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COMPREHENSIVE STUDIES ON THE EFFECT OF METAL IONS OF MIXED LIGAND COMPLEXES DERIVED FROM HYDROQUINONE AND 1,10-PHENANTHROLINE ON INTERACTION WITH HERRING SPERM DNA AND ANTIOXIDANT ACTIVITY

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ABSTRACT

Mixed ligand complexes of Copper(II), Nickel(II) and Cobalt(II) derived from 1,10 phenanthroline and 8-hydroxyquinoline were synthesized. The structural features have been arrived from their elemental analysis, magnetic susceptibility, molar conductance, Uv-Vis, i.r. and ^{1}H NMR studies. The binding properties of Cu(II) complexes with herring sperm DNA was comprehensively investigated by electronic absorption titration, viscosity, electrochemistry and different effects such as hypochromism, blue shift were observed in the resulting DNA structure. The protectivity activity was investigated by gel electrophoresis. The investigated interaction showed clear evidence of changes in the DNA structure and suggest a predominant intercalation mode leading damage to the biomolecule. The synthesized complexes were tested for their antioxidant activities and the results reveal significant antioxidant activity.

INTRODUCTION

Recent years have witnessed an unprecedented progress in biological application of inorganic pharmaceuticals because of their key role in clinical therapy[1-3]. Transition metals are particularly suitable for this purpose because they can adopt a wide variety of coordination numbers, geometries and oxidation states in comparison with carbon and main group element[4]. Metal complexes have also been reported to function as antibacterial antiviral and antifungal agents[5]. A further reason for using metal containing compounds as structural scaffolds relates to the kinetic stabilities of their coordination sphere in biological environment[6-9]. The interaction of DNA with transition metal complexes continue to attract attention for the design and development of synthetic restriction enzymes, chemotherapeutic drugs and DNA foot-printing agents, DNA cleavage agents and molecular light switches[10-14]. Transition metal chelation is an excellent way to increase the lyophillic character of organic moiety, on coordination ligands might improve their bioactivity profile while some inactive ligands may acquire pharmacological properties, consequently transition metal complexes have become an important class of structure selective DNA binding and cleavage agents for nucleic acids , so the development of synthetic sequence selective DNA binding and cleavage agents for DNA itself is essential for expected applications in molecular biology and related field[13,15,16]

Metal complexes of N, S, O – chelating ligands have been used as tools for understanding DNA structure, as agents for mediation of DNA cleavage or as chemotherapeutic agents. The chemistry of 1, 10- phenanthroline ligand and its complexes have intensely been investigated owing to coordination properties and diverse application [17-19]

In the present study we report the synthesis and DNA binding interaction of Cu(II) complex with herring sperm DNA (hs-DNA) and the antioxidant activity of the synthesized complexes.

Experimental

MATERIAL AND METHOD

All reagents chemicals and solvents were of analytical grade and were used as such. Doubly distilled water were used throughout the experiment. 1,10-phenanthroline, 8-hydroxyquinoline, NaCl, DPPH, NBT, Ascorbic acid, Tris and hs-DNA was purchased from Sigma Chemical Company. Metal Chlorides (Cu (II), Ni(II) and Co(II) were purchased from E-Merck (India) Ltd. DNA stock solution was prepared by dilution of hs-DNA with buffer (containing 25 mM Tris and 50

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mM NaCl, pH 7.2) followed by exhaustive stirring at 4 ^{0}C for three days and kept at 4 ^{0}C for no longer than a week. The stock solution of DNA gave a ratio of UV absorbance at 260 and 280 nm (A_{260}/A_{280}) of 1.88, indicating that DNA was free from protein contamination. The DNA concentration was determined by UV absorbance at 260 nm after 1: 20 dilution using $\varepsilon = 6600\ cm^{-1}$[20].

IR spectra of the complexes were recorded on a FTIR spectrometer with samples prepared in KBr pellets. Electronic spectra were recorded on Shamidzu-UV-3600 spectrophotometer. The NMR spectra were obtained on Bruker DRX 400 spectrometer operating at room temperature. Magnetic measurements were carried out on magnetic susceptibility balance of Sherwood Scientific (Cambridge U.K.) at room temperature. Elemental analysis were performed on Perkin Elmer 2408 elemental analyzer. Molar conductance was measured at room temperature on Systronic conductivity bridge. Cyclic Voltammetry were performed on SAS SP 150 Biologic Science Instruments carried out in 30 ml three electrode electrolytic cell. The working electrode was platinum disk, a separate Pt single sheet electrode was used as counter electrode and Ag/AgCl electrode saturated with KCl used as reference electrode. KNO_3 and buffer was used as supporting electrolyte. The Cyclic voltammogram of the complex was recorded in tris HCl buffer (pH= 7.2) at 100 mV/s. All electrochemical measurements were performed at room temperature. Hydrodynamic measurements were carried out from the observed flow time of DNA containing solution (t > 100) corrected for flow time of the buffer alone (t_0) using Ostwalds viscometer at 25 ± 0.01 ^{O}C. Flow time was measured with a digital stop watch with least count of 0.01 sec.

Hydroxyl radicals generated by Fenton reaction were used to induce oxidative damage to DNA. The reaction mixture (15 µL) contained 25 mg of DNA in 20 mM phosphate buffer saline (pH 7.4) and 500 µg of test compounds were added and incubated with DNA for 15 min at room temperature. The oxidation was induced by treating DNA with 1 µl H_2O_2 30 mM, 1 µl 20 mM ferric nitrate and 1 µl 100 mM ascorbic acid and incubated for 1 h at 37 ^{O}C. The reaction was terminated by the addition of loading dye (40% sucrose and 0.25% bromophenol blue) and the mixture was subjected to gel electrophoresis [21] using Hi Media LA666 in 0.7% agarose/TAE buffer run at 100 V. DNA was visualized by Gel Doc system.

The antioxidant activity was carried out using different assays. In DPPH assay quantitative measurement of radical scavenging properties of metal complexes were carried out according to the method by Blios *et al* [22]. Briefly 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of metal complex (100-300 µg/ml) and shikonin (300 µg/ml). α-tocopherol was used as a reference antioxidant. Discoloration of reaction mixture was measured at 517 nm after incubation for 30 min.

The Superoxide anion radical scavenging activity involves measurement of scavenging activity of all the metal complexes based on the method described by Liu *et al* [23] with slight modification. 100µl riboflavin solution (20µg), 200µl EDTA solution (12 mM), 200µl methanol and 100 µl nitrobluetetrazolium (NBT) solution (0.1 mg) were mixed in test tube and reaction mixture was diluted upto 3ml with phosphate buffer (50 mM). The absorbance of the solution was measured at 590 nm using phosphate buffer as blank after illumination for 5 minutes. Different concentrations (50 µl) i.e. 100 µg, 200 µg, 300 µg of complex solutions were taken and diluted upto 150 µl with methanol. To each solutions 100 µl riboflavin, 200 µl EDTA, 200 µl methanol and 100 µl NBT was mixed in test tube and further diluted upto 3ml with phosphate buffer. Absorbance was measured after illumination for 5 minutes at 590 nm. Decreased absorbance of the reaction mixture indicates increased super oxide anion scavenging activity.

In hydroxyl scavenging activity-deoxyribose assay [24] the colorimetric deoxyribose (TBARS) method was applied as the reference method of comparison for determining the hydroxyl radical scavenging activity of metal complexes. The reacting mixture for deoxyribose assay contained in a final volume of 1 ml, the following reagents: 200 µl KH_2PO_4-KOH (100 mM), 200 µl deoxyribose (15 mM), 200 µl Ferric Chloride (500 µM), 100 µl EDTA (1 mM), 100 µl Ascorbic acid (1 mM), 100 µl Hydrogen peroxide (10 mM) and 100 µl of complex (100-300 µg/ml). Reaction mixtures were incubated at 37 ºC for one hour. At the end of the incubation period, 1 ml of 1% (w/v) thiobarbituric acid (TBA) was added to each mixture followed by the addition of 1 ml of 2.8% (w/v) trichloroacetic acid (TCA). The solutions were heated on a waterbath at 80 ºC for 20 minutes to develop pink coloured malonaldehyde-thiobarbituric acid (MDA-TBA) adduct and the absorbance of the resulting solution was measured at 532 nm.

In Ferric thiocyanate method (FTC) [25] 2 ml of complex solution (100-300 µg/ml) was mixed with 2.88 ml of linoleic acid (2.51%, v/v in 4 ml of 99.9% ethanol), 0.05 M phosphate buffer (pH 7.0, 8 ml) and distilled water (3.9 ml). The whole reaction mixture was incubated at 40 ^{O}C for 96 hrs. To 100, 300 and 400 µl of this solution, 9.7, 9.4, 9.3 ml of 75% (v/v) ethanol was added respectively followed by 0.1 ml of 30% ammonium thiocyanate. Precisely after three minutes, 0.1 ml of 3.5% v/v HCl was added to the reaction mixture, the absorbance at 500 nm of the resulting solution was measured and it was recorded again after 24 hrs, until the day when the absorbance of the control reached the maximum value. α-tocopherol was used as reference antioxidant substance.

In thiobarbutiric acid assay, thiobarbitric acid was added to the reaction mixture which interacts with malanoaldhyde and TBARS produced was measured spectrophotometrically [26]. To 2 ml of reaction mixtures of ferric thiocyanate assay, 2 ml of TCA (20%) and 2 ml TBA (0.67%) was added and kept in boiling water for 10 min and were later on cooled under tap water. The reaction mixtures were centrifuged at 3000 rpm for 20 min and the supernatant was read at 500 nm. α- tocopherol was taken as reference antioxidant substance. The capacity to scavenge the radicals was calculated using the following equation:

$$\% \text{ Inhibition} = A_c - A_s/A_c \times 100$$

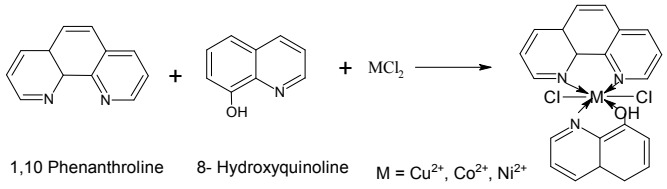
Where 'A_c' is the absorbance of the controlled reaction (reaction mixture without any antioxidant substance) and 'A_s'

is absorbance of reaction mixture with reference substance or complex. The experiments were repeated thrice.

General synthesis of complexes

To a solution of 1,10 phenanthroline (1.80g,10 mmol) and 8-hydoxyquinoline (1.45g, 10 mmol) in 50 ml absolute ethanol was added copper chloride solution (1.71g,10 mmol) and the mixture was refluxed for 1 h. A deep green precipitate was obtained metal which was isolated from the hot solution washed with ether and dried in vacuo. Similar procedure was adopted for the preparation of Co(II) and Ni(II) complexes.

$C_{21}H_{15}N_3OCuCl_2$Yeild; 80%, Anal. (%Calcd./Found) C; 54.83/54.90, H; 3.28/3.32, N; 9.13/9.25. FT-IR (KBr, cm^{-1}); 3328 ν(OH), 1560 ν(C = N), 1085 ν(C – O), 852ν(C - H), μ_{eff} = 1.86, Soluble in DMF, DMSO. $C_{21}H_{15}N_3ONiCl_2$ C; 55.66/55.72, H; 3.37/3.42, N; 9.27/9.35. FT-IR (KBr, cm^{1}) 3325 ν(OH), 1564 ν(C=N), 1089 ν(C -O), 850 ν(C - H), Soluble in ethanol. $C_{21}H_{15}N_3OCoCl_2$ 55.39/54.45, H; 3.32/3.36, N; 9.22/9.27. FT-IR (KBr, cm^{-1}); 3329 ν(OH),1563 ν(C=N), 1090 ν(C - O), 848 ν(C - H), soluble in DMF. ^{1}H NMR (400MHZ, DMSO-d_6) δ(ppm) 7.6-8.0(m), 8.3 (m)



Scheme 1

RESULT AND DISCUSSION

IR spectroscopy

In i.r. spectra of complexes the band due to ν(C=N) ring vibration of uncoordinated phenanthroline was shifted from 1588 to 1560 cm^{-1}. This shift by 28 cm^{-1} to lower frequency region shows that 1, 10-phenanthroline is coordinated to the metal center [27]. Similarly stretching bands in complex due to CH and C=C undergo coordination induced lower frequency shift of 10 cm^{-1} and 15 cm^{-1} respectively[28]. The low intensity bands at ca 512 cm^{-1} and 631 cm^{-1} are attributed to ν(M-N) and (M- O) vibration respectively[29].

Electronic spectra

The electronic spectra of copper(II) complex recorded in DMF at room temperature exhibits bands at 12820 cm^{-1}

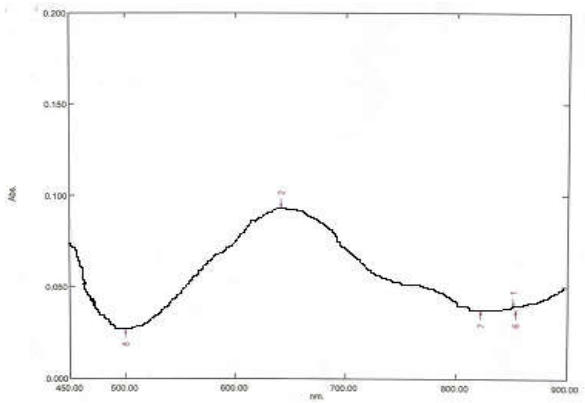


Figure 1 Electronic Spectrum of cobalt com

assigned to ^2E_g---$^2T_{2g}$ transition suggesting an octahedral geometry [30]. A charge transfer band is also observed at 25000 cm^{-1} [31].

The cobalt(II) complex shows two bands at 15552 and 12936 cm^{-1}which can be attributed to $^4T_{1g}$—$^4T_{2g}$(P) and $^4T_{1g}$(F)---$^4A_{2g}$(F) transitions for octahedral geometry [32].The nickel(II) complex shows bands corresponding to octahedral geometry with transitions $^3A_{2g}$(F) ----$^3T_{2g}$ and $^3A_{2g}$(F) ---$^3T_{1g}$(P) at ca 12642 cm^{-1} and 19000 cm^{-1}.

Magnetic Susceptiblity

Magnetic susceptibility values for the metal complexes at room temperature lies in the range compatible with octahedral geometry around the metal ion. The Cu(II) complex shows magnetic moment of 1.92 BM corresponding to octahedral geometry for Cu(II) complex.[33] The observed magnetic value (μ_{eff}) for Co(II) complex is 4.83 BM which is expected range for octahedral cobalt(II) complexes. For octahedral nickel(II) complexes, the magnetic moment lies in the range of 2.9 – 3.9 BM and Ni(II) complex has the magnetic moment of 3.91 BM in accordance with the octahedral geometry.

EPR studies

The EPR spectrum of copper(II) complex obtained at LNT with magnetic field strength 3000±1000 G displayed both parallel and perpendicular g values as $g_{\parallel}$= 2.380 and $g_{\perp}$= 2.132 and g_{avg} = 2.948 calculated from the formula $g_{avg} = g_{\parallel} - 2g_{\perp}/3$ suggesting an octahedral geometry[34] The value of $g_{\parallel} > g_{\perp} > g_e$ (2.0023) revealed that the unpaired electron was located in the d_x^2- d_y^2 orbital of the Cu(II) ion characteristic of axial symmetry. $G = (g_{\parallel}-2,0023)/(g_{\perp} -2.0023)$ measures the exchange interaction between metal centers in a poly crystalline solid. The value of G > 4 indicates negligible exchange interaction in complex and when G < 4, considerable exchange interactions which suggest that considerable exchange interaction occur in Cu(II) complex.

1H NMR

The ^{1}H NMR of Nickel(II) complex was recorded in dmso-d_6 medium. The complex shows signal at 7.1 - 7.9 attributed to aromatic protons. The signal due to OH proton is observed at 8.32

Electronic absorption spectroscopy

Electronic spectroscopy is one of the most useful technique for DNA binding studies of metal complexes [35, 36,37]. The absorption spectra of Cu(II) complex in presence of hs-DNA are shown in figure. As hs-DNA concentration is increased, the transition band of complex at 367 nm and 763 nm exhibit hypochromism and a blue shift of 20 nm. The spectral characteristics may suggest a mode of binding that involve strong stacking interaction between aromatic chromo phores and base pairs of hs-DNA. In order to elucidate the binding strength of complex, the DNA binding constant K_b were determined by monitoring the changes of absorbance in the MLCT band with increasing concentration of hs-DNA. The intrinsic binding constant K_b was calculated using the equation $[DNA]/[\varepsilon_a-\varepsilon_f]=[DNA]/\varepsilon_b-\varepsilon_f+1/k_b(\varepsilon_b-\varepsilon_f)$

Where ε_a, ε_b and ε_f are apparent, bound and free extinction coefficients respectively. In the plot of $[DNA]/[\varepsilon_a-\varepsilon_f]$ vs

[DNA], K_b is given by the ratio of slope to intercept. The binding constant for the complex is $4.9x10^4$ M^{-1}. From the binding constant, it is clear that the complex binds strongly to the complex.

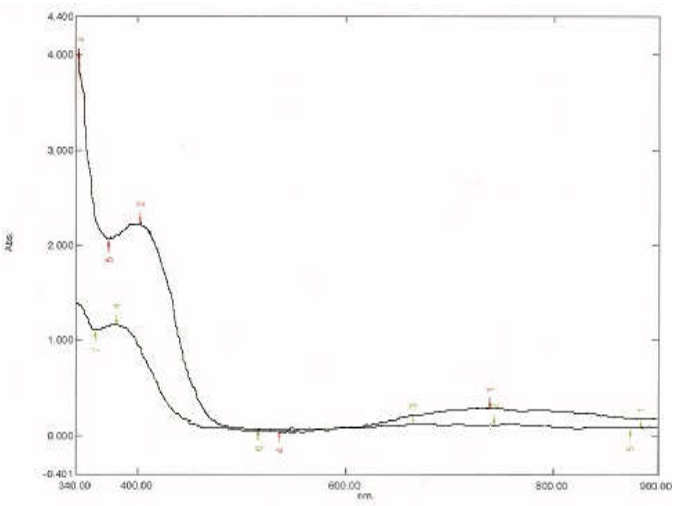


Figure 2 Absorption spectra of copper (II) complex showing shift after addition of hs-DNA

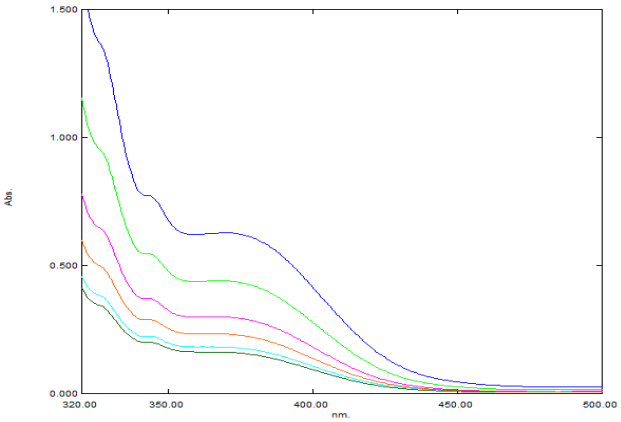


Figure 3 Electronic spectra showing hypochromism

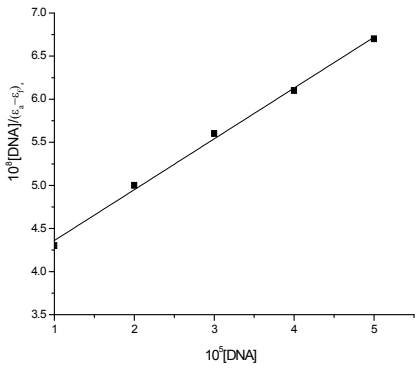


Figure 4 Plot of $[DNA]/\varepsilon_a-\varepsilon_f$ versus [DNA] for absorption titration of hs-DNA with copper(II) complex

Cyclic Voltammetry

The application of cyclic voltammetry to the investigation of binding of small molecules to hs-DNA provides useful compliment to the other methods of investigation. Figure 5 shows the cyclic voltammogram of the Cu(II) complex in presence and absence of hs-DNA. After addition of hs-DNA no new peak peak was formed but the original peak decreased in intensity suggesting the existence of interaction between the complex and hs-DNA[38-40], Bard and coworkers [41] have discussed the binding mode between small molecules and DNA, if the interaction is electrostatic binding mode, the formal potential shits to more negative potential while as intercalating binding mode shifts the potential to more positive potential. In presence of hs-DNA the Cu(II) complex experiences positive shift showing intercalative binding mode.

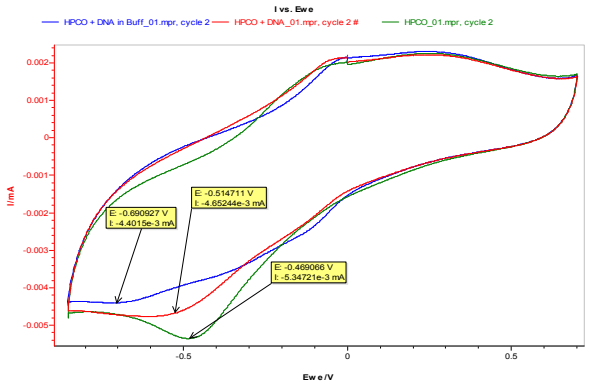


Figure 5 Cyclic Voltammogram of Cu(II) complex in the presence and absence of complex

Viscosity measurements

To further investigate the binging mode of complex to hs-DNA, viscosity measurements were carried out by varying concentration of the complex added to the DNA solution. Viscosity is particularly sensitive to the changes in length of DNA. The intercalative binding mode causes elongation of DNA helix as base pairs are pushed apart to accommodate the complexes, thus resulting in an increase in viscosity. In contrast a partial or non classical intercalation could bend or kink DNA resulting in decrease in the effective length with concomitant decrease in viscosity [42]. The relative specific viscosity (η/η_0) of DNA generally reflects the increase in contour length associated with separation of base pairs caused by intercalation. The results demonstrate that complex could bind to hs-DNA by intercalation mode, which is consistent with electronic and electrochemical results.

Gel electrophoresis

The principle of this method is that molecules migrate in the gel as a function of their mass, charge and shape. The ability of complex to interact with DNA was examined by gel electrophoresis. Binding of complex to DNA retards the movement of DNA under gel electro phoresis. Both Cu(II) and Co(II) complexes show protectivity activity

Lane1: Native ct-DNA; Lane 2: BHT + Reaction mixture + DNA; Lane 3: ct-DNA + Reaction mixture + Cu(II) complex (500 µg); Lane 4: ct-DNA + Reaction mixture + Co(II) complex (500 µg); Lane 5: ct-DNA + reaction mixture + Ni(II) (500 µg); Lane 6: BHT (500 µg) + Reaction mixture + ct-DNA

Antioxidant activity

The antioxidant activity of metal complexes was compared with a positive control (BHT) and Ascorbic acid which is known to protect tissues and cells against various oxidative stresses [43].

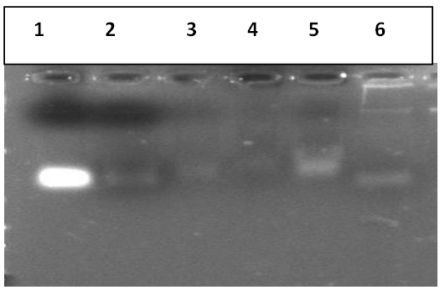


Figure 6 Protective DNA damage activity by metal complexes

The mechanism of activity of scavenging of radicals cannot be evaluated by a single method, therefore five different antioxidant models were used in this study [44] The antioxidant ability of metal complexes is determined in the DPPH method by a decrease in absorption strength as indicated by discoloration (yellow colour) from original radical scavenging activities of Cu(II), Co(II) and Ni(II) complexes, Ascorbic acid, and BHT at 500µgml^{-1} were observed to be 20%, 53%, 66%, 70% and 80% respectively (Fig.1a). It was found that the free radical scavenging activity of Ni(II) complex was stronger than other complexes however BHT was found to have higher activity. All the complexes exhibited moderate to high superoxide dismutase activity at variable concentrations. The Cu(II) complex exhibited higher scavenging activity (69%) followed by Co(II) and Ni(II) complexes at 500 µg/ml. However, BHT (80%) was found highly significant in scavenging the radicals (Fig.1b).

The hydroxyl radicals are known to cause DNA damage by degradation of deoxyribose moiety which contributes to carcinogenesis, mutagenesis The hydroxyl radicals are known to cause DNA damage by degradation of deoxyribose moiety which contributes to carcinogenesis, mutagenesis and cytotoxicity [45], however, the scavenging or chelation of radicals by any substance is due to the antioxidant capacity of that particular substance [46]. In our study, only Cu(II) and Ni(II) complexes exhibited high chelating activity of hydroxyl radicals at a concentration range of 100–500µg/ml. Overall the scavenging activity decreased in the order of Ascorbic acid (83%) > BHT (65%) > Cu(63%) > Ni(60%) at the concentration of 500µgml^{-1} (Fig.1c).

FTC evaluates the effect of a reference antioxidant and extracts on preventing peroxidation of polyunsaturated fatty acids and linoleic acid. The concentrations used were 100–300 µg/ml, per cent inhibition was recorded after every 24 h and results are given here for three consecutive days. The percentage protective effect of linoleic acid peroxidation was 80% for Cu(II) complex, 70% for Ni(II) complex and 40% for Co(II) complex at the 300µg/ml concentration (Fig. 1d). In the TBA method, formation of malanoaldehyde is the basis for evaluating the extent of lipid peroxidation. At low pH and high temperature malanoaldehyde, which is the end product of lipid peroxidation, binds TBA to form a red coloured complex. The concentrations used were 100–300 µg/ml, as for the FTC method, because this assay is based on the same samples. The FTC method measures the amount of peroxide produced during the initial stage of lipid peroxidation. Subsequently at later stages of oxidation, peroxides decompose to form carbonyl compounds that are measured by the TBA method. At a concentration of 300 µgml^{-1} the Cu complex exhibited 61% inhibition of radicals, followed by Ni(II) complex (65%) (Fig. 1e). Some of the reports mentioned the ferric reducing power of bioactive compounds such as phenolic substances and flavonoids [47-49]. The findings of this work confirm that the metal complex can be used as an alternative for the performing the various biological activities to combat various indigenous as well as exogenous stresses.

Table 1 IC_{50} determination of metal complexes

Complexes	IC_{50} µg/ml				
	DPPH assay	SOD	Hyrdoxyl scavenging assay	FTC	TBA
HPNI	260	-	220	98	80
HPCo	160	-	-	80	180
HPCu	120	80	180	220	275
ASCORBIC ACID	175	60	>300	70	100
BHT	117	40	97	70	160

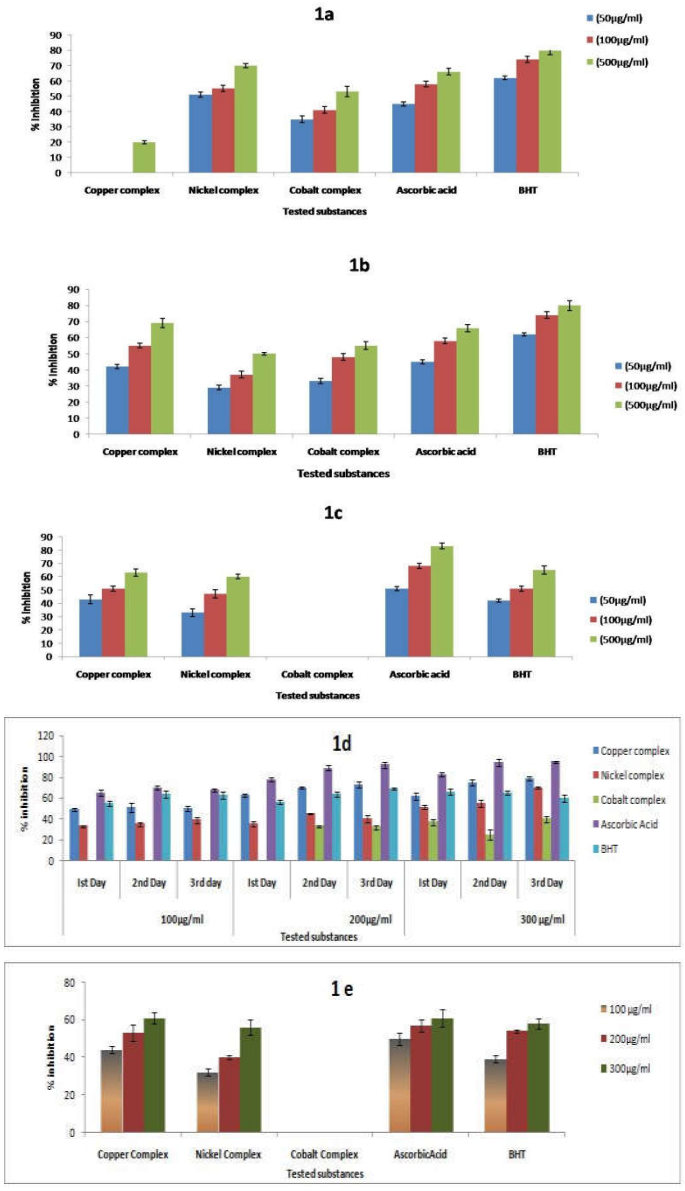


Figure 7 Antioxidant activity of the metal complexes

CONCLUSIONS

In summary the metal complex derived from 1,10-phenanthroline and 8-hydroxyquinolone were synthesized and

characterized by various physicochemical methods. The interaction of Cu(II) complex with hs-DNA was studied by absorption spectroscopy, viscosity and cyclic voltammetry under physiological conditions. The protective activity was studied by gel electrophoresis. The results reveal that Cu(II) complexes is capable of binding to DNA via intercalation mode. The metal complexes were also studied for antioxidant activity using different assays and the results reveal strong antioxidant activity.

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