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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, characterization, antibacterial activity and DNA interaction studies of drug-based mixed ligand copper(II) complexes with terpyridines

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Abstract A series of ternary copper(II) complexes have been derived using ciprofloxacin and four terpyridine derivatives. Complexes were characterized using metal estimation, IR spectroscopy, FAB-mass spectrometry and reflectance spectra. Compounds were screened for their in vitro antimicrobial activity against gram(+ve) and gram(−ve) bacterial species. Binding behaviour of the complexes towards DNA were determined using UV–Vis absorption titration, viscometric and DNA thermal denaturation experiment, where as the cleavage efficacy of the complexes towards pUC19 DNA was determined by gel electrophoresis in the presence of ethidium bromide. The catalytic activity of the copper(II) complexes towards the superoxide anion ($O_2^{\bullet -}$) dismutation was assayed by their ability to inhibit the reduction of nitroblue tetrazolium.

Keywords Drug-based complexes of Cu(II) · FAB-MS · Thermal denaturation · IC_{50}

Introduction

The study of the interaction between DNA with inorganic compounds is of primary scientific interest, since DNA is the main target molecule for most anticancer and antibacterial therapies according to cell biology. The development of metal complexes as drugs has been facilitated by the extensive knowledge of the inorganic chemists in the coordination and redox properties of metal ions (Sherman and Lippard 1987). Metal centers being positively charged are favored to bind with negatively charged molecules such as DNA. The constituents of proteins and nucleic acids offer adequate sites for metal binding in a specific way and it can be related to the pharmacological properties of the complexes. As a result, coordination compounds relevantly affect cellular processes such as cell division and gene expression, as well as others like carcinogenicity and antitumor chemistry (Singh *et al.*, 2005). The factors that lead the binding modes show that the most important factor is the molecular shape (Hong *et al.*, 2004).

The interaction of DNA with transition metal complexes has got intensive attention in the last few years in order to develop new novel nonradioactive probes of DNA structure (Erkkila *et al.*, 1999), new therapeutic agents that cleave DNA (Ren and Chaires 1999; Barton and Raphael 1984; Chaires *et al.*, 1982) and DNA-mediated electron transfer reactions (Barton and Raphael 1985). These complexes give an opportunity to discover the effects of central metal atom, ligands and coordination geometries on the binding event. As to the different ligands, it is possible to change the mode of interaction of the complex with nucleic acids that makes easy individual applications (Shields and Barton 1995; Krotz *et al.*, 1993; Sitlani *et al.*, 1992). The application of octahedral complexes has allowed the targeting of specific DNA sites by matching the shape, symmetry and functionality of the metal complex to that of the DNA target (Selvi *et al.*, 2005). Because of the unusual binding properties and general photo-activity, these coordination compounds will be available candidates as DNA secondary structure probe, photocleavers and antitumor drugs (Chan and Wong 1995; Liang *et al.*, 2004; Wang *et al.*, 2004).

The role of copper compounds as pharmaceutical drugs in the treatment of numerous chronic diseases is well-established. Numerous copper compounds are able to act as antioxidant (Naso *et al.*, 2009), antimicrobial (Klayman *et al.*, 1983), antiparasitic (Agrawal and Santorelli 1978),

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anti-inflammatory, anticonvulsant (Jones *et al.*, 1965) and antitumoral agents (Anthroline *et al.*, 1977; Urquiola *et al.*, 2008). Copper complexes with ligands containing nitrogenated aromatic rings have deserved a great interest since the complex of terpyridines proved its ability to break DNA chains (Zelenko *et al.*, 1998, Morehouse *et al.*, 2000, Bhirud and Srivastava 1991). The chelation of copper(II) with ligand reduces polarity of metal ion by overlap of the ligand orbital and partial sharing of the positive charge of the metal ion with the donor groups. Increase in delocalization of π-electrons over the whole ligand enhances the penetration of complexes into lipid membranes and blocking of the metal binding sites in the enzymes of microorganisms (Chohan *et al.*, 2005). The complexes may also disturb the respiration process of the cell and thus block the synthesis of proteins, which restricts further growth of the organism and thus results in the overall gain in the biological potency.

Herein, we represent the DNA interaction and biological properties of Cu(II) complexes with the second-generation quinolone ciprofloxacin and tridentate ligands.

Experiment

Reagent

All the reagents, chemicals and solvents used were of analytical grade; double distilled water was used throughout. Ciprofloxacin hydrochloride was generously supplied on demand by Bayer AG (Wuppertal, Germany). Cupric chloride dihydrate was purchased from E. Merck Ltd. (India). Pyridine, 2-acetyl pyridine, *p*-benzyloxy benzaldehyde, *m*-benzyloxy benzaldehyde, *p*-methyl benzaldehyde and *p*-methoxy benzaldehyde were purchased from Loba Chemie PVT. LTD. (India). Ethidium bromide and Luria Broth were purchased from Himedia (India). Nicotinamide adenine dinucleotide reduced (NADH), NBT and phenazin methosulphate (PMS) were purchased from Loba Chemie PVT. LTD. (India). Acetic acid and Na_2H_2EDTA were purchased from Sd Fine Chemicals (India). CT DNA was purchased from Sigma Chemical (India).

Physical measurement

Elemental analysis (carbon, hydrogen and nitrogen) were performed on a model Perkin-Elmer 240 elemental analyzer. The reflectance spectra were recorded on a UV-160A UV–Vis spectrophotometer, Shimadzu (Japan). Infrared spectra were recorded on a FT-IR Shimadzu spectrophotometer as KBr pellets in the range 4000–400 cm^{-1}. The ^1H NMR and ^{13}C NMR were recorded on a Bruker Avance (400 MHz). FAB-mass spectra were recorded on Jeol SX 102/Da–600 mass spectrometer/Data system using Argon/Xenon (6 kV, 10 mA) as the FAB gas, Massachusetts (USA). The accelerating voltage was 10 kV and spectra were recorded at room temperature. The metal contents of the complexes were analyzed by Na_2H_2EDTA titration (Vogel 1978) after decomposing the organic matter with a mixture of $HClO_4$, H_2SO_4 and HNO_3 (1:1.5:2.5). At room temperature magnetic measurement for the complexes was made using Gouy's method on Citizen Balance, (India). The Gouy tube was calibrated using mercury(II)tetrathiocyanatocobaltate(II) as the calibrant ($\chi_g = 16.44 \times 10^{-6}$ cgs units at 20°C). Photo quantization of the gel after electrophoresis was done using AlphaDigiDoc™ RT. Version V.4.0.0 PC–Image software, California (USA).

Synthesis of ligands

4′-(4-Benzyloxyphenyl)-2,2′:6′,2″-terpyridine [L^1]

An aqueous solution of NaOH (10 mL, 1.5 mmol) was added to a stirred solution of two moles of 2-acetylpyridine (l0 mmol) and one mole of 4-benzyloxybezaldehyde (10 mmol) in ethanol (20 mL) than added ammonia solution (10 mL). Stirring the solution for 4 h at room temperature, the yellow precipitate was isolated by filtration and recrystallized from methanol. Yield: 32.4% m.p.: 160–162°C, Anal. Calc. for $C_{28}H_{21}N_3O$ (415.49 g/mol): Calc. (%): C, 80.94; H, 5.09; N, 10.11 Found (%): C, 81.17; H, 4.82; N, 10.37. ^1H NMR ($CDCl_3$, 400 MHz) δ/ppm: 8.79, (s, 2H, $H_{3',5'}$); 8.76, (dd, 2H, $H_{3,3''}$); 8.69, (d, 2H, $H_{6,6''}$); 7.91, (dt, 2H, $H_{4,4''}$); 7.55, (d, 2H, $H_{Ph2,6}$); 7.48–7.37, (complex, 7H); 7.15, (d, 2H, $H_{Ph3,5}$); 5.17, (s, 2H, CH_2), ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm: 160.58, (C_{Ph4}); 156.15, ($C_{2',6'}$); 155.57, ($C_{2,2''}$); 149.8, ($C_{4'}$); 148.94, ($C_{6,6''}$); 137.27, (C_{Bz1}); 137.13, ($C_{4,4''}$); 130.75, (C_{Ph1}); 128.86, ($C_{Bz3,5}$); 128.64, ($C_{Ph2,6}$); 127.58, (C_{Bz4}); 127.13, ($C_{Bz2,6}$); 123.73, ($C_{5,5''}$); 121.58, ($C_{3,3''}$); 118.12, ($C_{3',5'}$); 114.64, ($C_{Ph3,5}$); 70.7, (CH_2).

4′-(3-Benzyloxyphenyl)-2,2′:6′,2″-terpyridine [L^2]

Similar procedure was followed by taking 3-benzyloxybezaldehyde. Yield: 30% m.p.:146–148°C, Anal. Calc. for: $C_{28}H_{21}N_3O$ (415.49 g/mol): Calc. (%): C, 80.94; H, 5.09; N, 10.11 Found (%): C, 80.76; H, 5.28; N, 10.35. ^1H NMR ($CDCl_3$, 400 MHz) δ/ppm: 8.76, (d, 2H, $H_{3,3''}$), 8.72, (s, 2H, $H_{3',5'}$), 8.67, (d, 2H, $H_{6,6''}$), 7.49–7.34, (complex, 11H), 7.28, (d, 1H, H_{Ph6}), 7.16, (d, 1H, H_{Ph4}), 5.17, (s, 2H, CH_2), ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm: 157.63, (C_{Ph3}), 156.13, ($C_{2',6'}$), 155.27, ($C_{2,2''}$), 151.7, ($C_{4'}$), 149.1, ($C_{6,6''}$), 148.28, (C_{Ph1}), 136.86, ($C_{4,4''}$), 136.75, (C_{Bz1}), 130.26, (C_{Ph5}), 129.03, ($C_{Bz3,5}$), 127.56, (C_{Bz4}), 127.08, ($C_{Bz2,6}$), 123.7, ($C_{5,5''}$), 121.34, ($C_{3,3''}$), 119.6, (C_{Ph6}), 118.06, ($C_{3',5'}$), 114.76, (C_{Ph2}), 113.17, (C_{Ph4}), 70.75, (CH_2).

4′-(4-Methoxyphenyl)-2,2′:6′,2″-terpyridine [L^3]

Similar procedure was followed by taking 4-methoxybezaldehyde. Yield: 34.31% m.p.: 158°C, Anal. Calc. for: $C_{22}H_{17}N_3O$ (339.39 g/mol): Calc. (%): C, 77.86; H, 5.05; N, 12.38 Found (%): C, 77.65; H, 4.78; N, 12.53. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: 8.795–8.76, (complex, 4H, $H_{3,3',5',3''}$), 8.71, (d, 2H, $H_{6,6''}$), 7.940–7.913, (complex, 4H), 7.396, (d, 2H, $H_{ph3,5}$), 7.055, (dd, 2H, $H_{5,5''}$), 3.90, (s, 3H, OCH_3), ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm: 160.57, (C_{ph4}), 156.15, ($C_{2',6'}$), 155.59, ($C_{2,2''}$), 149.81, ($C_{4'}$), 148.88, ($C_{6,6''}$), 137.08, ($C_{4,4''}$), 130.63, (C_{ph1}), 128.55, ($C_{ph2,6}$), 123.81, ($C_{5,5''}$), 121.48, ($C_{3,3''}$), 118.41, ($C_{3',5'}$), 114.35, ($C_{ph3,5}$), 55.38, (OCH_3).

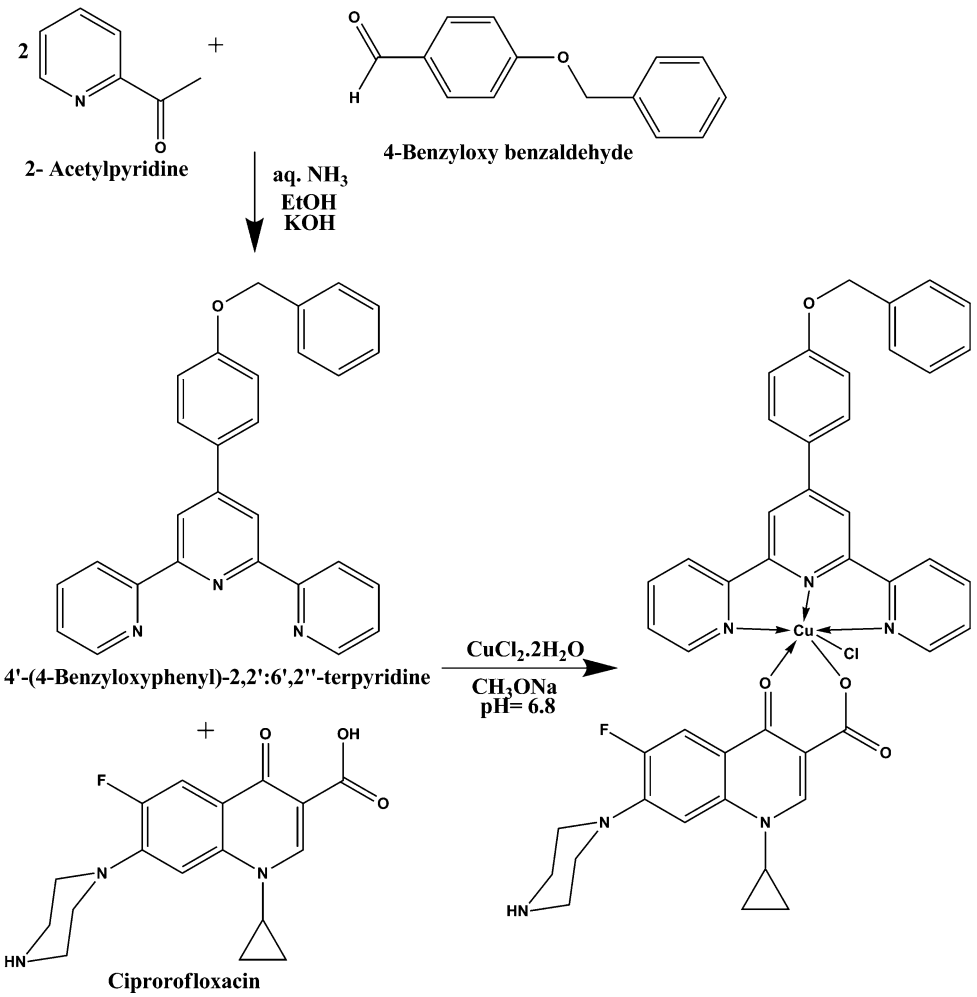
4′-(4-Tolyl)-2,2′:6′,2″-terpyridine [L^4]

Similar procedure was followed by taking 4-methylbezaldehyde. Yield: 1.26 g, 39% m.p.:151–152°C, Anal. Calc. for $C_{22}H_{17}N_3$ (323.39 g/mol): Calc. (%): C, 81.71; H, 5.30; N, 12.99 Found (%): C, 81.96; H, 5.13; N, 13.12. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: 8.798, (s, 2H, $H_{3',5'}$), 8.776, (d, 2H, $H_{3,3''}$), 8.721, (d, 2H, $H_{6,6''}$), 7.924, (dd, 2H, $H_{4,4''}$), 7.869, (d, 2H, $H_{ph2,6}$), 7.404, (dd, 2H, $H_{5,5''}$), 7.344, (d, 2H, $H_{ph3,5}$), 2.453, (s, 3H, CH_3), ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm: 156.26, ($C_{2',6'}$), 155.75, ($C_{2,2''}$), 150.18, ($C_{4'}$), 148.98, ($C_{6,6''}$), 139.1, (C_{ph1}), 136.92, ($C_{4,4''}$), 135.45, (C_{ph4}), 129.66, ($C_{ph3,5}$), 127.15, ($C_{ph2,6}$), 123.75, ($C_{5,5''}$), 121.42, ($C_{3,3''}$), 118.7, ($C_{3',5'}$), 21.25, (CH_3).

Synthesis of complexes

*[Cu(cpf)(L^1)Cl][**I**]*

Cupric chloride dihydrate (1.5 mmol), ciprofloxacin (1.5 mmol) and 4′-(4-benzyloxyphenyl)-2,2′:6′,2″-terpyridine (1.5 mmol) were added in methanol (20 mL) and pH was adjusted to 6.8 by 10% sodium methoxide solution. The resulting solution was refluxed for 2 h on a water bath, followed by concentrating it to half of its volume. A fine amorphous product of green colour obtained which was washed with ether/hexane and dried in vacuum desiccators. The proposed reaction is shown in Scheme 1.



Scheme 1 Reaction scheme for formation of complex [Cu(cpf)(L^1)Cl]

Yield: 62.1%, m.p.: 228°C, μ_{eff}: 1.86 B.M. Anal. Calc. for: $C_{45}H_{38}ClCuFN_6O_4$ (844.82): C, 63.98; H, 4.53; N, 9.95; Cu, 7.52. Found: C, 63.84; H, 4.42; N, 10.06; Cu, 7.44%.

[Cu(cpf)(L^2)Cl][**II**]*

It was prepared using 4′-(3-benzyloxyphenyl)-2,2′:6′,2″-terpyridine (1.5 mmol). Yield: 65.6%, m.p.: 218°C, μ_{eff}: 1.93 B.M. Anal. Calc. for: $C_{45}H_{38}ClCuFN_6O_4$ (844.82): C, 63.98; H, 4.53; N, 9.95; Cu, 7.52. Found: C, 64.10; H, 4.44; N, 10.08; Cu, 7.65%.

[Cu(cpf)(L^3)Cl][**III**]*

It was prepared using 4′-(4-methoxyphenyl)-2,2′:6′,2″-terpyridine (1.5 mmol). Yield: 65.6%, m.p.: 230°C, μ_{eff}: 1.88 B.M. Anal. Calc. for: $C_{39}H_{34}ClCuFN_6O_4$ (768.72): C, 60.93; H, 4.46; N, 10.93; Cu, 8.27. Found: C, 61.02; H, 4.59; N, 10.80; Cu, 8.14%.

[Cu(cpf)(L^4)Cl][**IV**]*

It was prepared using 4′-(4-tolyl)-2,2′:6′,2″-terpyridine (1.5 mmol). Yield: 61.0%, m.p.: 236°C, μ_{eff}: 1.82 B.M. Anal. Calc. for: $C_{39}H_{34}ClCuFN_6O_3$ (752.72): C, 62.23; H, 4.55; N, 11.16; Cu, 8.44. Found: C, 62.34; H, 4.66; N, 11.01; Cu, 8.38%.

Antibacterial activity

Here in, we performed antimicrobial activity by two fold serial dilution technique in liquid media containing 0.5–3,400 μM variation of test compound concentration (Alexious *et al.*, 2003), against two gram(+ve) organisms (*Bacillus subtilis* and *Staphylococcus aureus*) and three gram(−ve) organisms (*Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens*). A preculture of bacteria were grown in LB overnight at the optimal temperature (37°C) for each species. Minimum inhibition concentration of complexes, ciprofloxacin and metal salt was determined by their influence on growth of the microorganism at various concentrations. The lowest concentration that inhibited bacterial growth was considered as the MIC value. All the equipment and culture media used were sterile.

In addition, minimum inhibitory concentration in terms of colony forming unit was carried out by colony count. All the prepared complexes were screened for their activity against three gram(−ve) and two gram(+ve) bacteria. The inoculums were prepared by diluting an overnight culture, grown in LB than culture was diluted 10^6 times. Bacteria were exposed to concentrations of 0.25–1.75 μg/mL of compounds. The final volume of bacterial culture was 1 mL and cultures were incubated at 37°C for 2 h. The 100 μL bacterial culture from above was taken and spread over prepared agar plate. These were incubated for 24 h at 37°C and the visual colonies were calculated in order to check bactericidal activity of metal complexes, yielding 30–250 colonies.

DNA interaction activity

Hydrodynamic measurements

Hydrodynamic volume change was measured using Ubbelohde viscometer immersed in a thermostatic bath maintained at 27 (±0.1)°C. Flow times were measured with a digital stopwatch, each sample was measured three times, and an average flow time was calculated. Mixing of test compound and DNA was done by bubbling air to it. Data are presented as $(\eta/\eta_0)^{1/3}$ versus [complex]/[DNA], where η is the viscosity of DNA in the presence of complex and η_0 is the viscosity of DNA alone. Viscosity values were calculated from the observed flow time of DNA–containing solutions (t) corrected for that of the buffer alone (t_0), $\eta = (t - t_0)$ (Basili *et al.*, 2007).

Absorption titration

The ability of Cu(II) complexes to bind DNA was measured via DNA-mediated hypochromicity and hyperchromicity of the ligand or Cu(II) complex UV–Vis absorbance spectra (Reichmann *et al.*, 1954; Trommel and Marzilli 2001; Mudasir and Inoue 1999; Jin and Yang 1997). A solution of CT DNA in 5 mM Tris-HCl buffer (pH 7.2) gave A_{260}/A_{280} ratio of 1.9. UV absorbance indicated that DNA was sufficiently free of protein. So, no further effort was made on purifying the commercially obtained DNA. The concentrated stock solution of CT DNA was prepared, and the concentration of DNA in nucleotide phosphate was determined by UV absorbance at 260 nm. The molar absorption coefficient of CT DNA was taken as 6600 mol^{-1} cm^{-1} L^{-1} (Cohen and Eisenberg 1969). Spectroscopic titrations were carried out at room temperature to determine the binding affinity between DNA and the metal complex. The solutions of varying concentration of DNA (50–150 μM) and metal complexes (15 μM) are prepared along with blank sample of buffered DNA solution. After the solutions had been mixed for 10 min, the absorption spectra were recorded. The titration processes were repeated until there was no change in the spectra for four titrations at least, indicating that binding saturation had been achieved. The intrinsic binding constant, K_b was determined using equation below (Wolfe *et al.*, 1987).

$$[\mathrm{DNA}]/(\varepsilon_a - \varepsilon_f) = [\mathrm{DNA}]/(\varepsilon_b - \varepsilon_f) + 1/K_\mathrm{b}(\varepsilon_b - \varepsilon_f)$$

where, [DNA] is the concentration of CT-DNA in terms of nucleotide phosphate [NP], the apparent absorption coefficient ε_f, ε_a and ε_b correspond to the extinction coefficient of the free complex, the extinction coefficient for each addition of CT-DNA to the complex and the extinction coefficient for the complex in the fully bound form, respectively, and K_b is the ratio of the slope to the y intercept.

DNA thermal denaturation

DNA melting experiments were carried out by monitoring the absorption intensity of CT DNA (100 μM) at 260 nm in the temperature range from 35 to 90°C with temperature increments of 0.5°C, both in the absence and presence of the copper(II) complex (20 μM). Measurements were performed with an Agilent 8453 UV–Vis spectrophotometer. The melting temperature (T_m) of DNA was determined as the midpoint of the optically detected transition curves. The ΔT_m value was defined as the difference between T_m of the free DNA and T_m of the bound DNA.

DNA cleavage study

Gel electrophoresis of pUC19 DNA was carried out in TAE buffer (0.04 M Tris–Acetate, pH 8, 0.001 M EDTA). 15 μL reaction mixture containing plasmid DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) 300 μg/mL and 200 μM complex. Reactions were allowed to proceed for 3 h at 37°C. The reactions were satiated by addition of 3 μL loading buffer (0.25% bromophenol blue, 40% sucrose, 0.25% xylene cyanole and 200 mM EDTA). The aliquots were loaded directly on to 1% agarose gel and electrophoresed at 50 V in 1× TAE buffer. Gel was stained with 0.5 μg/mL ethidium bromide and was photographed on a UV illuminator. The percentage of each form of DNA was quantities. The degree of DNA cleavage activity was expressed in terms of the percentage of cleavage of the SC-DNA according to the following equation (Yang *et al.*, 2000)

$$\%\ \text{DNA clevage} = \frac{(\%\ \text{of SC} - \text{DNA})\ \text{control} - (\%\ \text{of SC} - \text{DNA})\ \text{sample}}{(\%\ \text{of SC} - \text{DNA})\ \text{control}} \times 100$$

Determination of SOD-like activity

The photoreduction of nitroblue tetrazolium (NBT) was taken as indicator of $O_2^{\bullet -}$ production (Ponti *et al.*, 1978). Solutions containing the complex (0.5–3.0 μM), NBT (75 μM), NADH (79 μM) and PMS (30 μM) in phosphate buffer at pH 7.8, were used for assaying SOD-like activity of the investigated complexes. The constant flux of superoxide anion was photogenerated by this system and spectrophotometrically detected by monitoring the formation of monoformazan which absorbs at 560 nm. The NBT reduction rate was measured in the presence and the absence of the investigated complex for 3 min. The IC_{50} (the concentration of SOD or the complex which causes 50% inhibition of NBT reduction) of the complex was calculated.

Result and discussion

Reflectance spectra and magnetism

The magnetic moments of the copper(II) complexes lie in the 1.82–1.93 B.M. range. The observed magnetic moments of all the complexes correspond to typical high-spin octahedral complexes. However, the values are slightly higher than the expected spin-only values due to spin–orbit-coupling contribution (Cotton and Wilkinson, 1988). Copper(II) complexes with octahedral geometry are mock to demonstrate the absorption spectra in visible region and show one broad band at about 660 nm (Figgis and Lewis, 1960). Herein our case, the broad band obtained at $\lambda_{max} = 645$ nm points toward distorted octahedral geometry.

Mass spectrometry

FAB-mass spectra of all complexes were obtained using *m*-nitro benzyl alcohol as matrix. A mass spectrum of complex [Cu(cpf)(L^1)Cl] **[1]** is shown in Fig. 1. Peaks at 136, 137, 154, 289 and 307 *m/z* values are of matrix. Peaks at 843 and 845 *m/z* are assigned to (M) and (M + 2) molecular ion peak of complex **1**. It can be clearly seen in Fig. 1 that the peak at 513,515; and 415,417 *m/z* for the fragments of complex having single Cl atom. Loss of chlorine atom gave a fragment ion peak at $m/z = 808$, which also confirm that chlorine atom attached to metal ion with covalent bond. The other fragment corresponds to peaks at 691, 478, 428 and 331 *m/z* value. The corresponding mass fragments are shown in supplementary materials.

IR spectra

Ring mode vibrations of the ligands are affected by coordination to Cu(II), corresponding to shift in energy for bands in the 1700–500 cm^{-1} region of IR spectra. In the IR spectra of complexes, these bands are also slightly sensitive to the substituents of ligand. The prominent IR spectral data of the complexes are shown in Table 1.

The complete elimination of broad band at 3010 cm^{-1} due to –OH stretching of ciprofloxacin carboxylic group

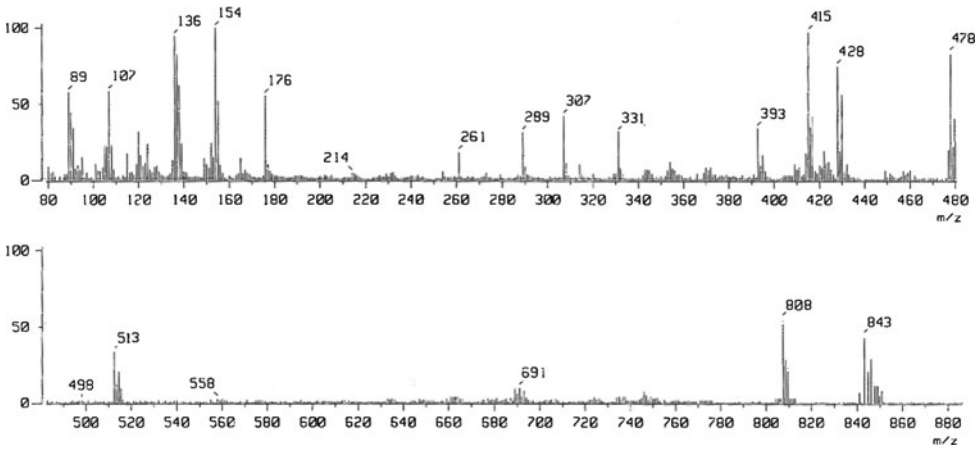


Fig. 1 FAB-mass spectrum of complex **1**, that is [Cu(cpf)(L^1)Cl], obtained using *m*-nitro benzyl alcohol

Table 1 IR spectra data (cm^{-1})

Compounds	ν(C=O) pyridone	$\nu(COO)_{as}$	$\nu(COO)_s$	$\Delta\nu$	ν(M–N)	ν(M–O)
Ciprofloxacin	1708	1624	1340	284	–	–
[Cu(cpf)(L^1)Cl] (**I**)	1617	1564	1363	201	537	505
[Cu(cpf)(L^2)Cl] (**II**)	1620	1574	1372	202	542	503
[Cu(cpf)(L^3)Cl] (**III**)	1622	1582	1376	206	539	510
[Cu(cpf)(L^4)Cl] (**IV**)	1621	1586	1384	202	540	512

can be ascribed to presence of covalent bond between copper and $–COO^-$ of ciprofloxacin. The shifting of ν(C=O) stretching vibration band from 1708 to 1617–1622 cm^{-1}, confirms ciprofloxacin as coordinating unit in complexes and the carbonyl oxygen of pyridine ring as coordination site (Chohan *et al.*, 2005). The characteristic band of carbonyl group, i.e. ν(COO) asymmetric and symmetric vibrations, observed as strong absorption band at 1624 and 1340 cm^{-1} in ciprofloxacin, also shift to 1564–1586 and 1363–1384 cm^{-1}, respectively, in metal complexes. The difference $\Delta\nu = \nu_{as}(COO) - \nu_s(COO)$ is greater than 200 cm^{-1}, suggests the monodentate coordination behaviour of carboxylato group (Chohan *et al.*, 2005; Nakamoto, 1986) of the ciprofloxacin. The band at 537–542 cm^{-1} are due to the N–Cu stretch and another at 503–512 cm^{-1} are due to the O–Cu stretch (Freedman, 1961). In the investigated ligands, the ν(C=N) band of terpyridine derivatives is observed at about 1584 cm^{-1}. This band shift to higher frequency at $\sim$1626 cm^{-1} in complexes suggests the tridentate N–N coordination of the ligands (Patel, 1974).

Antibacterial activity

In vitro antimicrobial screening

Synthesized complexes, ciprofloxacin and metal salt were checked for their in vitro antibacterial activity in terms of minimum inhibitory concentration (MIC) against bacterial strain such as *E. coli*, *P. aeruginosa*, *S. marcescens*, *S. aureus* and *B. subtilis.* Table 2 comprises the antibacterial activity data from which following conclusion can be drawn:

1. *S. aureus*: All the complexes are more bacteriostatic compare to ciprofloxacin.
2. *B. subtilis*: Except complexes **IV** all are more potent than ciprofloxacin.
3. *S. marcescens*: All the complexes are more bacteriostatic compare to ciprofloxacin.
4. *P. aeruginosa*: Complexes **I**, **II** and **III** exhibit good activity compare to ciprofloxacin.
5. *E. coli*: Complexes **I**, **II** and **III** has rather more potency than ciprofloxacin.

From the data of Table 2, one can conclude that the complex **I**, **II** and **III** is more active. The compounds show quite similar antibacterial activity than the Cu(II) complexes synthesized and reported by Eleni *et al.* against *E. coli*, *P. aeruginosa* and *S. aureus* (Efthimiadou *et al.*, 2007a, b).

The good antimicrobial activity is observed may be studied under following five principles (Dendrinou-Samara *et al.*, 2001; Russell 1991).

I The chelate effect, i.e. ligands that are bound to metal ions in a bidentate fashion, such as the quinolones and phenanthroline, bipyridine or bipyridylamine show

Table 2 MIC data of the compounds (μM)

	S.aureus	*B. subtilis*	*S. marcescens*	*P. aeruginosa*	*E. coli*
$CuCl_2{\cdot}2H_2O$	2698.00	2815.00	2756.00	2404.00	3402.00
Ciprofloxacin	1.6	1.1	1.6	1.4	1.4
$[Cu(cpf)(L^1)Cl]$ (**I**)	0.9	0.9	0.5	0.5	0.8
$[Cu(cpf)(L^2)Cl]$ (**II**)	1.2	0.9	0.7	0.6	1.0
$[Cu(cpf)(L^3)Cl]$ (**III**)	1.1	1.0	1.0	0.8	1.3
$[Cu(cpf)(L^4)Cl]$ (**IV**)	1.5	1.8	1.8	1.6	1.6

higher antimicrobial efficiency towards complexes with N-donor ligands.

II Nature of the ligands.

III The total charge of the complex; generally the antimicrobial efficiency decreases in the order cationic > neutral > anionic complex.

IV The nature of the ion neutralizing the ionic complex

V The nuclearity of the metal center in the complex; dinuclear centers are usually more active than mononuclear ones.

Thus, first two factors may be considered for increase in the activity, i.e. chelate effect.

The study regarding bactericidal activity in terms of CFU/mL of complexes against three gram(−ve) and two gram(+ve) microorganisms, which revealed decrease in number of colonies with increasing the concentration of compounds. The 1.75 μg/mL was the maximum concentration for all the complexes, which gave lesser number of colonies. Colonies counted in this technique was 30–250. The results are shown in Fig. 2 for all the complexes against *S. aureus.*

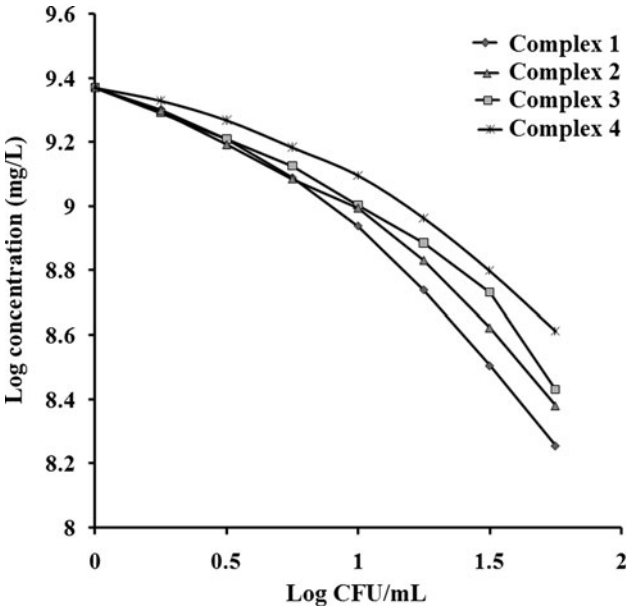


Fig. 2 Relationship between concentration and bactericidal activity of all complexes against *S. aureus*

Metal complex DNA interaction

Hydrodynamic measurement

The geometry of a supercoiled molecule may be altered by any factor which affects the intrinsic twisting of the DNA helix. One important factor is the presence of an intercalator, e.g. ethidium bromide; the positively charged polycyclic aromatic compound, which binds to DNA by inserting itself between the base pairs. Intercalators introduce strong structural perturbations in DNA. The axial flexibility of DNA in the sense of dynamic flux of changes in axial and helical parameters allows the compound to stack between adjacent DNA base pair. The complex is thought to be stabilized by $\pi-\pi^*$ stacking interactions between the compound and DNA bases.

Optical photophysical probes generally provide necessary, but insufficient clues to support a binding mode. Hydrodynamic measurements such as viscosity which is sensitive to length changes are regarded as the least ambiguous and the most critical tests of binding modes in solution (Patel *et al.*, 2009). The possibility of bending the helix with larger ring systems and the steric restrictions on intercalation is supported by viscosity study. A classical intercalation model demands that the DNA helix lengthens as base pairs are separated to accommodate the complex, leading to the increase of DNA viscosity. In contrast, decrease in DNA viscosity is noted in the partial or non-classical intercalator, because it may bend DNA helix, thereby decreasing its effective length and therefore the viscosity decreases like in ciprofloxacin shown in Fig. 3. For all the complexes, as increasing the amounts of complexes, the viscosity of DNA increases steadily suggesting classical intercalative mode of binding.

UV–Vis absorption spectra

It is a general observation that the binding of intercalative molecules to DNA is accompanied by a red shift and hypochromism in the absorption spectra. The extent of spectral change is related to the strength of binding

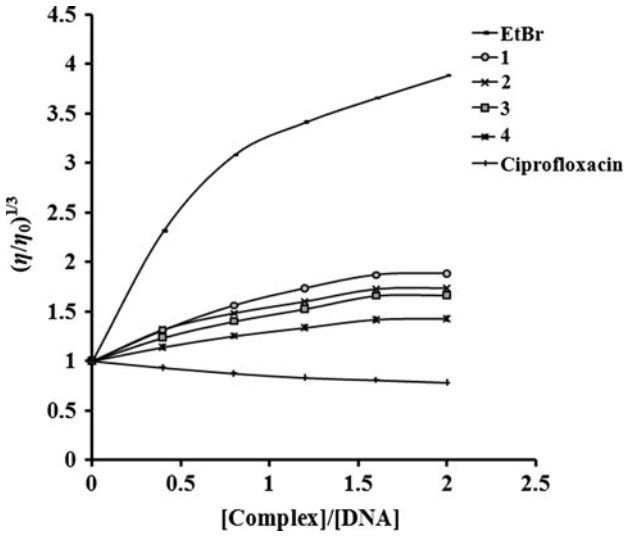


Fig. 3 Effect on relative viscosity of DNA under the influence of increasing amount of complexes at 27 ± 0.1°C in 5 mM Tris-HCl buffer (pH 7.2) as a medium

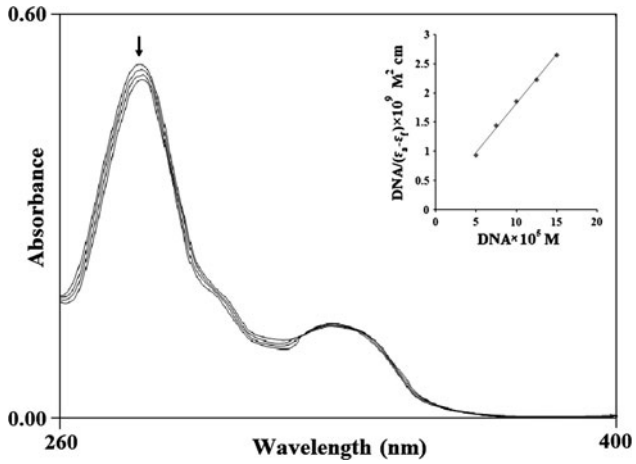


Fig. 4 Electronic absorption titration curve of [Cu(cpf)(L^1)Cl] in the absence and in the presence of increasing amount of DNA; 50–150 μM in 5 mM Tris-HCl buffer (pH 7.2), [complex] = 15 μM, [DNA] = 50–150 μM with incubation period of 30 min. at 37°C, *Inset* Plot of [DNA]/(ε_a– ε_f) versus [DNA]

(Long and Barton, 1990; Le Pecq and Paoletti, 1967; Li *et al.*, 2008; Hirohama *et al.*, 2005). The absorption spectra of the complex in absence and presence of DNA is illustrated in Fig. 4. In order to compare quantitatively the binding strength of the complexes, the intrinsic binding constant K_b was obtained by monitoring the changes in absorbance for complexes with increasing concentration of DNA. From the K_b value (Table 3) and red shift, it is clear that complexes bind via intercalation mode. K_b value of all complexes is found to be in range of 1.05×10^4–1.30×10^4 M^{-1}. The quinolone Cu(II) complexes with quite similar K_b value have been reported. (Efthimiadou *et al.*, 2007a, b). These spectral characteristics are consistent with a mode of interaction that involves a stacking interaction between the complex and the base pairs of DNA, which means that the complexes can intercalate into the double helix structure of DNA.

Table 3 Binding constant (K_b) and IC_{50} values of copper(II) complexes

Complexes	K_b (M^{-1})	IC_{50}
[Cu(cpf)(L^1)Cl] (**I**)	3.01×10^4	0.65
[Cu(cpf)(L^2)Cl] (**II**)	2.33×10^4	0.78
[Cu(cpf)(L^3)Cl] (**III**)	1.75×10^4	1.05
[Cu(cpf)(L^4)Cl] (**IV**)	1.57×10^4	1.25

DNA melting experiment

The melting temperature (T_m) of double-stranded DNA changes with different binding modes. In general, the melting temperature increases when metal complexes bind to DNA by intercalation, as intercalation of the complexes into DNA base pairs causes stabilization of base stacking and hence raises the melting temperature of the double-stranded DNA. DNA melting experiments are useful in establishing the extent of intercalation (Patel *et al.*, 2009). A large change in the T_m of DNA is indicative of a strong interaction with DNA. The effect of complexes on the melting temperature of CT DNA in buffer is shown in Fig. 5. At the melting temperature, the double helix denatures into single-stranded DNA. The thermal

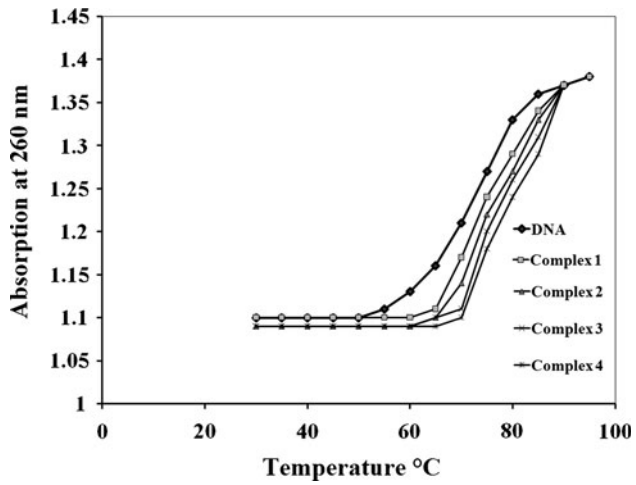


Fig. 5 Melting curves of CT DNA in the absence and the presence of complexes **1–5**

denaturation experiment carried out for CT DNA in the absence of complex gave a T_m 74.2 ± 1°C under our experimental conditions. The observed melting temperatures in the presence of complexes were 79.3 ± 1°C, 78.8 ± 1°C and 78.2 ± 1°C and 77.9 ± 1°C for complexes **I**, **II**, **III** and **IV**, respectively. The ΔT_m values of the

DNA were increases in presence of the complexes and comparable to those observed for classical intercalation and suggest that the complex has a moderate DNA-binding affinity.

Gel electrophoresis

There has been considerable interest in DNA cleavage reactions that are activated by transition metal complex (Hertzberg and Dervan 1982; Kumar *et al.*, 2008). The ability of the complexes to interact double stranded DNA was examined by agarose gel electrophoresis. The principle of this method is that molecules migrate in the gel as a function of their mass, charge and shape, with supercoiled DNA migrating faster than open circular molecules of the same mass and charge. When circular plasmid DNA is subject to electrophoresis, relatively fast migration is generally observed for the intact supercoiled form. When scission occurs on one strand (nicking), the supercoil (SC) relaxes to generate a slower-moving, open-circular (OC) form. When both strands are cleaved, a linear form (L) is generated, which migrates between SC form and OC form DNA. Figure 6 illustrates the gel electrophoretic separations that show cleavage of pUC19 DNA induced by the complexes under aerobic conditions. Results clearly show that the relative binding efficiency of complexes to DNA is much higher than the binding efficacy of metal salt or ciprofloxacin (Table 4). The similar behaviour of Cu(II) complexes with plasmid DNA is shown by compounds of type [Cu(Hpr-norf)(phen)Cl], [Cu(Hpr-norf)(bipy)Cl], [Cu(erx)(phen)Cl], [Cu(erx)(bipy)Cl] reported by Katsaros et al. (2008).

SOD-like activity

The measured IC_{50} values for SOD-like activity at biological pH ranges from 0.65 to 1.25 μM (Table 3). Figure 7 represents plot of absorbance values (Abs_{560}) against time (t) with varying concentration of complex **I** from 0.5 to 3 μM. Figure 8 illustrate that chromophore requires to yield 50% inhibition of the reduction of NBT (IC_{50}) value of complexes are higher than exhibited by the copper salt.

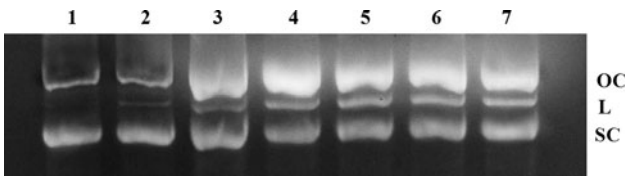


Fig. 6 Photogenic view of interaction of pUC19 DNA (300 μg/mL) with series of copper(II) complexes (200 μM): *Lane 1* DNA control; *Lane 2*, $CuCl_2{\cdot}2H_2O$; *Lane 3* ciprofloxacin; *Lane 4* [Cu(cpf)(L^1)Cl]; *Lane 5* [Cu(cpf)(L^2)Cl]; *Lane 6* [Cu(L^3)(cpf)Cl]; *Lane 7* [Cu(cpf)(L^4)Cl]

Table 4 Gel electrophoresis data

Compounds	%SC	%OC	%LC	% Cleavage
DNA Control	75	25	–	
DNA +Metal salt	72	28	–	4.00
DNA +CPFH	67	33	–	10.66
DNA +**I**	17	50	33	77.33
DNA +**II**	18	51	31	76.00
DNA +**III**	20	50	30	73.33
DNA +**IV**	27	58	15	64.00

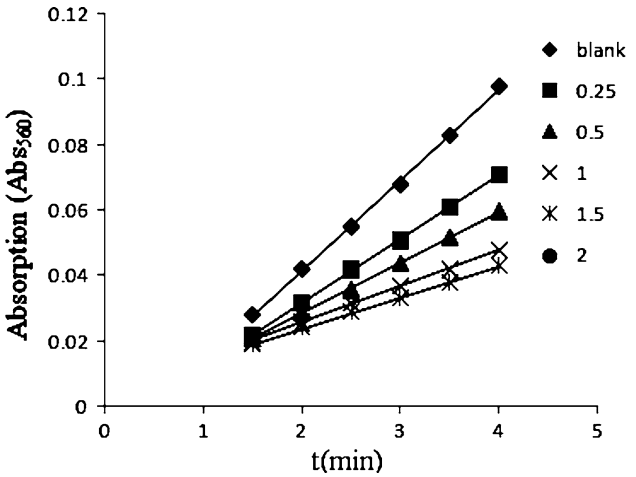


Fig. 7 Plot of absorbance (Abs_{560}) as a function of time (t) for different concentration complex **1** ranging from 0.5–3 μM

The complexes show the different extent of superoxide scavenging ability. The higher IC_{50} can be accredited to the vacant coordination site facilitating the binding of superoxide anion, electrons of aromatic ligands that stabilize $Cu–O_2^-$ interaction and not only to the partial dissociation of complex in solution (Roberts and Robinson 1985). Difference in electron negativity on terpyridine can also be taken under consideration for difference in affinity of complexes towards reactive specie.

Structure–activity-relationship

The SAR studies revealed that, the fluorine atom and the 1-alkyl, 1,4-dihydro-4-oxo-quinoline-3-carboxylic acid skeleton of fluoroquinolones is responsible for potency represented in binding with DNA. Different substituents on the neutral tridentate ligands are also responsible for increase and decrease in biological activities. The result concerning biological aspects of metal complexes show that electron-withdrawing substituent on the intercalative ligand can improve the DNA interaction affinity, free radical scavenging activity and antibacterial activity of the original complex, whereas the case is reversed for

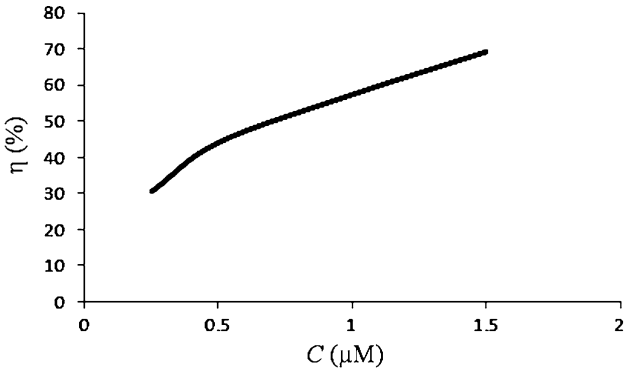


Fig. 8 Plot of percentage of inhibiting NBT reduction with an increase in the concentration of complex **1**

electron-releasing substituents. Such a trend suggests that the DNA interaction affinity, free radical scavenging activity and antibacterial activity of the complex can be effectively controlled by the substituents. Furthermore benzyloxy group at fourth position can contribute more biological activity rather than at third position. Bulky substituents on the benzene ring, such as a lipophilic methyl group or a hydrophilic methoxy group, did not improve the biological activity of the parent analogue. This support that electron withdrawing substituent at position 4 more effectively facilitated the complex structure to be attacked by reactive oxygen species in case of SOD-like activity. The ascending order of complexes towards the biological activities is **I** > **II** > **III** > **IV**.

Conclusion

From the results of MIC, it is clearly observed that the potency of ciprofloxacin is lifted up higher due to coordination with metal ion. The slight difference in binding behaviour of complexes may be attributed to the electro negativity of the substituted group on basic terpyridine skeleton. The order for the biological behaviour of synthesized complexes is **IV** < **III** < **II** < **I** on the bases of antibacterial activity, K_b values, change in relative viscosity, value of ΔT_m and plasmid cleavage study. The similar fashion is also true for the SOD-like activity (IC_{50} value) of all metal complexes since ligand which can facilitate the stabilization of binding between metal centre and oxygen radical anion favours enhancement in enzymatic behaviour.

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Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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