ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

NNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 3, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

DNA CLEAVAGE ACTIVITY OF NOVEL SCHIFF BASE COPPER(II) COMPLEXES WITH SULFUR CONTAINING-LIGANDS: (2-(2-METHYLTHIO)PHENYLIMINO)METHYLPHENOL, N-BENZYLIDINE-2-METHYLTHIOANILINE

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Received: 16 Febuary 2016, Revised and Accepted: 22 Febuary 2016

ABSTRACT

Objective: To study the DNA cleavage activity of all copper(II) complexes using supercoiled pUC19 DNA by agarose gel electrophoresis.

Methods: In this study, six new schiff base mixed ligand copper(II) complexes containing [Cu(L_1)(8Hq)Cl_2], [Cu(L_2)(8Hq)Cl_2], [Cu(L_1)(phen)Cl_2], [Cu(L_2)(phen)Cl_2], [Cu(L_1)(Bpy)Cl_2], and [Cu(L_2)(Bpy)Cl_2] (where, phen=1,10-phenanthroline, Bpy=2, 2'-bipyridyl, 8Hq=8-hydroxy quinoline, ligand L_1=(2-(2-methylthio)phenylimino)methylphenol, ligand L_2=N-benzylidine-2-methylthioaniline) were synthesized. The six novel schiff base ligands were characterized by molar conductance, ultraviolet, infrared (IR), and cyclic voltammetry (CV) measurements. Low molar conductance values of copper(II) complexes showed non-electrolyte in nature. The IR spectral data indicate that nitrogen, sulfur, and azomethine nitrogen coordinate to the central metal ion. The electrochemical properties such as anodic, cathodic peak potential, and electron transfer process of the complexes were studied using CV measurements. Based on these spectral data, octahedral geometry was proposed to all copper(II) complexes.

Results: The result confirmed that binding constant value of $13.95 \times 10^3\ M^{-1}$ for complex (VI) suggested good intercalative binding with calf thymus (CT) DNA. When DNA (pUC19) cleavage analyses was performed, copper(II) complexes - I, II, V, VI showed cleavage activity.

Conclusion: The complexes - III and IV did not exhibit DNA binding as well as DNA cleavage potential which may be due to the presence of O-phenanthroline.

Keywords: (2-(2-methylthio)phenylimino)methylphenol, N-benzylidine-2-methylthioaniline, Copper(II) complexes, Calf thymus DNA, pUC19 DNA, DNA binding and cleavage activity.

INTRODUCTION

One of the most rapidly developing areas of pharmaceutical research is the discovery of drugs for cancer therapy [1]. Cis-platin is one of the foremost and widely used metal-based anticancer drugs. Although it acts as an effective drug against cancer, it has encountered many side effects such as hair loss, a decrease in bone marrow effectiveness, neurotoxicity and damage due to the drug resistance phenomenon and limited activity and selectivity in cancer cells [2]. Therefore, considerable attempts are being made to replace this drug with suitable alternatives and numerous transition metal complexes such as Cu(II), Ru(II), Co(II), Ni(II), and Zn(II). Several schiff base copper(II) complexes have been now proposed as potential anticancer substances, demonstrating remarkable anticancer activity, and showing general toxicity lower than platinum compounds [3-5]. Number of copper(II) complexes have been found to be active both *in-vitro* and *in-vivo*. Many copper(II) complexes are known to bind to DNA *via* both covalent and non-covalent interactions. In covalent binding, the labile ligand of the complexes is replaced by a nitrogen base of DNA. On the other hand, the non-covalent DNA interactions include intercalative, electrostatic, and groove (surface) binding of cationic metal complexes outside of the DNA helix, major or minor groove. DNA molecules are prone to be damaged under various conditions such as interactions with some molecules. This damage may cause various pathological changes in living organisms, which is due to their possible application as new therapeutic agents and their photochemical properties which make them potential probes of DNA structure and conformation [6]. It has been recently found that mixed ligand copper(II) complexes exhibit strong DNA binding and cleavage activity and induce apoptosis in cancer cells [7-9]. Previous studies also reported that mixed ligand copper(II) complexes with sulfur-containing ligands shown better nuclease activity [10,11]. The present work stems from the interest in evaluating DNA binding and cleavage activities of novel schiff base mixed ligand copper(II) complexes with sulfur-containing ligands for cancer treatment. The novelty of the present study is the copper(II) complexes, which are not yet prepared in combination with the ligand.

METHODS

Reagents and materials

All the reagents and chemicals were purchased from commercial sources (Sigma-Aldrich, Merck) and used without purification. The commercial solvents were distilled and then used for preparation of ligands and copper(II) complexes and spectroscopic measurements. The calf-thymus DNA and double-stranded supercoiled pUC19 DNA were purchased from Bangalore Genei (India) and were stored at 4°C. Agarose (molecular biology grade) and ethidium bromide were purchased from Sigma (USA). Tris-HCl/NaCl (pH 7.2) buffer solution was prepared using Millipore water.

Physical measurements

Molar conductance of the copper(II) complexes was measured using a Toshniwal digital conductivity meter cell constant, 1.00. Approximately 1×10^{-3} M solution of the complexes in DI methyl formamide (DMF) was employed for conductivity measurements. Electronic absorption spectral measurements were made as DMF solutions on a JASCO V-550 ultraviolet (UV)-visible spectrophotometer in the range of 200-800 nm. The infrared (IR) spectra of all the complexes and the ligands were recorded on an FTIR-8400S SHIMADZU spectrometer in the range of 400-4000 cm^{s}. Potassium bromide disc method was employed for sample preparation. Cyclic voltammetry (CV) measurements were performed on CHI 6036C electrochemical analyzer equipped with a three electrode system consisted of glassy carbon (working), platinum wire (auxiliary), and a silver-silver chloride (reference) electrode using DMF as a solvent.

Synthesis of ligands

Synthesis of (2-(2-methylthio)phenylimino)methylphenol (ligandL$_1$)

To a solution of salicylaldehyde (1 mmol, 1.221 g) in ethanol (100 ml), 2-methylthioaniline (1 mmol, 1.392 g) in ethanol (100 ml) was added dropwise. The above mixture was stirred for 1 hr. The solid product was not obtained, and hence, the complexes of this ligand were prepared using template method.

Synthesis of N-benzylidine-2-methylthio aniline (ligand L$_2$)

To a solution of benzaldehyde (1 mmol, 1.061 g) in ethanol (100 ml), 2-methylthioaniline (1 mmol, 1.392 g) in ethanol (100 ml) was added dropwise. The above mixture was stirred for 1 hr. The solid product was not obtained, and hence, the complexes of this ligand were prepared using template method.

Synthesis of complexes

Preparation of [dichloro(8-hydroxy quinoline)2-(2-methylthio) phenyliminomethylphenolcopper(II)]

To the ethanolic solution, ligand L$_1$ was added slowly with constant stirring for 1 hr. To the above solution, copper(II)chloride dihydrate (1 mmol, 1.704 g) in ethanol (100 ml) was added dropwise with constant stirring for 30 minutes. This above mixture was added to 8-hydroxy quinoline (1 mmol, 1.451 g) in ethanol (100 ml). The complex was settled and kept undisturbed for 24 hrs. The solid product obtained was filtered and dried in air (yield=78%).

Preparation of [N-benzylindine-2-methylthioanilinedichloro (8-hydroxyquinoline) copper(II)]

To the ethanolic solution, ligand L$_2$ was added slowly with constant stirring 1 hr. To the above solution, copper(II) chloride dihydrate (1 mmol, 1.704 g) in ethanol (100 ml) was added dropwise with constant stirring for 30 minutes. This above mixture was added to 8-hydroxy quinoline (1 mmol, 1.451 g) in ethanol (100 ml). The complex was settled and kept undisturbed for 24 hrs. The solid product obtained was filtered and dried in air (yield=76%).

Preparation of [dichloro2-(2-methylthio) phenyliminomethylphenol(1,10, phenanthroline)copper(II)]

To the ethanolic solution, ligand L$_1$ was added slowly with constant stirring for 1 hr. To the above solution, copper(II) chloride dihydrate (1 mmol, 1.704 g) in ethanol (100 ml) was added dropwise with constant stirring for 30 minutes. This above mixture was added to 1,10-phenanthroline (1 mmol, 1.98 g) in ethanol (100 ml). The complex was settled and kept undisturbed for 24 hrs. The solid product obtained was filtered and dried in air (yield=71%).

Preparation of [N-benzylindine-2-methylthioanilinedichloro (1,10, phenanthroline)copper(II)]

To the ethanolic solution, ligand L$_2$ was added dropwise with constant stirring for 1 hr. To the above solution, copper(II) chloride dihydrate (1 mmol, 1.704 g) in ethanol (100 ml) was added dropwise with constant stirring for 30 minutes. This above mixture was added to 1,10-phenanthroline (1 mmol, 1.98 g) in ethanol (100 ml). The complex was settled and kept undisturbed for 24 hrs. The solid product obtained was filtered and dried in air (yield=72%).

Preparation of [(2,2'-bipyridyl)dichloro2-(2-methylthio) phenyliminomethylphenolcopper(II)]

To the ethanolic solution, L$_1$ was added slowly with constant stirring for 1 hr. To the above solution, copper(II)chloride dihydrate (1 mmol, 1.704 g) in ethanol (100 ml) was added dropwise with constant stirring for 30 minutes. This above mixture was added to 2,2-bipyridyl (1 mmol, 1.56 g) in ethanol (100 ml). The complex was settled and kept undisturbed for 24 hrs. The solid product obtained was filtered and dried in air (yield=70%).

Preparation of [N-benzylindine-2-methylthioaniline(2,2'-bipyridyl)dicholorocopper(II)]

To the ethanolic solution, ligand L$_2$ ethanol was added slowly with constant stirring for 1 hr. To the above solution, copper(II)chloride dihydrate (1 mmol, 0.170 g) in ethanol was added dropwise with constant stirring for 30 minutes. This above mixture was added to 2,2-bipyridyl (1 mmol, 1.56 g). The complex was settled and kept undisturbed for 24 hrs. The solid product obtained was filtered and dried in air (yield=66%).

DNA binding studies

Preparation of DNA stock solution

Concentrated solutions of calf thymus (CT)-DNA were prepared in 50 mM NaCl/5 mM Tris-HCl in water at pH 7.2 gave a ratio of UV absorbance at 260-280 nm, A260/A280 of 1.9 indicating that the DNA was free from protein. The concentration of DNA was determined by the UV absorbance at 260 nm after 1:100 dilutions. The stock solutions were stored at 4°C and used within 4 days [12].

Absorption spectra

Absorption spectra for the DNA binding studies were recorded on Thermo Scientific Helious Alpha UV-visible spectrophotometer using 1 cm quartz micro cuvettes. Absorption titration was performed by keeping the concentration of the complex constant at 200 μM and varying the concentration of CT-DNA from 0 to 20 μM. The DNA concentration per nucleotide was determined by knowing the molar extinction coefficient value of 6600 $cm^{-1}mol\ L^{-1}$. The binding constants (Kb) for the binding of the complex with DNA were determined using the following equation [10-12].

$$[DNA]/(\varepsilon a-\varepsilon f)=[DNA]/(\varepsilon b-\varepsilon f)+1/Kb(\varepsilon b-\varepsilon f)$$

The apparent extinction coefficient (Ɛa) was obtained by calculating A_{obsd}/[Cu]. The terms Ɛf and Ɛb correspond to the extinction coefficients of free (unbound) and of the fully bound complex. Kb, the ratio of the slope to the intercept, was obtained from a plot of [DNA]/(Ɛb-Ɛf) versus a slope 1/(Ɛb-Ɛf) and an intercept 1/kB(Ɛb-Ɛf).

Nuclease activity

DNA cleavage studies with pUC19 DNA

The cleavage of pUC19 DNA was monitored by agarose gel electrophoresis. The electrophoresis experiments were performed with samples containing 1.5 μl of pUC19 DNA, 5 μl (200 μM and 150 μM) of copper complex, 4 μl (100 μM) of ascorbic acid, and diluted to 20 μl with 50 mM Tris-HCl buffer containing 50 mM NaCl (pH 7.2). The samples were incubated for 1 hr at 370°C. After incubation, the samples added loading buffer containing 25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, and electrophoresed for 2 hrs at 50 V on 0.7% agarose gel containing 1.0 μg/ml of ethidium bromide. After electrophoresis, the bands were observed under Spectroline UV transilluminator and photographed to determine the extent of DNA cleavage. Then, the results were compared with standard DNA marker [10-13].

RESULTS AND DISCUSSION

The ligand form octahedral complexes - I, II, III, IV, V, and VI with $CuCl_2.2H_20$ in ethanol. The proposed structures of the complexes are shown in Fig. 2a-f. All the six complexes were colored, stable in air and atmosphere. Complexes I and II are dark brown, complexes III and IV were dark green, and complexes V and VI were green. These complexes were insoluble in water, moderately soluble in chloroform, acetonitrile, completely soluble in DMF. The molar conductance values

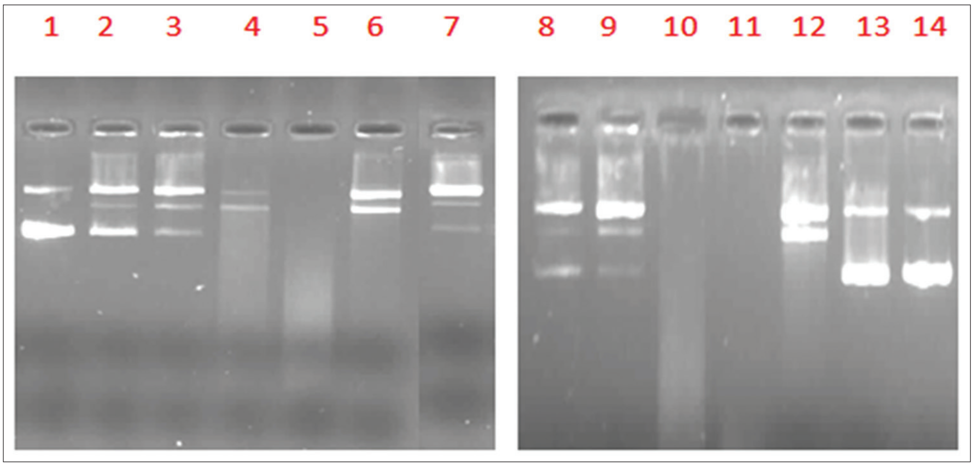

Fig. 1: Agarose gel (Lane 2-7 at 50 µM, Lane 8-13 at 100 µM) showing the cleavage of SC pUC19 DNA by the copper complexes in DMF in buffer containing 50 mM Tri-HCl/50 mM NaCl (pH 7.2) in the presence of ascorbic acid at 37°C

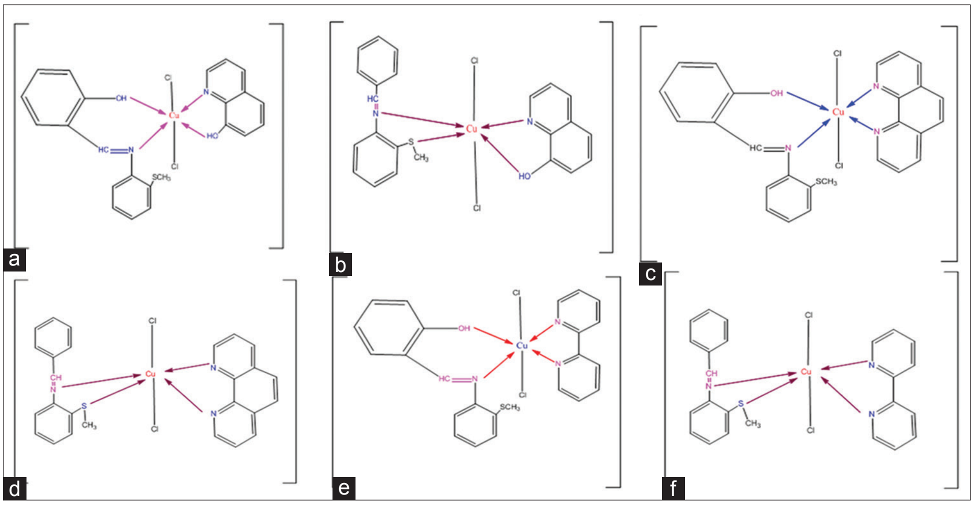

Fig. 2: Proposed structure of the complexes. (a) $[Cu(L_1)(8Hq)Cl_2]$, (b) $[Cu(L_2)(8Hq)Cl_2]$, (c) $[Cu(L_1)(phen)Cl_2]$, (d) $[Cu(L_2)(phen)Cl_2]$, (e) $[Cu(L_1)(Bpy)Cl_2]$, (f) $[Cu(L_2)(Bpy)Cl_2]$

of all the complexes were too, indicating the non-electrolyte nature of the complexes.

UV spectral analysis

The electronic spectrums of all the metal complexes were recorded in DMF solution at approximately the 10^{-3} M concentration. The electronic absorption spectral bands of the copper complexes were recorded over the range 200-800 nm in DMF. UV of copper complexes for six complexes showed two bands, one of 420, 390, 398, 404, 490, and 414 nm that corresponds to the charge transfer band (L→M) and another band at 656, 646, 642, 650, 654, and 625 nm in the visible region, assigned to $2E_g \rightarrow 2T_{2g}$ which was characteristic of distorted octahedral geometry [14].

IR spectral analysis

The coordination sites of the ligand were inferred by IR spectroscopy, which is considered a useful technique for probing the structure of Schiff base complexes. The band at 1615 cm^{-1} which is characteristics of azomethine nitrogen atom presents in the free ligand [14]. This is shifted to lower frequency 1581, 1582, 1581, 1579, 1566, and 1566 cm^{-1} indicating the involvement of azomethine nitrogen in coordination with the metal ion. Coordination of nitrogen to the metal ion would reduce the electron density of azomethine nitrogen and thus cause shifts in the ν (HC=N) group. The IR spectra of the copper complexes show some new bands in the region 600-640 cm^{-1} and 420-622 cm^{-1} which are due to Cu-S and Cu-N.

CV analysis

The redox properties of Cu(II) complexes have been studied by CV, using glassy carbon working electrode in DMF at a scan rate 100mVs^{-1}. The cyclic voltammogram of the complexes in DMF solution recorded over a potential range of +1.5 V to −1.5 V has shown a well-defined redox process corresponding to the Cu(II)/Cu(I) couple. The ΔEp values of six complexes (0.3759, 0.4388, 0.6575, 0.6306, 0.5163, and 0.3683) for Cu(II)/Cu(I) couple were found to be greater than the Nernstian values (ΔEp ⊐ 0.059 V). This indicated that Cu(II) complexes exhibited quasi-reversible electron transfer process involving Cu(II)/Cu(I) couple [15].

Biological studies

DNA binding activity

Electronic absorption spectroscopy was used to determine the binding of the complexes with DNA. Complex bound to DNA through intercalation usually results in hypochromism and red shift (bathochromism), due to the intercalative mode, involving a strong stacking interaction between the aromatic chromophore and the base pairs of DNA. The absorption spectra of all the complexes in the absence and the presence of CT-DNA were taken. In the UV region, all the mixed ligand copper(II) complexes exhibited an intense band around 400 nm which is attributed to the ligand to metal charge transfer absorption. In addition, of CT-DNA, the absorption bands of the complexes were affected, resulting in the obvious tendency of hypochromism and the insignificance of

Table 1: Intrinsic binding constant Kb, of the synthesized mixed ligand schiff base copper(II) complexes

Complexes	Intrinsic binding constant (Kb×10^3 M^{-1})
[Cu (L_1)(8Hq) Cl_2]	1.675
[Cu (L_2)(8Hq) Cl_2]	2.952
[Cu (L_1)(Bpy) Cl_2]	2.70
[Cu (L_2)(Bpy) Cl_2]	13.95

L_1: (2-(2-methylthio) phenylimino) methylphenol,
L_2: N-benzylidine-2-methylthioaniline, 8Hq: 8Hydroxy quinoline,
Bpy-2: 2'bipyridyl, Kb: Intrinsic binding constant

redshift [16-18]. The copper(II) complexes can bind to the double-stranded DNA in different binding modes on the basis of their structure, charge, and type of ligands. The hypochromism for the band of around 400nm for complexes I, II, III, IV, and V suggested that the complex binds to DNA strongly. However, complexes III and IV [dichloro2-(2-methylthio)phenylimino)methylphenol(1,10-phenanthroline) copper(II)], [N-benzylidine-2-methylthioanilinedichloro(1,10-phenanthroline)copper(II)] failed to bind to DNA. The binding constant obtained for all the complexes was shown in Table 1. The Kb value obtained by the plot of [DNA]/(Ɛb-Ɛf) versus a slope 1/(Ɛb-Ɛf) and an intercept 1/Kb (Ɛb-Ɛf).

Nuclease activity

Cleavage of pUC19 DNA

The cleavage efficiency of all the copper(II) complexes was measured by determining the ability of the complex to convert supercoiled (SC) DNA to nicked form (NC). When the plasmid DNA is subjected to electrophoresis, fastest migration is observed to supercoiled form (Form I). If one strand is cleaved, the supercoiled will relax to produce a nicked circular form (Form II) which migrates slowly. If both the strands are cleaved, linear conformation (Form III) is produced, which migrates between the other two forms [17,19,20]. Fig. 1 illustrates the gel electrophoretic separation showing the cleavage of plasmid pUC19 DNA induced by the six complexes under identical reaction conditions using ascorbic acid as a reducing agent.

In this study, all the complexes have shown good nuclease activity except complexes (III and IV) as indicated by slight conversion of SC form (Form I) into nicked circular form (Form II). The cleavage efficiency of the complexes compared to that of the control DNA is due to their efficiency of DNA binding ability.

When pUC19 DNA was treated with the complexes I and II at 50 μM and 100μM concentration. At 50 μM complexes I and II exhibit partial and moderate DNA cleavage activity. At 100 μM both complexes exhibit very good nuclease activity.

When pUC19 DNA was treated with the complexes III and IV in 50 μM and 100 μM concentration. No bands were observed for complexes III (50 μM) and IV (50, 100 μM), and only faint bands were observed for complex III (50 μM) in the gel. This may be due to the presence of O-Phenanthroline. Thus, at both the concentrations (50, 100 μM) complex IV have the capacity to completely degrade the DNA.

When pUC19 DNA was treated with the complexes V and VI (50 μM and 100 μM), complete DNA cleavage was observed for complex V at both the concentrations, whereas complex VI (50 μM) exhibited complete DNA cleavage activity and no cleavage activity was observed at 100 μM concentration. Thus, the complexes V and VI exhibit good nuclease activity at 50 μM concentrations.

DNA cleavage studies

Lane 1: DNA control + Ascorbic acid
Lane 2: DNA + [Cu (L_1) (8Hq) Cl_2] (50 μM) + Ascorbic acid
Lane 3: DNA + [Cu (L_2) (8Hq) Cl_2)] (50 μM) + Ascorbic acid
Lane 4: DNA + [Cu (L_1) (phen) Cl_2] (50 μM) + Ascorbic acid
Lane 5: DNA + [Cu (L_2) (phen) Cl_2] (50 μM) + Ascorbic acid
Lane 6: DNA + [Cu (L_1) (Bpy) Cl_2] (50 μM) + Ascorbic acid
Lane 7: DNA + [Cu (L_2) (Bpy) Cl_2] (50 μM) + Ascorbic acid
Lane 8: DNA + [Cu (L_1) (8Hq) Cl_2)] (100 μM) + Ascorbic acid
Lane 9: DNA + [Cu (L_2) (8Hq) Cl_2)] (100 μM) + Ascorbic acid
Lane 10: DNA + [Cu (L_1) (phen) Cl_2)] (100 μM) + Ascorbic acid
Lane 11: DNA + [Cu (L_2) (phen) Cl_2)] (100 μM) + Ascorbic acid
Lane 12: DNA + [Cu (L_1) Bpy) Cl_2] (100 μM) +Ascorbic acid
Lane 13: DNA + [Cu (L_2) (Bpy) Cl_2)] (100 μM) + Ascorbic acid
Lane 14: DNA control.

DISCUSSION

In this study, new complexes with sulfur-containing ligands such as (2-(2-methylthio)phenylimino)methylphenol, N-benzylidine-2-methylthio aniline, and DNA binder, such as 8-hydroxy quinoline, 1,10-phenanthroline, 2,2'-bipyridyl, were synthesized and characterized by various methods. The electrochemical studies of the complexes indicated quasi-reversible electron transfer process involving redox couple. The electronic and IR spectral data suggested that these complexes have octahedral geometry. The DNA binding studies suggested that the prepared complexes have the ability to bind with DNA as indicated by their high binding constant values. The DNA cleavage studies of the complexes with SC pUC19 DNA in the presence of ascorbic acid have shown that they have the ability to convert the SC form of DNA into nicked circular form.

Proposed structure of the complexes

Based on the spectral data gathered, the following structures were proposed for the complexes. (Fig. 2)

CONCLUSION

The complexes - I, II, V, and VI exhibit binding as well as cleavage potential those complexes can be further used for cytotoxic studies to prove whether they exhibit anticancer activity.

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