 260 $[L+Cu]^{2+}$: IR (KBr, cm^{-1}): 1662 (C=O), 1606 (C=N), 1109, 623 (-ClO_4), 473 (M-O), 424 (M-N); [λ/nm (ε/M^{-1} cm^{-1})] in acetonitrile: 239 (117100), 265 (99800), 345 (65000), 590 (35000). ESR: g_n = 2.182, $g_\perp$ = 2.064 and g_{iso} = 2.123.

D. Ni(II) complex: Yield: 76%, Anal.(%) Calc. for $[C_{31}H_{26}ClNiN_2O_6^-]$ C, 60.38; H, 4.25; N, 4.54: Found: C, 60.31; H, 4.28; N, 4.56: ESI-MS: 615 $[L+Ni+ClO_4]^+$; 258 $[L+Ni]^{2+}$: IR (KBr, cm^{-1}): 1667 (C=O), 1611 (C=N), 1107, 620 (-ClO_4), 476 (M-O), 431 (M-N); [λ/nm (ε/M^{-1} cm^{-1})] in acetonitrile: 241 (113600), 259 (99650), 358 (63500), 565 (32000).

E. Zn(II) complex: Yield: 79%, Anal.(%) Calc. for $[C_{31}H_{26}ClZnN_2O_6^-]$ C, 59.72; H, 4.20; N, 4.49: Found: C, 59.68; H, 4.22; N, 4.53: ESI-MS: 621 $[L+Zn+ClO_4]^+$; 260 $[L+Zn]^{2+}$: IR (KBr, cm^{-1}): 1661 (C=O), 1613 (C=N), 1111, 627 (-ClO_4), 471 (M-O), 427 (M-N).

F. DNA binding experiments. Absorption spectral studies

Electronic absorption spectra of the complex were recorded before and after addition of DNA in the presence of 50 mM Tris-HCl buffer (pH 7.5). A fixed concentration of metal complexes (10 µM) was titrated with incremental amounts of CT-DNA over the range (0 – 100 µM). The equilibrium binding constant (K_b) values for the interaction of the complex with CT-DNA were obtained from absorption spectral titration data using the following equation [26].

$$[DNA]/ (\varepsilon_a - \varepsilon_f) = [DNA]/ (\varepsilon_b - \varepsilon_f) + 1/K_b (\varepsilon_b - \varepsilon_f)$$

Where ε_a is the extinction coefficient observed for the charge transfer absorption at a given DNA concentration, ε_f the extinction coefficient at the complex free in solution, ε_b the extinction coefficient of the complex when fully bound to DNA, K_b the equilibrium binding constant, and [DNA] the concentration in nucleotides. A plot of [DNA]/ (ε_a - ε_f) versus [DNA] gives K_b as the ratio of the slope to the intercept. The non-linear least squares analysis was done using Origin lab, version 6.1.

G. Fluorescence spectral studies

Experiments were carried out at pH 7.2 in the buffer containing Tris – HCl buffer 50 mM by keeping EB-DNA solution containing [EB] = 4µM and [DNA] = 50 µM as constant and varying the concentration of complex (0-100 µM). Fluorescence spectra were recorded using excitation wavelength of 496 nm and the emission range set between 520 and 650 nm. The quenching constant K_{sv} was deduced from Stern-Volmer method where the ratio of fluorescence of the compound alone (I_0) over the fluorescence of the compound in the presence of CT-DNA (I) is presented as a function of CT-DNA concentration.

$$I_o/I=1 + K_{sv}[r].$$

Where I_0, is the ratio of fluorescence intensities of the complex alone, I is the ratio of fluorescence intensities of the complex in the presence of CT-DNA. K_{sv} is a linear Stern – Volmer quenching constant and r is the ratio of the total concentration of quencher to that of DNA, [M] / [DNA]. A plot of I_0 / I *vs.* [complex]/ [DNA], K_{sv} is given by the ratio of the slope to the intercept. The apparent binding constant (K_{app}) was calculated using the equation K_{EB}[EB] / K_{app}[complex], where the complex concentration was the value at a 50% reduction of the fluorescence intensity of EB and K_{EB} = 1.0 x 10^7 M^{-1} ([EB] = 3.3 µM) [27].

H. Viscosity measurements

The binding mode of the complexes to CT-DNA, viscosity measurements were carried out on CT-DNA (0.5 mM) by varying the concentration of the complex (0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM, 0.05 mM). Data were presented as (η/η_o) versus binding ratio of concentration of complex to that of concentration of CT-DNA , where η is the viscosity of DNA in the presence of complex and η_o is the viscosity of DNA alone.

I. Circular dichoric spectral studies

Circular dichoric spectra of CT-DNA were obtained by using a JASCO J-715 spectropolarimeter equipped with a Peltier temperature control device at 25 ± 0.1 °C with a 0.1 cm path length cuvette. Incubation of the DNA with the Cu(II), Ni(II) and Zn(II) complexes induced slight changes in CD spectrum.

J. DNA cleavage experiments

The DNA cleavage experiments were performed by agarose gel electrophoresis. pBR322 DNA (0.1µg/µl) in Tris-buffer (pH 7.2) was treated with complex (50 µM) in the presence of additives. The sample was incubated for 3h at 37 º C and the reaction was quenched by 1 µl of loading buffer pBR322 DNA bands were stained by EB, visualized under UV light and photographed. The extent of cleavage of SC DNA was determined by measuring the intensities of the bands using a UVITECH Gel Documentation System.

III. Result and Discussion

A. Synthesis and characterization

The Schiff base ligand 1,2,8,9-Tetraphenyl-3,7-diazaduohepta -2,7-diene-1,9-dione was synthesized according to the literature procedure [25] as represented in Scheme I. The corresponding metal complexes [Cu(II), Ni(II) and Zn(II)] were synthesized (Scheme II) and characterized the complexes by physiochemical techniques like IR, NMR, elemental analysis, absorption and mass spectral analysis. The analytical data for the prepared complexes were in good agreement with the molecular formulae of the complexes.

B. FT-IR Spectra

The comparative IR spectra of the ligand L and its complexes provide intricate details about the binding behavior of the ligand with metal ions. The ligand showed a strong intensity band centered at 1632cm-1 assigned to the azomethine group ν(-C=N) confirming an effective Schiff base condensation between butylene diamine and benzil. Meanwhile, the appearance of band at 1680 cm-1 which is assignable to carbonyl group ν(-C=O) confirms presence of free carbonyl group. These two bands undergoes a negative shift in the complexes on comparing with the free ligand may be explained on the basis of a drift of lone pair density of azomethine nitrogen and carbonyl oxygen towards the metal atom indicating that co-ordination takes place through nitrogen of ν(-C=N) and ν(-C=O). Other bands present in the range of 3035, 1475, 765 cm-1 which were assignable to ν(-C-H) ν(-C=C) ν(-C-H) bending moieties in the free ligand did not show any shift in their frequencies in complex which shows the un co-ordination of these moieties towards metal ion. The mode of co-ordination is further supported by the presence of new bands at 470-480 and 420-430 cm-1 owing to ν(-M-N) ν(-M-O) vibrations. The strong intense bands around 620-630 and 1100-1110 cm-1 due to presence of coordinated perchlorate ion. These observations were in accordance with those reported by others workers [28-30].

C. UV-Visible spectra

The UV-Visible spectra of the complexes and the free ligand were recorded in acetonitrile. In the electronic spectra of the complex, the d-d transitions showed a broad band centered at 595 nm for Cu(II) and 565 nm for Ni(II) as depicted in Fig 1, suggesting a square-pyramidal geometry around Cu(II) [31] and Ni(II) [32]. In the UV region, three transitions were observed two intense bands below 300 nm assignable to the intraligand charge-transfer transition and a band assignable to ligand to metal charge transfer (LMCT) was observed around 360-380 nm.

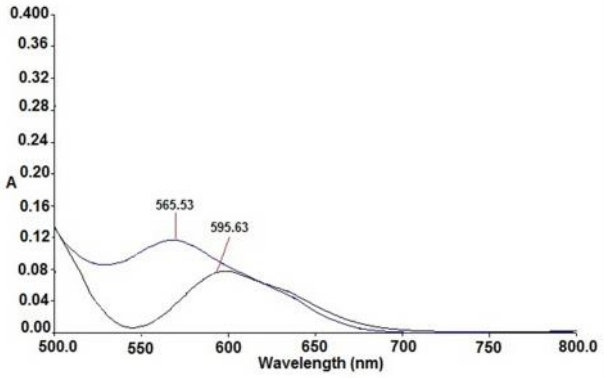

Fig 1. UV-Visible spectra

D. NMR Spectra

The ^{1}H and ^{13}C NMR spectra of the ligand and its Zn(II) complex exhibits well resolved signals and significant differences in their chemical shifts in DMSO d6. The ^{1}H NMR spectrum of the ligand exhibited proton resonance signal at 8.02-8.06 ppm corresponding to the protons of –CH=N(2H) of the macrocyclic skeleton, which confirms the condensation between the amine and benzyl moiety. The multiplet in the region 7.24-8.12 ppm (m, Ar-H) may reasonably due to the aromatic protons of the benzil moiety. A triplet observed in the region of 2.80-2.88 ppm may be attributed to the methylene protons (N-CH2-C: 8H) adjacent to the imine moiety of macrocyclic framework. The multiplet at 1.52 ppm corresponds to the middle methylene protons (C-CH2-C: 4H) of the diaminopropane moiety. The 1H NMR spectra of the Zn complex, exhibited almost the same values as the ligand. Although we expected a shift on the position of CH=N signal for the NMR spectra of the complexes, no significant shift could be observed. However, the CH=N signal is observed in low intensity compared to that of the ligand [33] as depicted in Fig 2.

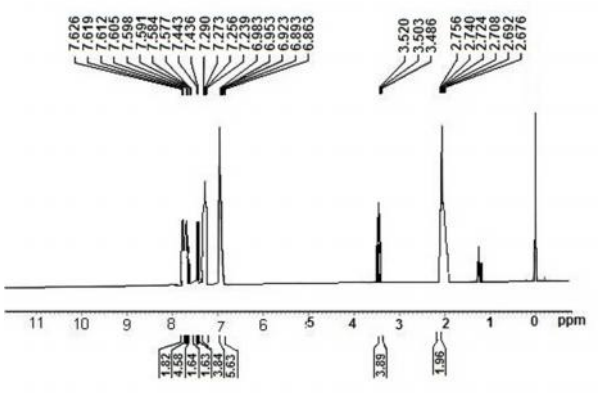

Fig 2. NMR Spectra

The ^{13}C NMR of the ligand displayed a resonance signals at 164 ppm and 189 ppm assigned to C=N and C=O group as represented in Fig (S1). In Zinc complex the imine and carbonyl carbon signals are deshielded compared to that of free ligand suggesting the coordination of azomethine nitrogen and carbonyl oxygen to metal ion. On comparing with all other carbon peaks of the ligand with these of Zinc complex showed some up-field and down-field shifts which are not much significant.

E. Mass spectra

The elemental and analytical data of the ligand and its complex suggested the empirical formula $M[C_{31}H_{26}ClN_2O_6^-]$. The base peak in the spectrum at m/z of 620(Cu), 615(Ni) and 621 (Zn) corresponds to the molecular ion $[M+L+ClO_4]$. The peak at m/z of 260 Cu, 522 (Zn) and 516 (Ni) complexes corresponds to the four coordinate species $[ML]^{2+}$ obtained, apart from these the spectrum showed few peaks which are due to various fragments of the complexes as shown in Fig (3) and (S2 and S3). The mass spectrometric data provides evidence for the formation of monomeric five coordinate complexes.

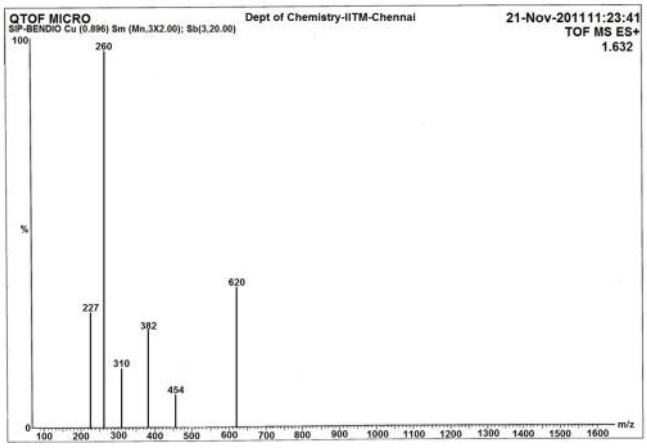

Fig 3. Mass spectra

F. ESR Spectral analysis

The room temperature solid state X-band ESR spectra of the Cu(II) complex showed four well resolved lines with nuclear hyperfine spin 3/2 due to hyperfine splitting as represented in Fig (4). The Cu(II) complex exhibits an axial EPR spectrum with $g_{\pi} = 2.182$, $g_{\perp} = 2.064$ and $g_{iso} = 2.123$. The parameters obtained from the ESR spectrum is consistent with square pyramidal coordination sphere [34] around Cu(II) and the single unpaired electron is located in an essentially $d_{x^2-y^2}$ orbital.

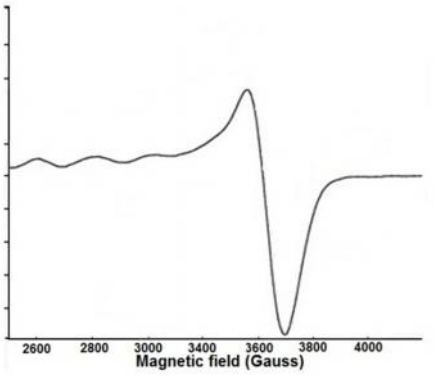

Fig 4. ESR Spectral analysis

G. DNA binding properties

The binding of metal complexes with CT DNA was measured by absorption, fluorescence spectrophotometric titrations, viscosity measurements and Circular dichoric spectral studies.

H. Absorption spectral studies

The binding of metal complex to DNA was monitored classically through absorption titration method. Metal complexes bound to DNA through intercalation is characterized by the change in absorbance (hypochromism) and red shift in wavelength, due to the intercalative binding mode involving a stacking interaction between the DNA pairs. The electronic absorption spectra of the complexes Cu(II), Ni(II) and Zn(II) consists of two well resolved bands in the range of 200-500nm. The UV –Visible absorption spectra of the metal complexes are significantly perturbed by the addition of increasing amounts of DNA. With increasing concentration of CT-DNA (0 – 200 µM), a considerable drop hypochromism in the absorption bands at about 220-260nm for the Cu (II), Zn(II) and Ni(II) complexes was observed accompanied by a moderate red shift of not more than 5-8 nm, suggesting of stabilation of the DNA Helix as represented in Fig (5) (S4) and (S5). In order to compare quantitatively the binding affinity of complexes Cu(II), Ni(II) and Zn(II) with CT-DNA the intrinsic binding constants K_b of the complexes were determined. The binding Constants (K_b) of the metal complexes were determined to be 8.6 x 10^5 M^{-1}, 8.3x 10^5 M^{-1} and 7.8x 10^5 M^{-1} respectively. These results suggest that upon addition of DNA to complexes, Cu(II) complex has marginally higher binding affinities than that of Ni(II) and Zn(II) complex. The significant difference in DNA binding affinity of the three metal (II) complexes could be understood as a result of the fact that the complex with higher number of metal (II) chelates showed stronger binding affinity with CT-DNA [35].

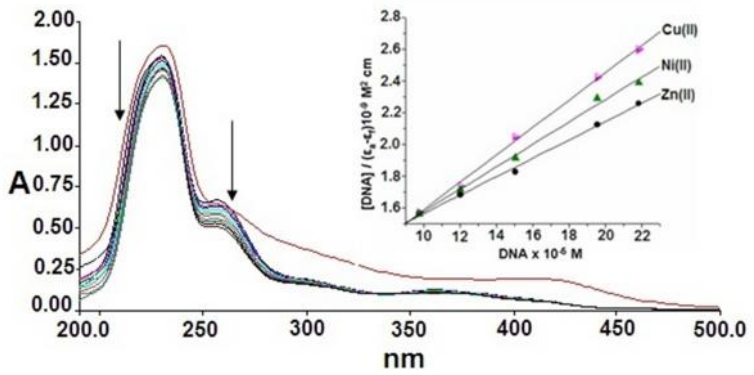

Fig 5. Absorption spectral studies

I. Fluorescence spectral studies

The binding of Cu, Ni and Zn complexes to the CT DNA has been examined by fluorescence spectral method. No apparent emission change was observed for all the complexes either in tris buffer or in the presence of DNA. Steady-state competitive binding experiments using Cu, Zn and Ni complexes as quenches were undertaken to get further proof for the binding of complexes to DNA. It is well known that free EB displays a decrease in emission intensity in tris-HCl buffer medium because of quenching by solvent molecules. However, EB emits intense fluorescent light in the presence of DNA due to its strong intercalation between the adjacent DNA base pairs. The emission spectra of EB-DNA in the absence and presence of complexes are presented in Fig (6), (S6) and (S7). The emission band at 623 nm of the DNA-EB system decreased in intensity with the increasing concentration of the complex. The observed quenching of the DNA-EB fluorescence intensity for the compounds suggested that they can display EB from the DNA- EB system and interact with DNA probably *via* the intercalative mode [36]. The extend of reduction of the emission intensity gives a measure of the binding propensity of the complex to DNA. The apparent binding constants (K_{sv}) were calculated to be 7.2 x 10^5 M^{-1} (Cu), 6.5 x 10^5 M^{-1} (Ni) and 5.3 x 10^5 M^{-1} (Zn) complexes. The maximum value obtained for copper complex is in agreement with its highest K_b value, i.e., it binds to CT DNA very strongly, hence, maximum quenching. High K_{sv} of the Cu(II) complex reveals that it can bind to CT DNA avidly [37].

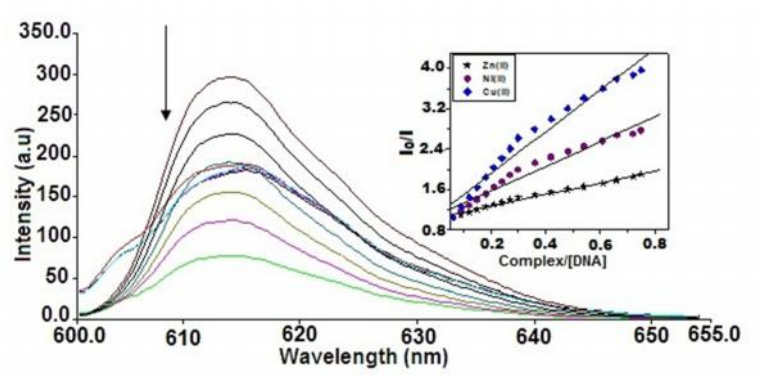

Fig 6. Fluorescence spectral studies

J. Viscosity measurements

In the absence of any crystallographic data viscosity measurement are regarded as the most critical and least ambiguous method to determine the binding mode of the DNA solution [38]. Since the relative specific viscosity of DNA gives a measure of the increase in contour length associated with separation of DNA base pairs caused by intercalation, a classical intercalative interaction causes a significant increase in viscosity of DNA solution due to the increase in separation of base pairs at intercalation sites and hence an increase in overall DNA length. [39]. Upon steadily increasing the amount of the complex the relative viscosity of DNA also increases steadily which reveals that the complex binds to DNA by intercalation. The results of the viscosity measurements are shown in Fig (7). The result suggests that the complex may bind to DNA by intercalation.

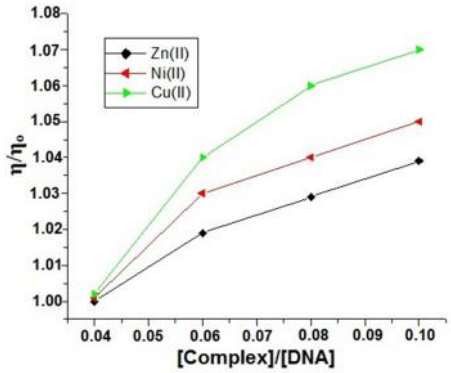

Fig 7. Viscosity measurements

K. Circular dichoric spectral studies

Circular dichoric spectral technique is useful in diagnosing the conformational variations in DNA morphology during drug-DNA interactions. The CD spectrum of the CT-DNA recorded in the absence of the metal complexes showed a positive band due to base stacking (274 nm) and a negative band due to right-handed helicity (242 nm) are quite sensitive to the three main DNA binding modes, namely intercalative (negative band), outside groove binding (positive CD), and outside stacking (bisignate CD). Simple groove binding and electrostatic interaction of small molecules with DNA shows little or no perturbations on the base stacking and helicity bands, intercalation enhances the intensities of both the bands, stabilizing the right handed B conformation of the CT-DNA. On incubation of the metal complexes, both the positive and negative bands undergoes marginal changes in intensity on addition of complexes, which corresponds to induced CD on the hydrophobic interaction of metal complexes with DNA. However, there were no evident shifts in the band positions as shown in Fig (8). These observations clearly rule out the effect of strong intercalation of the compounds on the base stacking and decreased right-handedness of CT-DNA as well [40-41].

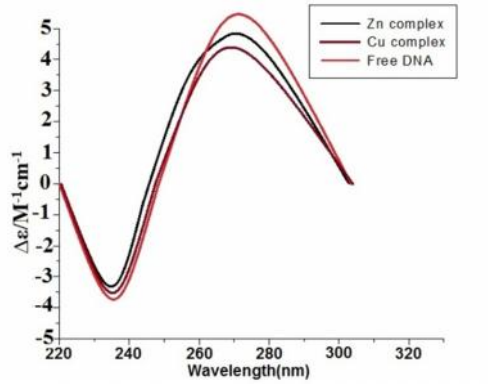

Fig 8. Circular dichoric spectral studies

L. DNA cleavage activity

The irradiation of pBR 322 plasmid DNA in the presence of the complexes was studied so as to validate the efficiency with which it sensitizes DNA cleavage. The entire complex does not transmit about the DNA cleavage in the absence of any coreagent. All the complexes are found to exhibit nuclease activity as shown in Fig (9) in the presence of H_2O_2. DNA cleavage activities of the complexes were assayed from the conversion of supercoiled DNA (Form I) to nicked circular DNA (Form II) or linearized DNA (Form III). In the presence of H_2O_2, 50µM concentration of Cu (II) complex converted 96% of SC DNA to NC Form, compared to 88% conversion by Ni(II) and 81% conversion by Zn(II) complexes. In order to see the role of hydroxyl radical (OH.) scavenger an experiment was conducted with DMSO [42], No inhibition of nuclease activity was observed. Addition of singlet oxygen scavengers like NaN_3 and L- histidine [43] showed complete inhibition of nuclease as represented in Fig (10).

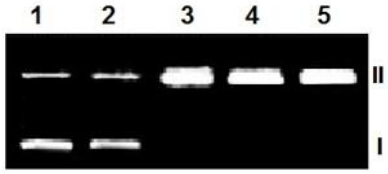

Fig 9. DNA cleavage activity

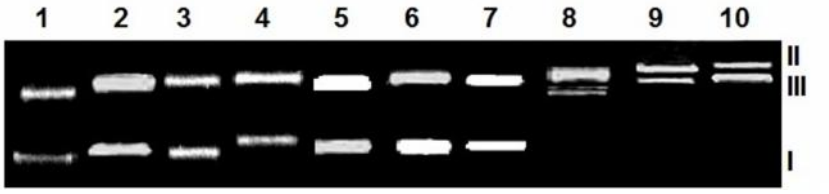

Fig 10. DNA cleavage activity

IV. CONCLUSION

In this work, the synthesis and characterization of three new Cu(II), Ni(II) and Zn(II) complexes have been reported. The complexes were characterized by elemental analysis, IR, UV-Vis, NMR and Mass spectral analysis. Electronic, emission, viscosity and circular dichoric measurements suggest that the metal complexes

bind with CT-DNA through intercalative mode. The binding strength of the complexes with CT-DNA calculated with absorption and emission spectroscopic titrations have shown that Cu(II) complex exhibits the highest intrinsic binding constant values (K_b = 8.6 x $10^5 M^{-1}$, K_{SV} = 7.2 x 10^5 M^{-1}) among the complexes investigated. The results of agorose gel electrophoresis indicate that the complexes exhibit cleavage capability of pBR322 DNA in the presence of hydrogen peroxide. The mechanism studies revealed that singlet oxygen ($^1O_2$) play an important role in the DNA cleavage.

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