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# Synthesis, Characterisation, DNA-Binding Studies and antimicrobial activity of Copper(II) Complex with 1,10 Phenanthroline, L-Tyrosine and Urea as Ligands

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**ABSTRACT**

The interaction of transition metal complexes with DNA has long been a subject of intensive investigation with the perspective of development of newer materials for application in biotechnology and medicine. These investigations have resulted in the synthesis of many new metal complexes, which bind to DNA by non-covalent interactions such as electrostatic binding, groove binding and intercalative binding [1,2]. Hence, the current growing interest in small molecules that are capable of binding and cleaving DNA is related to their utility in the design and development of synthetic restriction enzymes, new drugs, DNA foot-printing agents, etc., and also to their ability to probe the structure of DNA itself [3].



## Introduction

The interaction of transition metal complexes with DNA has long been a subject of intensive investigation with the perspective of development of newer materials for application in biotechnology and medicine. These investigations have resulted in the synthesis of many new metal complexes, which bind to DNA by non-covalent interactions such as electrostatic binding, groove binding and intercalative binding [1,2]. Hence, the current growing interest in small molecules that are capable of binding and cleaving DNA is related to their utility in the design and development of synthetic restriction enzymes, new drugs, DNA foot-printing agents, etc., and also to their ability to probe the structure of DNA itself [3].

Copper complexes of 1,10-phenanthroline and its derivatives are of great interest since they exhibit numerous biological activities such as antitumour[4], anti-Candida[5], antimycobacterial [6], antimicrobial [7], activities etc. Moreover, considerable attention has been focused on the use of phenanthroline complexes as intercalating agents of DNA [8] and as artificial nucleases [9-11].

Copper(II) is known to play a significant role in biological systems and also as a pharmacological agent. Synthetic copper(II) complexes have been reported to act as potential anticancer and cancer inhibiting agents [12-14], and a number of copper complexes [15,16] have been found to be active both in vitro and in vivo.. Very recently, Reedijk and co-workers have found that the complex [$Cu^{II}$(pyrimol)Cl] brings about efficient self-activated DNA cleavage and cytotoxic effects towards L1210 murine leukaemia and A2780 human ovarian carcinoma cell lines [17]. Sadler and co-workers have observed [4] that mixed ligand bis (salicylato) copper(II) complexes with diimines as co-ligands exhibit cytotoxic and antiviral activities. Very recently, Ng and co-workers have prepared ternary copper(II) complexes of ethylene diamine diacetic acid ($H_2$edda) and 1,10-phenanthroline, that strongly bind to DNA and also regulate apoptosis [18]. Palaniandavar, et. al., recently reported that mixed ligand copper(II) complexes of diimines, that bind and cleave DNA and also exhibit anticancer activity which is more efficient than that of cisplatin[19]. The use of phenanthroline as the co-ligand in the above ternary complexes is of considerable interest because some of the phenanthroline containing copper complexes exhibit biological as well as pharmacological properties[20]. Palaniandavar and co-workers also reported that mixed ligand copper(II) [19] and ruthenium(II) [21-22] complexes exhibit prominent anticancer activities in which the diimine co-ligands play a pivotal role in the mechanisms underlying the induction of cell death. The mixed ligand palladium(II) and platinum(II) complexes containing amino acids have been shown to act as potential anticancer agents [23-24].

The study of ternary copper(II) complexes of diimines and amino acids as anticancer drugs is of interest because copper is a bio essential element but only in traces, and also, amino acids are present in all biological systems. Also, these mono positive complexes are expected to be more lipophilic than the corresponding dicationic complexes, thereby providing for greater uptake by cells and hence an enhanced cytotoxicity. Very recently, Guo and co-workers have found the ternary copper(II) complex of phenanthroline and L-threonine to cleave DNA oxidatively and exhibit cytotoxicity [25].

Further, some ternary copper(II) complexes are having macrocyclic bases such as 1,10 phenanthroline and its derivatives which are capable of DNA intercalation/binding in the presence of auxiliary ligands, were prepared and

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characterised. These complexes bind and cleave DNA effectively in the presence of a reductant or upon irradiation with UV or visible light [26-28].

Certain amino acid complexes are found to exhibit potent anti-tumour and artificial nuclease activity. The focus was shifted towards the development of ternary copper(II) complex of phenanthroline with amino acids and their DNA cleavage activity was investigated.

In this paper, the synthesis and characterisation of $[Cu(Phen)(L\text{-}Tyr)(U)]ClO_4$ complex by elemental analysis, conductivity measurement, IR and EPR spectra have been reported. The binding property of the complex to calf thymus DNA has been studied using different physico-chemical methods and the binding modes are discussed. The experimental results show that the copper(II) complex sample binds effectively with DNA-binding agents and cleaves its super-coiled form into nicked and linear forms. We have also reported the antimicrobial activities of copper(II) complex against gram positive, gram negative bacteria as well as fungus.

It is generally believed that molecules which damage DNA and block DNA synthesis indirectly through inhibition of biosynthesis of precursor molecules for nucleic acids or disrupt hormonal regulation of cell growth would make them better candidates for development as anticancer agents [29]. Also, DNA has been identified as the possible primary molecular target [30] of metal-based anticancer agents such as cisplatin[31]. Therefore, we propose to investigate whether the complex exhibits DNA-binding and cleavage properties. The introduction of chirality via the amino acid may enhance the pharmacological behaviour of the copper complex by adopting a specific conformation and may also confer selective binding affinity for the chiral DNA. Furthermore, the carboxylate group of L-tyrosine has the potential to interact with sugar hydroxyl groups of DNA to enhance the DNA binding affinity and to provide recognition element, thus leading to the selective control of the metal-chelate nucleo base recognition process. Also, there has been considerable attention focused on the use of the small metal complexes containing diimines as recognition elements of DNA [32-35] and metal-based synthetic nucleases [35,10,9].

## Experimental

### Materials and Methods

The reagents such as ethanol, anhydrous ether, $Cu(NO_3)_2.3H_2O$, NaOH, $NaClO_4.H_2O$ and 1,10 phenanthroline of analytical grade (sd fine chemical) are used in the same condition as received. L- Tyrosine was purchased from Aldrich. Disodium salts of calf-thymus DNA (CT DNA) was purchased from Aldrich. Ascorbic acid, tris HCl, NaCl and ethidium bromide were purchased from sd fine chemicals. The spectroscopic titration was carried out in the buffer (50 mM NaCl–5 mM Tris–HCl, pH 7.1) at room temperature. A solution of calf thymus DNA in the buffer gave a ratio of UV absorbance 1.8–1.9:1 at 260 and 280 nm, indicating that the DNA was sufficiently free of protein [36]. Milli-Q water was used to prepare the solutions. Absorption spectral study was carried out by using UV–VIS–NIR Cary 300 spectrophotometer which is having cuvettes of 1 cm path length, and emission spectral study was carried out by using JASCO FP 770 spectrofluorimeter. The complex, $[Cu(Phen)(L\text{-}Tyr)(H_2O)]ClO_4$, was prepared as reported earlier in the literature [37].

Element analysis was performed by SAIF, Lucknow, India. The conductivity study was carried out by using an aqueous solution of complex with an Elico conductivity bridge type CM82 and a dip-type cell with cell constant 1.0. Absorption spectral study was carried out by using UV–VIS–NIR Cary 300 spectrophotometer having cuvettes of 1-cm path length, and emission spectral study was carried out by using JASCO FP 770 spectrofluorimeter. IR spectra were recorded on an FT-IR Perkin Elmer spectrophotometer.

Electron paramagnetic resonance spectra of the Copper(II) complex was obtained on a Brukker EMX EPR spectrometer. The spectra were recorded for solution of the Copper(II) complex in acetonitrile : acetone (4 :1 *v*/*v*) solution at room temperature (RT) as well as at liquid nitrogen temperature (77 K). DPPH was used as the field marker.

The antimicrobial screening studies were carried out at micro labs, Arcot, India. The bacteria and fungus species were obtained from National Chemical Laboratory (NCL), Pune, India. Electrochemical measurements were recorded on an Electrochemical analyser CH Instrument version 5.01 and model-600C. A three-electrode system comprising a glassy carbon working electrode, a platinum wire auxiliary electrode and a saturated calomel reference (SCE) electrode was used for voltametric work. The buffer solution (50 mM NaCl-5 mM Tris–HCl) was used as the supporting electrolyte. Agarose gel electrophoresis method was carried out at micro labs, Arcot, India. Water purified using a Milli-Q system was used for all the present studies.

**Synthesis of $[Cu(Phen)(L\text{-}Tyr)(U)]ClO_4$.**

1.6241 g (0.003mM) of $[Cu(Phen)(LTyr)(H_2O)]ClO_4$ complex is dissolved in 15 ml water and mixed with equimolar concentration of urea i.e., 0.1801 g (0.003mM) and stirred for one hour to get the blue coloured precipitate which was then filtered and washed with ethanol and then dried. Yield, 12% (0.071 g); Found (%): C 45.12, H 3.91, N 11.94; Calc. (%): C 45.29, H 3.80, N 12.00.

**DNA Binding activity**

The DNA binding experiments were performed at 30.0 ± 0.2$^o$ C. The DNA concentration per nucleotide was determined by electronic absorption spectroscopy using the known molar extinction coefficient value of 6600 $M^{-1}$ $cm^{-1}$ at 260 nm [38]. Absorption titration experiments of copper(II) complex samples in buffer solution (50 mM NaCl-5 mM Tris–HCl, pH 7.2) were performed by using a fixed complex concentration to which increments of the DNA stock solutions were added. Copper(II) complex–DNA solutions were allowed to incubate for 10 minutes before the absorption study was carried out.

For fluorescence-quenching experiments, DNA was pre-treated with ethidium bromide (EB) for 30 minutes. The copper(II) complex samples were then added to this mixture and their effect on the emission intensity was measured. Samples were excited at 450 nm and emission was observed between 500 nm and 800 nm.

Viscosity measurements were carried out using an Ubbelodhe viscometer maintained at a constant temperature of 30.0±0.1 °C in a thermostatic water-bath. Calf-thymus DNA samples approximately 200 base pairs in average length were prepared by sonicating in order to minimize complexities arising from DNA flexibility [39]. Flow time was measured with a digital stopwatch and each sample was measured three times and an average flow time was calculated. Data were presented as $(\eta/\eta_0)^{1/3}$ versus binding ratio [40], where $\eta$ is the viscosity of CT DNA in the presence of complex, and $\eta_0$ is the viscosity of CT DNA alone.

**DNA cleavage**

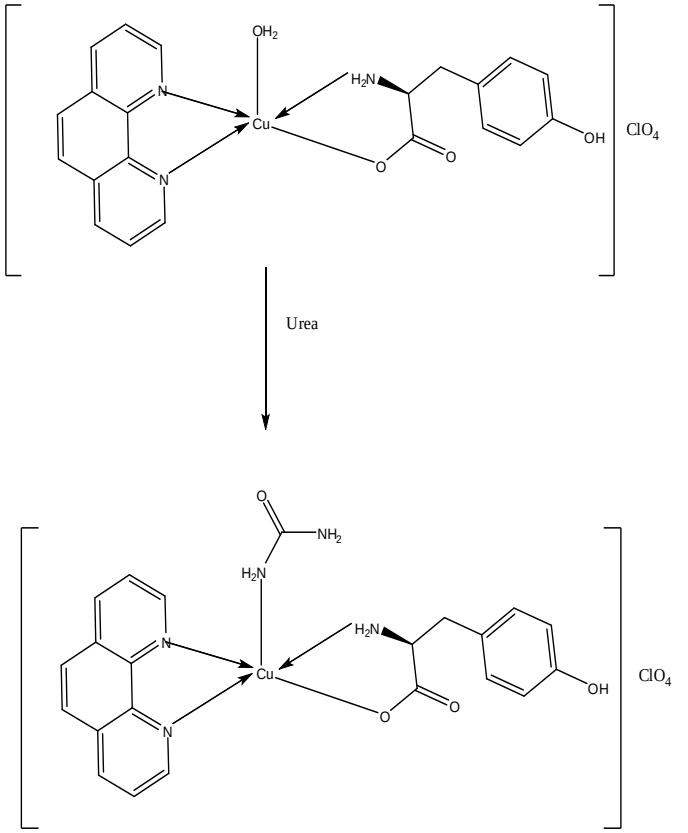
For the gel electrophoresis study, super coiled pBR322 DNA (0.1 µg) was treated with the copper(II) complex in 50 mM Tris–HCl-18 mM NaCl buffer, pH 7.2. The samples were electrophoresed for 3 hours at 50 V on a 0.8 % agarose gel in tris–acetic acid–EDTA buffer. The gel was stained with 0.5 µg of ethidium bromide and photographed under UV light.

**Microbial Assay**

Antimicrobial analysis was followed using standard agar well diffusion method to study the antimicrobial activity of essential oils [41-43]. Each bacterial and fungal isolate was suspended in Brain Heart Infusion (BHI) broth and diluted to approximately $10^5$ colony forming unit (CFU) per mL. They were flood inoculated onto the surface of BHI agar and then dried. 5mm diameter wells were cut from agar using a sterile cork-borer and 30 µL (5µg compound in 500µL DMSO) of the sample solution were poured into the wells. The plates were incubated for 18 hours at 37°C for bacteria and at room temperature for fungi. Antimicrobial activity was evaluated by measuring the zone of inhibition in mm against the test microorganisms. DMSO was used as solvent control. Ciprofloxacin was used as reference antibacterial agent. Ketoconazole was used as a reference antifungal agent. The tests were carried out in triplicates.

**Results And Discussion**

The elemental analysis data were found to be in good agreement with those of the calculated values. The $\Lambda_M$ value of the complex in water is 45 $Ohm^{-1}$ $cm^2$ $mol^{-1}$, which indicated that the complex is a 1:1 electrolyte [44]. The synthetic strategy of the complex is outlined in Scheme 1.




**Scheme-1: Synthetic strategy of Cu (II) complex**

In the IR region the bands around 1627 $cm^{-1}$ and 1386 $cm^{-1}$ can be attributed to the ring stretching frequencies [ν (C=C) and ν (C=N)] of 1,10-phenanthroline. The IR values, δ (C–H) 720$cm^{-1}$ and 626 $cm^{-1}$ observed for phenanthroline are shifted to 848 $cm^{-1}$ and 817 $cm^{-1}$. These shifts can be explained by the fact that each of the two nitrogen atoms of phenanthroline ligands donates a pair of electrons to the central copper metal forming a coordinate covalent bond. The broad band observed around 3404 $cm^{-1}$ is assigned to the N–H stretching of L- tyrosine and the band around 1087 $cm^{-1}$ has been assigned to ν (Cl–O) of perchlorate anion.

The fig 1 shows EPR spectra of the copper(II) complex which exhibits well-defined single isotropic feature near $g_{\perp r}$ (perpendicular) value of 2.12 and $g_{\Pi}$ (parallel) value of 2.08. Such isotropic lines are usually a result of intermolecular spin exchange which broadens the lines. This intermolecular type of spin exchange is caused due to the strong spin coupling which occurs during a coupling of two paramagnetic species.

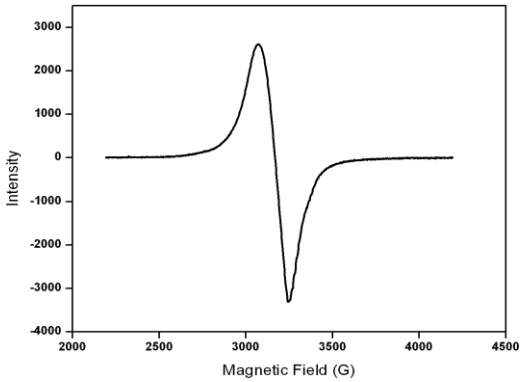



**Fig 1**

**DNA Binding – Electronic absorption study**

Electronic absorption spectroscopy was an effective method to examine the binding mode of DNA with metal complexes [45-47]. In general, hypochromism and red-shift are associated with the binding of the complex to the helix by an intercalative mode involving strong stacking interaction of the aromatic chromophore of the complex between the DNA base pairs.

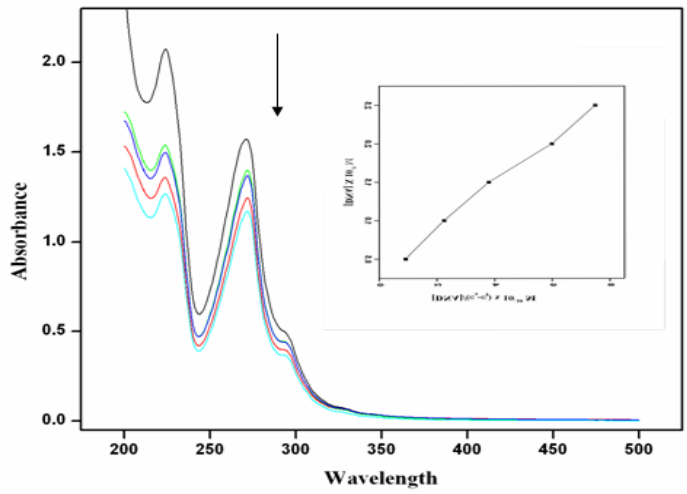



**Fig 2**

Fig. 2 shows the UV absorption spectra of copper(II) complex in the absence and presence of DNA. In the ultraviolet region from 240 to 300 nm, the complex had strong absorption peak at 265 nm, besides a shoulder band around 294 nm. The absorption intensity of the copper(II) complex sample decreased (hypochromism) evidently after the addition of DNA, which indicated the interactions between DNA and the complex. We

have observed minor red shift along with significant hypochromicity for the complex.

The copper(II) complex can bind to the double stranded DNA in different binding modes on the basis of their structure, charge and type of ligands. As DNA double helix possesses many hydrogen bonding sites which are accessible both in the minor and major grooves. The binding propensity of the phenanthroline complex is due to the presence of the extended planar aromatic ring in phenanthroline. The binding constant, $K_{b,}$ was determined by using the following equation [48]:

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

Where [DNA] is the concentration of DNA in base pairs, $\varepsilon_a$, $\varepsilon_f$ and $\varepsilon_b$ correspond to Aobsd/[Cu], the extinction coefficient of the free copper complex and the extinction coefficient of the complex in the fully bound form, respectively, and $K_b$ is the intrinsic binding constant. The ratio of the slope to intercept in the plot of [DNA]/($\varepsilon_a$ - $\varepsilon_f$) versus [DNA] gives the value of $K_b$ and for our copper(II) complex it is 3.47 X $10^{-5}$ M.

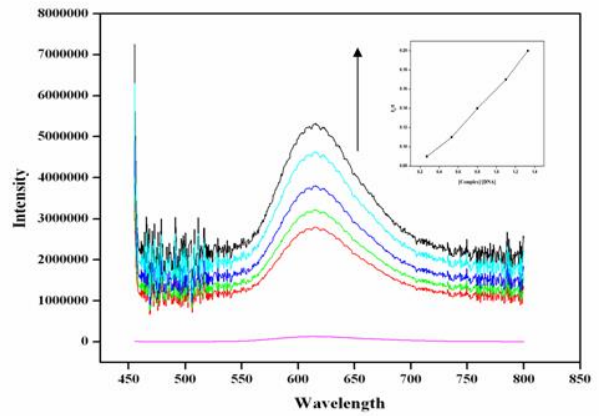



**Fig 3**

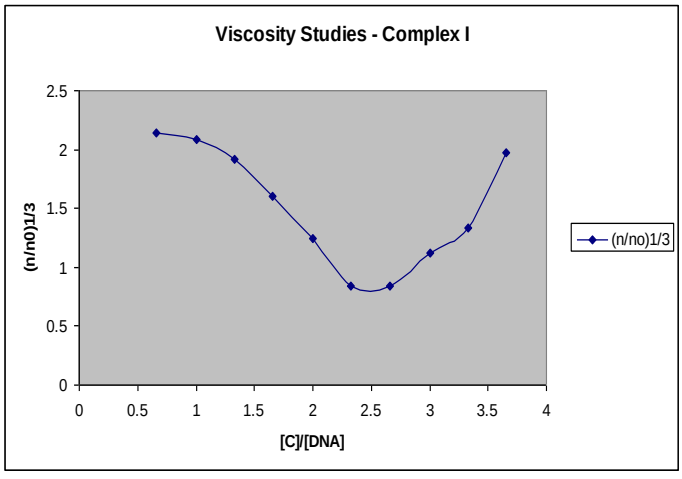



**Fig 4**

The $K_b$ values obtained for our copper(II) complex is very similar than those for any other known simple mononuclear or binuclear copper(II) complexes including complexes such as $[Cu_2phen_2Cl_4]$ ($K_b$=4.75 X $10^4$ $M^{-1}$) [49], $[Cu(Phen)_2Cl_2]$ ($K_b$ = 2.70 X $10^3$ $M^{-1}$ )[50], $[Cu(Phen)(L\text{-}Thr)(H_2O)]ClO_4$ ($K_b$ = 6.35 X $10^3$ $M^{-1}$)[25] and $[Cu(L\text{-}Tyr)(phen)]^+$ ($K_b$ = 4 X $10^3$ $M^{-1}$ ) [37].

**DNA Binding – Emission spectral study**

As the present copper(II) complex is non-emissive, ethidium bromide(EB) binding study was undertaken to gain support for the extent of binding of the complex with DNA. Ethidium bromide (EB) was shown to emit intense fluorescence light in the presence of DNA, due to its strong intercalation between the adjacent DNA base pairs. It was previously reported that the fluorescent light could be quenched by the addition of a second molecule [51]. The quenching extent of the fluorescence of EB binding to DNA is used to determine the extent of binding between the second molecule and DNA. The addition of the complex to DNA pretreated with EB causes appreciable change in the emission intensity. This behaviour can be analysed through the Stern–Volmer equation [52], $Io/I = 1 + K_{sv}r$, where Io and I are the fluorescence intensities in the absence and the presence of complex respectively. $K_{sv}$ is a linear Stern–Volmer quenching constant, r is the ratio of the total concentration of complex to that of DNA. The quenching plot (Fig. 3) illustrates that the quenching of EB bound DNA by the copper(II) complex is in good agreement with the linear Stern–Volmer equation, which also indicates that the complex binds to DNA. In the plot of Io/I versus [Complex]/[DNA], $K_{sv}$ is given by the ratio of the slope to intercept. The $K_{sv}$ value for copper(II) complex thus obtained is 1.79. This suggest that our copper(II) complex binds strongly with DNA, which is also consistent with our absorption spectral result.

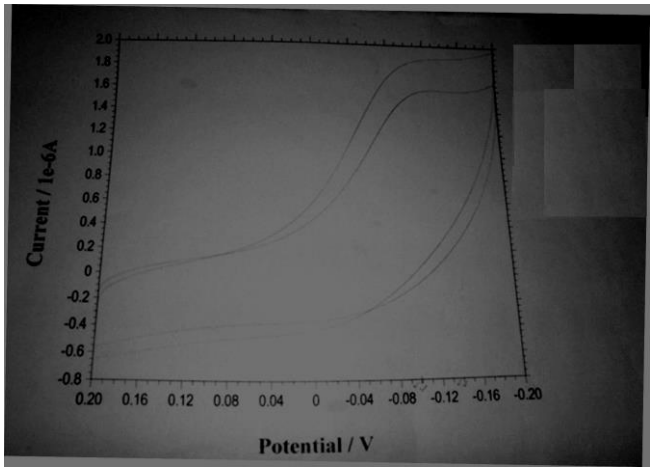



**Fig 5**

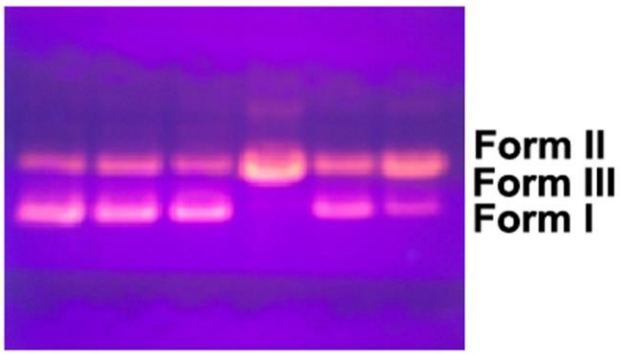



**Fig 6**

**DNA Binding – Viscosity study**

To explore further the interaction between the copper(II) complex and DNA, viscosity measurements were carried out on CT- DNA by varying the concentration of the complex. Spectroscopic data are necessary but insufficient to support an intercalative binding mode. Hydrodynamic measurements which are sensitive to length-increase (i.e. viscosity, sedimentation, etc.) are regarded as the least ambiguous and the most critical tests of binding on solution in the absence of crystallographic structure data [46]. A classical intercalation mode causes a significant increase in the viscosity of DNA solution due to the increase in separation of the base pairs at intercalation sites and hence to an increase in overall DNA contours length. A partial intercalation of ligand would reduce the DNA viscosity [53].

The effects of the copper(II) complex on the viscosity of CT DNA solution are given in figure 4. The plot shows that the complex had a reverse effect on the relative viscosity of the CT DNA. With the addition of the complex, the relative viscosity of DNA changed. Since the change is far less than that observed for an intercalator such as EB, this observation leads us to support the above spectral studies which suggest that the complex interact with DNA via partial intercalation between DNA base pairs, which is similar to the interaction of $[Cu(phen)_2]^{2+}$ with DNA [54-55].

**DNA Binding – Cyclic voltametric study**

Cyclic voltametric technique was employed to study the interaction of the present redox active metal complex with DNA with a view to further exploring the DNA binding modes assessed from the above spectral and viscometric studies. Typical cyclic voltametry (CV) behaviours of our copper(II) in the absence and presence of CT-DNA are shown in Fig 5. The cyclic voltammogram of copper(II) in the absence of DNA featured reduction of copper(II) to the copper(I) form at cathodic potential, $E_{pc}$ of -0.10 V and anodic peak potential, $E_{pa}$ of -0.14 V. The separation of the anodic and cathodic peak potentials, $E_p$ = -0.040 V. The formal potential $E_{1/2}$ was taken as the average of $E_{pc}$ and $E_{pa}$ is -0.14 V in the absence of DNA. The presence of DNA in the solution at the same concentration of copper(II) causes a considerable decrease in the voltametric current coupled with a slight shift in the potential ($E_{1/2}$ = -0.112 V). The drop of the voltametric currents in the presence of CT-DNA can be attributed to diffusion of the metal complex bound to the large, slowly diffusing DNA molecule. Obviously, $E_{1/2}$ undergoes a positive shift after forming aggregation with DNA, suggesting that the copper complex binds to DNA mainly by intercalation binding mode [56] and this result also confirms the results obtained from viscosity and absorption spectrum studies again.

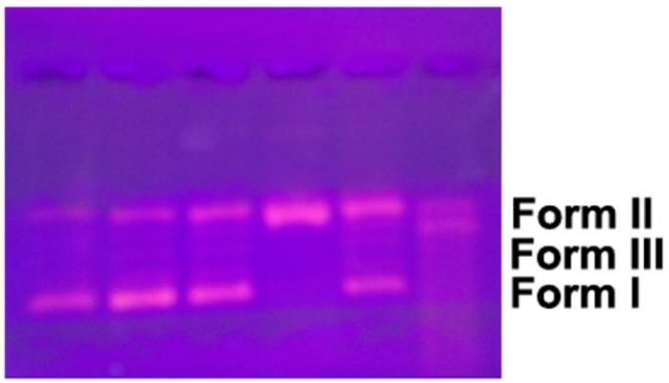



**Fig 7**

**DNA Cleavage - Electrophoresis**

The characterisation of DNA recognition by transition metal complex has been aided by the DNA cleavage chemistry that is associated with redox-active or photo activated metal complexes [57]. DNA cleavage is controlled by relaxation of super coiled circular form of pBR322 DNA into nicked circular form and linear form. When circular plasmid DNA is subjected to electrophoresis study, the fastest migration will be observed for the super coiled form (Form I). If one strand is cleaved, the super coils will relax to produce a slower-moving open circular form (Form II). If both strands are cleaved, a linear form (Form III) will be generated which migrates in between.

DNA cleavage was analysed by monitoring the conversion of super coiled DNA (Form I) to nicked DNA (Form II) and linear DNA (Form III) in aerobic condition. Interestingly, we have found that this copper complex can cleave the super coiled DNA to nicked and linear DNA at the same time.

As shown in Fig. 6, with the increase of the complex concentration, the intensity of the circular supercoiled DNA (Form I) band was found to decrease, while that of nicked (Form II) and linear DNA (Form III) bands increase apparently. When the complex concentration was up to 20 μM (lane 4), the circular supercoiled DNA (Form I) band disappeared completely. When it is more than 40 μM (lane 6), the circular supercoiled DNA (Form I) band becomes extremely faint.

In order to establish the reactive species responsible for the cleavage of the plasmid DNA, we carried out the experiment in the presence of ascorbic acid as reducing agent (Fig. 7). Compared with the control experiments using only the copper (II) complex or ascorbic acid (lane 2, lane 3 and lane 5), the experiment using both copper (II) complex and the same concentration of ascorbic acid (lane 4 and lane 6) showed that the supercoiled DNA (Form I) apparently convert to nicked (Form II) and linear DNA (Form III). Although the ascorbic acid concentration in lane 5 was fivefold of that in lane 3, there is little difference between these two bands. When we add the same concentration of the copper (II) complex to them, an obvious difference occurred. Compared with lane 4, the supercoiled DNA (Form I) completely disappeared and the linear DNA (form III) apparently appeared in lane 6. These results are similar to that observed for some Cu-salen complexes as chemical nucleases [58,10]. It is likely the generation of hydroxyl radical and/or activated oxygen mediated by the copper complex results in DNA cleavage. Further studies are being pursued to clarify the cleavage mechanism.

**Antimicrobial Screening of $[Cu(Phen)(LTyr)(U)]ClO_4$ Complex**

Table 1 shows that the copper(II) complex exhibits significant activity against the gram positive and gram negative bacteria. In our biological experiments using copper complex, we observed considerable antibacterial activity against gram positive bacteria Micrococcus luteus and Bacillus cereus and gram negative bacteria E. coli and Klebsiella pneumoniae. The copper complex has shown a good activity against gram positive than against gram negative bacteria. The complex also show considerable activity against fungi. It may be concluded that our copper(II) complex inhibits the growth of bacteria and fungi to a good extent.

**Conclusion**

In this paper, we have described new copper(II) complex. Further characterisation of the complex was achieved through physico-chemical and spectroscopic methods. The effectiveness of the binding of complex is being confirmed by means of hypochromism in the electronic spectral studies and change in intensity of emission in the case of emission spectral studies. Besides, the effectiveness of binding is also confirmed by the viscometric and cyclic voltametric studies. This shows that the complex interacts with DNA base pairs effectively. The super-coiled DNA is being cleaved in the electrophoresis by the complex which confirms that the complex is having the ability to act as a potent DNA cleavaging agent. The copper(II) complex exhibits good antimicrobial activity.

**Acknowledgements**

We are grateful to IIT Madras, Chennai, for providing instrumental facilities such as IR spectroscopy, UV-Visible Absorption spectroscopy and Emission spectroscopy.

**Table 1: Antimicrobial activities of $[Cu(Phen)(L\text{-}Tyr)(U)]ClO_4$ complex**

| Complex | Diameter of zone inhibition (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $[Cu(Phen)(L\text{-}Tyr)(U)]ClO_4$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | - | 30 | 20 | 8 | 14 | 5 | 17 | 20 | - |
| | | | | | | | | | |

1.Staphylococcus aureus; 2.Micrococcus luteus; 3.Bacillus cereus 4.Escherichia coli;
5.Klebsiella pneumoniae; 6.Pseudomonas aeruginosa; 7.Aspergillus niger; 8.Aspergillus flavus; 9.Candida albicans; Solvent, DMSO (showed nil effect against the microorganisms under test).
Ciprofloxacin was used as reference antibacterial agent. Ketoconazole was used as a reference antifungal agent.

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