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Copper(II) complexes with norfloxacin and neutral terpyridines: Cytotoxic, antibacterial, superoxide dismutase and DNA-interaction approach

Mohan N. Patel *, Hardik N. Joshi, Chintan R. Patel

Department of Chemistry, Sardar Patel University, Vallabh Vidyanagar, 388120 Gujarat, India

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ABSTRACT

Cu(II) complexes of the type [Cu(NFL)(L^n)Cl] (L^n = substituted terpyridines, NFL = norfloxacin) were synthesised and characterized. Antibacterial activity was assayed against selective Gram$^{(+ve)}$ and Gram$^{(-ve)}$ microorganisms using the double dilution technique. The binding behavior of the complexes toward Herring Sperm (HS) DNA was determined using absorption titration and hydrodynamic measurements, whereas the cleavage efficacy of the complexes toward pUC19 DNA was determined by the gel electrophoresis technique. The brine shrimp bioassay was carried out to study the *in vitro* cytotoxic properties of the synthesized metal complexes. The superoxide dismutase (SOD) like activity of the complexes was measured using an NBT/NADH/PMS system and is expressed in term of the concentration of complex which terminates the formation of formazan by 50% (IC_{50} value). The IC_{50} values were observed to be in the range 0.653–1.344 μM.

1. Introduction

The study of the interaction between drugs and transition metal ions is an important and active research area in bioinorganic chemistry [1–3]. It is well known that the action of many drugs depends on the coordination with metal ions and/or the inhibition on the formation of metalloenzymes. Therefore, metal ions might play a vital role during the biological process of drug utilization in the body.

Quinolones, a commonly used term for quinolonecarboxylic acids or 4-quinolones, are a group of synthetic antibacterial agents containing a 4-oxo-1,4-dihydroquinoline skeleton [4–6]. They can act as antibacterial drugs that effectively inhibit DNA replication and are commonly used in the treatment of many infections [7,8]. Ciprofloxacin and norfloxacin are amongst the most common and widely used quinolones. The study of the biological properties of quinolones has been focused on their interaction with DNA [9], antibacterial activity tests on diverse microorganisms [10,11], cytotoxicity [11,12] and potential antitumor activity [13]. The study of the biological properties of quinolone metal complexes comprises mainly their antibacterial activity against diverse microorganisms [14–16] and, in some cases, their interaction with DNA [17,18].

The superoxide radical anion ($O_2^{\cdot -}$) is an inevitable by-product of aerobic metabolism which if not eliminated may cause significant cellular damage; consequently, the superoxide radical has been implicated in numerous medical disorders [19,20]. To avoid such harmful consequences, all oxygen metabolizing organisms possess metalloenzymes, known as superoxide dismutases (SODs). SODs are dedicated to keeping the concentration of $O_2^{\cdot -}$ in controlled low limits, thus protecting biological molecules from oxidative damage. These SODs disproportionate the toxic $O_2^{\cdot -}$ radical to molecular oxygen and hydrogen peroxide [21,22]. All SODs employ the two step ping-pong mechanism shown in Eqs. (1) and (2):

$$O_2^{\cdot -} + Cu^{II} \rightarrow O_2 + Cu^{I} \quad (1)$$

$$O_2^{\cdot -} + Cu^{I} + 2H^{+} \rightarrow H_2O_2 + Cu^{II} \quad (2)$$

The synthesis and characterization of new metal complexes with norfloxacin and other quinolone antibacterial agents are of great importance for understanding the drug–metal ion interaction and pharmacological applications [23–30].

In continuation of our previous work [31,32], we report here the synthesis and characterization of some new copper (II) complexes with their *in vitro* antimicrobial activity against two Gram$^{(+ve)}$ and three Gram$^{(-ve)}$ microorganisms. The DNA binding and cleavage properties of the complexes have been investigated using absorption titration, viscosity measurements and the gel electrophoresis method. The cytotoxic activities of all the complexes have been measured. The antioxidant properties of the complexes have been checked using the non-enzymatic NBT/NADH/PMS system.

2. Experiments

2.1. Reagents

All chemicals and solvents used were of analytical grade. The drug norfloxacin was purchased from Bayer AG (Wuppertal,

* Corresponding author. Tel.: +91 2692 226856x218.
E-mail address: jeenen@gmail.com (M.N. Patel).

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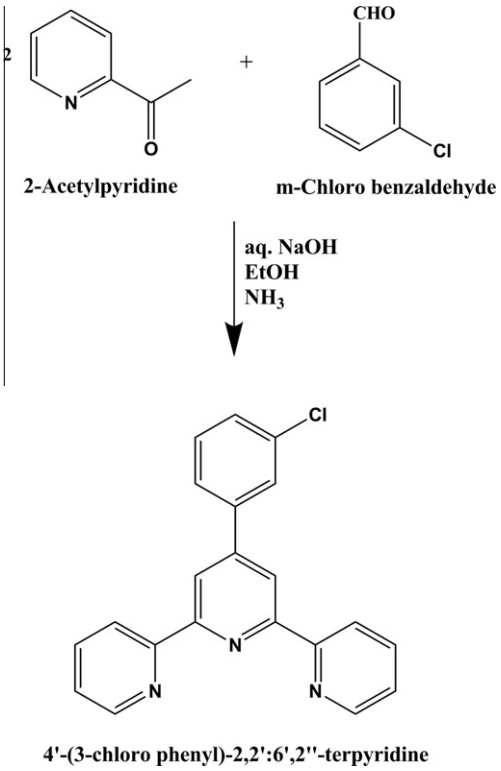
Germany). 2-Acetylpyridine, *m*-chloro benzaldehyde, *p*-methoxy benzaldehyde, *m*-bromo benzaldehyde, benzaldehyde, pyridine-3-carbaldehyde, 9-anthraldehyde and *m*-benzyloxy benzaldehyde were purchased from Loba Chemie Pvt. Ltd. (India). Ethidium bromide and Luria Broth (LB) were purchased from Himedia, India. Acetic acid and EDTA were purchased from Sd fine Chemicals (India).

2.2. Physical measurements

The elemental analyses (C, H and N) of the synthesized complexes were performed with a model 240 Perkin Elmer elemental analyzer. The metallic content of the complexes was determined after decomposing them in an acid mixture by heating and then titrating against an EDTA solution volumetrically [33]. Room temperature magnetic measurements of the complexes were carried out using a Gouy magnetic balance. 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) The electronic spectra were recorded on a UV-160A UV–Vis spectrophotometer, Shimadzu, Kyoto (Japan). Infrared spectra were recorded on a FT-IR ABB Bomen MB 3000 spectrophotometer as KBr pellets in the range 4000–400 cm^{-1}. The LC–MS were recorded using a Thermo Scientific mass spectrophotometer (USA). The minimum inhibitory concentration (MIC) study was performed by means of a laminar air flow cabinet Toshiba, Delhi (India).

2.3. Synthesis of the ligands

All the tridentate ligands were synthesized by following literature procedures [34]. 2-Acetylpyridine (20.0 mmol) was added to an ethanolic solution of the various aldehydes (10.0 mmol). KOH pellets (26.0 mmol) and 30 mL aqueous NH_3 were added to the solution, followed by stirring at room temperature for 8 h. An off-white solid formed, which was collected by filtration, followed by washings with H_2O (3 × 10 mL) and EtOH (2 × 5 mL). Crystallization from the $CHCl_3$/MeOH system gave a white crystalline solid. The proposed reaction is shown in Scheme 1.



Scheme 1. Synthesis of 4′-(3-chloro phenyl)-2,2′:6′,2″-terpyridine.

2.4. Synthesis of the complexes

2.4.1. [Cu(NFL)(L^1)Cl]

A methanolic solution of $CuCl_2 \cdot 2H_2O$ (1.5 mmol) was added to a methanolic solution of 4′-(4-methoxy phenyl)-2,2′:6′,2″-terpyridine (L^1) (1.5 mmol), followed by the addition of a previously prepared methanolic solution of norfloxacin (1.5 mmol) in the presence of CH_3ONa (1.5 mmol). After mixing the above solutions, the pH of solution was in the basic range (>6.8). Therefore, the pH of the reaction mixture was adjusted to ~6.8 by addition of dilute hydrochloric acid. The resulting solution was refluxed for 2 h on a water bath, followed by concentrating it to half of its volume. The fine, green, amorphous product thus obtained was washed with ether/hexane and dried in a vacuum desiccator (Scheme 2).

Yield: 67.8%, m.p.: >300 °C, μ_{eff}: 1.96 B.M. *Anal.* Calc. for $C_{38}H_{34}ClFCuN_6O_4$ (756.71): C, 60.31; H, 4.53; N, 11.11; Cu, 8.40. Found: C, 60.42; H, 4.40; N, 11.00; Cu, 8.49%.

2.4.2. [Cu(NFL)(L^2)Cl]

This was synthesized using 4′-(3-bromo phenyl)-2,2′:6′,2″-terpyridine as the ligand (L^2) (1.5 mmol). Yield: 65.6%, m.p.: 286 °C decomposed, μ_{eff}: 1.91 B.M. *Anal.* Calc. for $C_{37}H_{31}BrClFCuN_6O_3$ (805.58): C, 55.16; H, 3.88; N, 10.43; Cu, 7.89. Found: C, 55.02; H, 3.75; N, 10.30; Cu, 7.75%.

2.4.3. [Cu(NFL)(L^3)Cl]

This was synthesized using 4′-(3-chloro phenyl)-2,2′:6′,2″-terpyridine (1.5 mmol) as the ligand (L^3). Yield: 64.7%, m.p.: >300 °C decomposed, μ_{eff}: 1.89 B.M. *Anal.* Calc. for $C_{38}H_{31}Cl_2FCuN_6O_3$ (761.13): C, 58.39; H, 4.11; N, 11.04; Cu, 8.35. Found: C, 58.28; H, 4.01; N, 11.15; Cu, 8.24%.

2.4.4. [Cu(NFL)(L^4)Cl]

This was synthesized using 4′-(benzaldehyde)-2,2′:6′,2″-terpyridine (1.5 mmol) as the ligand (L^4). Yield: 67.8%, m.p.: 279 °C decomposed, μ_{eff}: 1.93 B.M. *Anal.* Calc. for $C_{37}H_{32}ClFCuN_6O_3$ (726.69): C, 61.15; H, 4.44; N, 11.56; Cu, 8.74. Found: C, 61.05; H, 4.34; N, 11.45; Cu, 8.63%.

2.4.5. [Cu(NFL)(L^5)Cl]

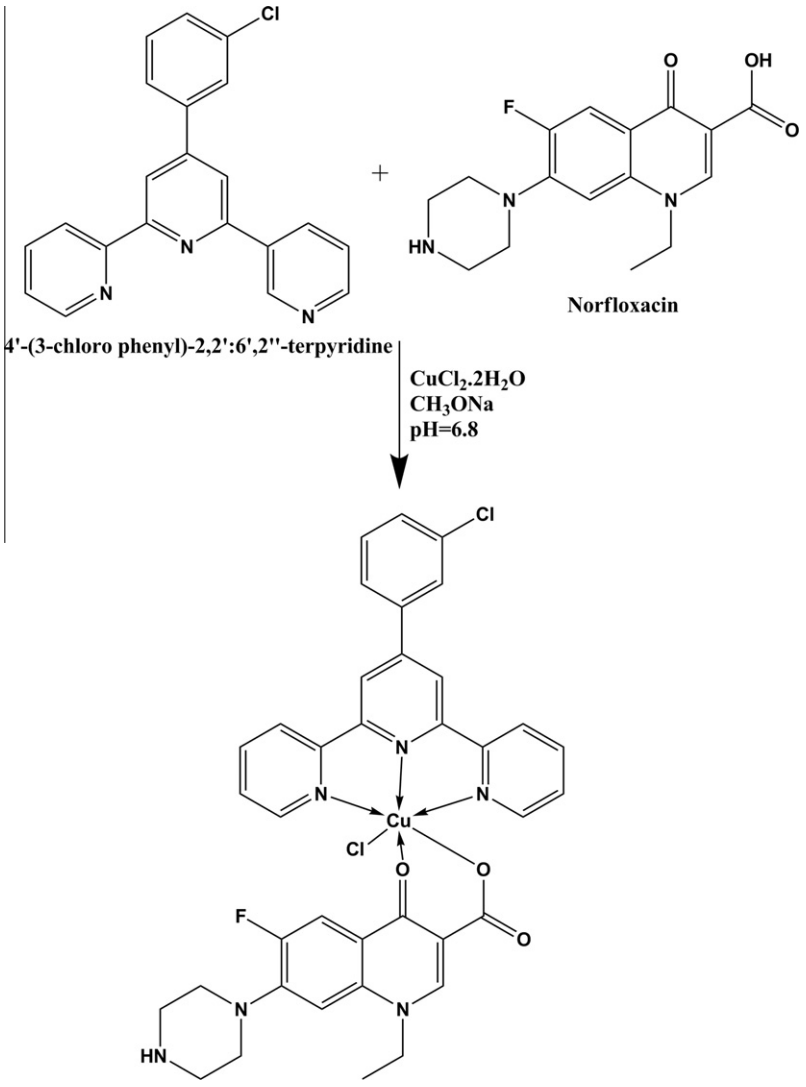
This was synthesized using 4′-(3-pyridyl)-2,2′:6′,2″-terpyridine (1.5 mmol) as the ligand (L^5). Yield: 65.0%, m.p.: >300 °C decomposed, μ_{eff}: 1.94 B.M. *Anal.* Calc. for $C_{36}H_{31}ClFCuN_7O_3$ (727.67): C, 59.42; H, 4.29; N, 13.47; Cu, 8.73. Found: C, 59.29; H, 4.17; N, 13.32; Cu, 8.60%.

2.4.6. [Cu(NFL)(L^6)Cl]

This was synthesized using 4′-(anthracene-9-yl)-2,2′:6′,2″-terpyridine (1.5 mmol) as the ligand (L^6). Yield: 65.0%, m.p.: 291 °C decomposed, μ_{eff}: 1.90 B.M. *Anal.* Calc. for $C_{45}H_{36}ClFCuN_6O_3$ (826.80): C, 65.37; H, 4.39; N, 10.16; Cu, 7.69. Found: C, 65.25; H, 4.28; N, 10.02; Cu, 7.57%.

2.4.7. [Cu(NFL)(L^7)Cl]

This was synthesized using 4′-(3-benzyloxyphenyl)-2,2′:6′,2″-terpyridine (1.5 mmol) as the ligand (L^7). Yield: 62.5%, m.p.: >300 °C, μ_{eff}: 1.95 B.M. *Anal.* Calc. for $C_{44}H_{38}ClFCuN_6O_4$ (832.81): C, 63.46; H, 4.60; N, 10.09; Cu, 7.63%. Found: C, 63.34; H, 4.49; N, 9.98; Cu, 7.51%.



Scheme 2. Synthesis of [Cu(NFL)(L^3)Cl].

2.5. In vitro antimicrobial screening

The antibacterial activities of the compounds were studied against various microorganisms, i.e. *Escherichia coli* (MTCC 433), *Pseudomonas aeruginosa* (MTCC P09), *Serratia marcescens* (MTCC 7103), *Bacillus subtilis* (MTCC 7193) and *Staphylococcus aureus* (MTCC 3160). Screening was performed by determining the minimum inhibitory concentration (MIC) using LB as a medium. Cultures for $Gram^{(+ve)}$ and $Gram^{(-ve)}$ microorganisms were incubated at 37 °C. Since the compounds are water insoluble, the samples were dissolved in DMSO. A control test without any active ingredient was also performed [35]. The MIC was determined using two-fold serial dilutions in liquid media containing the test compound. A preculture of bacteria was grown in LB overnight at the optimal temperature for each species. Bacterial growth was monitored by measuring the turbidity of the culture after 18 h. If a certain concentration of a compound inhibited the bacterial growth, half the concentration of compound was then tested. This procedure was carried out until a concentration was reached where the bacteria grew normally. The lowest concentration which inhibits bacterial growth was determined as the MIC value. All equipment and culture media used were sterile.

The bactericidal action of all the compounds was evaluated against the same microorganisms. The inoculum was prepared by diluting an overnight culture of microorganisms grown in LB to obtain 10^6 viable bacteria/mL. The bacteria were exposed to various concentrations of the compounds. Control tubes without compound were included in each run. The final volume was 1 mL. The cultures were incubated at 37 °C for 2 h. One hundred microliters bacterial culture from each dilution was taken, spread over a previously prepared agar plate and then the plates were incubated for 24 h. The numbers of colonies present on the plates were counted. The number of colonies should be in the range 30–300.

2.6. DNA interaction study

2.6.1. Absorption titration

DNA-mediated hypochromic and bathochromic shifts under the influence of the complexes were measured with the help of UV–Vis absorbance spectra [36–39]. After addition of an equivalent amount of DNA to a reference cell, it was incubated for 10 min at room temperature, followed by absorbance measurement. The DNA-mediated hypochromism (decrease in absorbance) for the test compounds was calculated. Absorption titration experiments were done using different concentrations of HS DNA, by keeping the concentration of the complexes constant, with due correction for the absorbance of the HS DNA itself. Samples were equilibrated

before recording each spectrum. The intrinsic binding constant K_b was determined using the following equation [40]:

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \quad (3)$$

where [DNA] is the concentration of HS DNA in base pairs, ε_a is the apparent extinction coefficient obtained by calculating $A_{obs.}$/[complex], ε_b refers to the extinction coefficient of the complex in the fully bound form and ε_f corresponds to the extinction coefficient of the complex in its free form. The data obtained were plotted in accordance with the above equation to give a straight line with a slope value corresponding to the term $1/(\varepsilon_a - \varepsilon_f)$ and a y intercept of $1/K_b(\varepsilon_b - \varepsilon_f)$. The K_b value was determined from the ratio of the slope to the intercept.

2.6.2. Viscosity titration

Viscosity measurement is regarded as a reliable tool to determine the binding mode in a solution state in the absence of crystallographic structural data and NMR data [41]. Viscometric titrations were performed using an Ubbelohde viscometer (Cannon, PA, USA) immersed in a thermostatic bath maintained at 27 ± 0.1 °C. The flow time was measured with a digital stopwatch. Each sample was measured in triplicate manner and an average flow time was then calculated. The data are represented 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. The viscosity values were calculated from the observed flow time of the solutions containing DNA (t) and corrected for that of the buffer alone (t_0), using the following equation [42]:

$$\eta = (t - t_0) \quad (4)$$

2.6.3. Gel electrophoresis: photo quantisation technique

For the gel electrophoresis experiment, a total volume of 15 μL containing 300 μg/mL of pUC19 DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) was treated with different complexes (200 μM). The mixture was incubated for 24 h at 37 °C. Then the samples were analyzed on the basis of their charge and size difference on a 1% agarose gel bed consisting of 0.5 μg/mL of ethidium bromide at 50 V, after quenching the reaction with 5 μL loading buffer (40% sucrose, 0.2% bromophenol blue). The whole bed was immersed in 1× TAE buffer (0.04 M Tris–acetate, pH 8, 0.001 M EDTA). The bands were visualized using UV light, then photographed. An estimation of the intensity of the DNA bands was done using the AlphaDigiDoc™RT. Version V.4.0.0 PC-Image software gel documentation system.

2.7. Brine shrimp assay

A brine shrimp assay was carried out according to the method given by Meyer et al. [43]. Brine shrimp (Artemia cysts) eggs were hatched in a shallow rectangular plastic dish (22 × 32 cm), filled with artificial seawater. Artificial seawater was prepared using a commercial salt mixture and double distilled water. An unequal partition was made in the plastic dish with the help of a perforated device. Approximately 50 mg of eggs were sprinkled into the large compartment which was opened to ordinary light. After 48 h nauplii were collected by a pipette. A sample of the test compounds was prepared by dissolving 10 mg of each compound in 10 mL of DMSO. Solutions were transferred from the stock solutions to 18 vials in such an amount to make final concentrations of 2, 4, 8, 10, 12 and 16 μg/mL (three sets for each dilution were run for all test samples and the mean of the three sets was taken for the calculation of LC_{50}). One vial was kept as a control, having the same amount of DMSO only. When the shrimp nauplii were ready, 1 mL of artificial seawater and 10 shrimps were added to each vial (30 shrimps/dilution). The volume was adjusted to 2.5 mL per vial using the artificial seawater. The number of survivors was counted after 24 h. Data were analyzed by a simple method to determine the LC_{50} values, in which the log values of the concentration of the samples were plotted against the percentage of mortality of nauplii [44].

2.8. SOD-like activity

A non-enzymatic system containing 30 μM PMS, 79 μM NADH, 75 μM NBT and phosphate buffer (pH 7.8) was used to produce the superoxide anion ($O_2^{\cdot -}$). The scavenging rate of $O_2^{\cdot -}$ under the influence of 0.5–3.0 μM tested compounds was determined by monitoring the reduction in the rate of transformation of NBT to monoformazan dye [45]. The reactions were monitored at 560 nm with a UV–Vis spectrophotometer and the rate of absorption change was determined. The percentage inhibition of NBT reduction was calculated using following equation [46]:

$$(\%\ \text{InhibitionofNBTreduction}) = (1 - k'/k) \times 100 \quad (5)$$

where k' and k represent slopes of the straight line of absorbance values as a function of time in the presence and absence of SOD mimic compounds, respectively. IC_{50} values of the complexes were determined by plotting a graph of percentage inhibition of NBT reduction against increasing concentrations of complex. The concentration of the complex which causes 50% inhibition of NBT reduction is reported as IC_{50}.

3. Result and discussion

3.1. Electronic spectra and magnetism

The magnetic moments of the copper(II) complexes lie in the range 1.89–1.96 B.M. These values are typical for mononuclear copper(II) compounds having a d^9 electronic configuration. 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 a spin–orbit coupling contribution [47]. The UV–Vis spectra of the complexes in the solid and solution states show a similar pattern suggesting that the complexes retain their structures in solution. The complexes exhibited one broad band along with a shoulder at ~14,000 and ~11,000 cm^{-1} respectively, which was attributed to the d–d transition for the Cu(II) atom in a distorted octahedral geometry [48–51].

3.2. IR spectra

In the IR spectrum of norfloxacin the valence vibration of the carboxylic stretch $\nu(C{=}O)_{carb}$ is found at 1730 cm^{-1} and the pyridone stretch $\nu(C{=}O)_p$ at 1642 cm^{-1} (Table 1). The characterization of quinolone metal complexes can be achieved by studying the most typical vibrations that are characteristic of the coordination type of quinolones.

In the IR spectra of the complexes **1**–**7** the absorption of the $\nu(C{=}O)_{carb}$ has disappeared. Two very strong characteristic bands are observed in the range 1565–1595 cm^{-1} and 1364–1381 cm^{-1}, assigned as ν(O–C–O) asymmetric and symmetric stretching vibrations, respectively, whereas $\nu(C{=}O)_p$ is shifted from 1642 cm^{-1} to 1618–1632 cm^{-1} upon bonding. The difference $\Delta\nu = \nu(CO_2)_{asym} - \nu(CO_2)_{sym}$ is a useful characteristic for determining the coordination mode of the ligands. The Δ values fall in the range 201–215 cm^{-1}, indicating a monodentate coordination mode of the carboxylato group of norfloxacin [52,53]. The overall changes of the IR spectra suggest that norfloxacin is coordinated to copper via the pyridone (O_p) and one carboxylate (O_c) oxygen.

Table 1
Change in IR bands for the interaction of norfloxacin with Cu(II) in addition to terpyridines (4000–400 cm^{-1}).

Compounds	ν(C=O) (cm^{-1}) pyridone	$\nu(COO)_{asym}$ (cm^{-1})	$\nu(COO)_{sym}$ (cm^{-1})	$\Delta\nu$ (cm^{-1})	ν(M–N) (cm^{-1})	ν(M–O) (cm^{-1})
Norfloxacin	1642	1730[a]	–	–	–	–
[Cu(NFL)(L^1)Cl]	1632	1565	1364	201	535	520
[Cu(NFL)(L^2)Cl]	1618	1591	1381	210	545	509
[Cu(NFL)(L^3)Cl]	1623	1575	1362	213	532	514
[Cu(NFL)(L^4)Cl]	1626	1582	1373	209	539	517
[Cu(NFL)(L^5)Cl]	1630	1595	1380	215	548	506
[Cu(NFL)(L^6)Cl]	1627	1579	1367	212	530	503
[Cu(NFL)(L^7)Cl]	1620	1568	1365	203	542	511

[a] As ν(COOH).

3.3. Mass spectra

The mass spectrum of complex **1** shows molecular ion peak at m/z = 755.21 and 757.21 due to the presence of one chlorine atom and it also confirms that chlorine atom is attached to metal atom through covalent bond. The peak observed at m/z = 720.10, 402.16, 340.18 and 320.23 are due to loss of one chlorine atom, copper-terpyridine moiety, terpyridine moiety and norfloxacin, respectively. The m/z values, i.e. 437.11, 439.12, 416.27, 418.27, 381.12, 262.14 and 156.31 correspond to peaks for particular fragments are duly highlighted in fragmentation pattern of complex **1**. Figure of mass spectrum and the proposed fragmentation pattern of complex **1** are kept in Supplementary material.

3.4. Antibacterial activity

The antibacterial activities of cupric chloride dihydrate, nor floxacin and the complexes were tested against two $Gram^{(+ve)}$ microorganisms, *S. aureus* and *B. subtilis*, and three $Gram^{(-ve)}$ microorganisms, *S. marcescens*, *E. coli* and *P. aeruginosa*, using the double dilution method. The antimicrobial activities of all the complexes against the five microorganisms are much higher than those of the metal salt (Table 2). It is clear from the data that complex **3** is active compared to NFLH against all the bacterial species employed. Complexes **1**, **2**, **4**, **5**, **6** and **7** are moderate to less active compared to NFLH against all the bacterial species employed. Out of all the active complexes, complex **3** is the most active one. All the complexes show better antibacterial activity than [Cu(pr-norf)$_2$(H_2O)] and [Cu(pr-norf)(bipyam)Cl] against *S. aureus* and *P. aeruginosa* [54]. All the complexes are less active than [Cu(pr-norf)$_2$(H_2O)], [Cu (pr-norf)(bipy)Cl] and [Cu(erx)$_2$(H_2O)] against *E. coli* [55,56]. The complexes show better activity than [Cu(oxo)$_2$(H_2O)], [Cu(oxo)(bipyam)Cl], [Cu(oxo)(bipy)Cl] and [Cu(oxo)(phen)Cl] against *S. aureus*, *P. aeruginosa* and *E. coli* [57].

Chelation could facilitate the ability of a complex to cross a cell membrane [58] and can be explained by Tweedy's chelation theory [59]. Chelation considerably reduces the polarity of the metal ion because of partial sharing of its positive charge with donor groups and possible π-electron delocalization over the whole chelate ring. Such a chelation could enhance the lipophilic character of the central metal atom, which subsequently favors its permeation through the lipid layers of the cell membrane [60] and blocking the metal binding sites on the enzymes of microorganisms. The different compounds exhibit microbial activity with small variations against the bacterial species, and this difference in activity could be attributed to the impermeability of the cell of the microbes, which in the case of $Gram^{(+ve)}$ is single layered and in the case of $Gram^{(-ve)}$ is a multilayered structure [61], or differences in the ribosomes of the microbial cells.

In addition to our study regarding MIC, we measured the bactericidal activity in terms of CFU/mL of the above metal complexes against the same microorganisms (two $Gram^{(+ve)}$ and three $Gram^{(-ve)}$). The results reveal decrease in the number of colonies with increasing concentration of the compounds. The results are shown in Fig. 1 for all the complexes against *S. aureus*. The number of colonies counted in this technique is in the range 30–300. From the minimum inhibitory concentration (MIC) values and colony forming units (CFU), it was found that complex **3** is more potent against all the five microorganisms.

Table 2
MIC in terms of μg/mL.

Compounds	Gram positive		Gram negative		
	S. aureus	*B. subtilis*	*S. marcescens*	*P. aeruginosa*	*E. coli*
$CuCl_2{\cdot}2H_2O$	459.95	479.90	469.84	409.83	579.97
Norfloxacin	0.77	0.80	1.25	1.18	0.86
[Cu(NFL)(L^1)Cl]	0.98	1.02	1.66	1.59	1.21
[Cu(NFL)(L^2)Cl]	0.87	0.97	1.57	1.45	1.13
[Cu(NFL)(L^3)Cl]	0.61	0.65	1.14	0.99	0.76
[Cu(NFL)(L^4)Cl]	1.24	1.31	2.14	2.03	1.53
[Cu(NFL)(L^5)Cl]	1.60	1.67	2.62	2.51	1.86
[Cu(NFL)(L^6)Cl]	2.81	2.98	4.22	3.97	3.22
[Cu(NFL)(L^7)Cl]	2.50	2.66	4.00	3.66	2.83

3.5. DNA interaction study

3.5.1. Absorption titration

Mixed-ligand complexes can bind to DNA via three distinct binding sites namely, groove-binding, binding to the phosphate group and intercalation [62]. These behaviors are of great importance with respect to the relevant biological role of quinolone antibiotics in the body [63,64]. The changes observed in the UV spectra of the complexes after mixing them with DNA (either increase or decrease in the intensity or shift in the wavelength) indicate the interaction of the complexes with DNA, due to the formation of a new complex with the double-helical DNA [65]. With an increasing concentration of HS DNA, the absorption bands

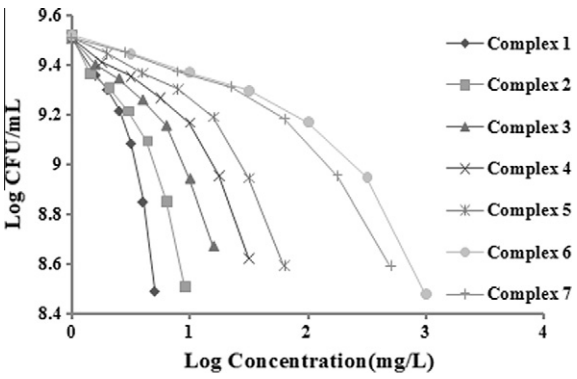


Fig. 1. Relationship between the concentration and bactericidal activity of all the complexes against *S. aureus*.

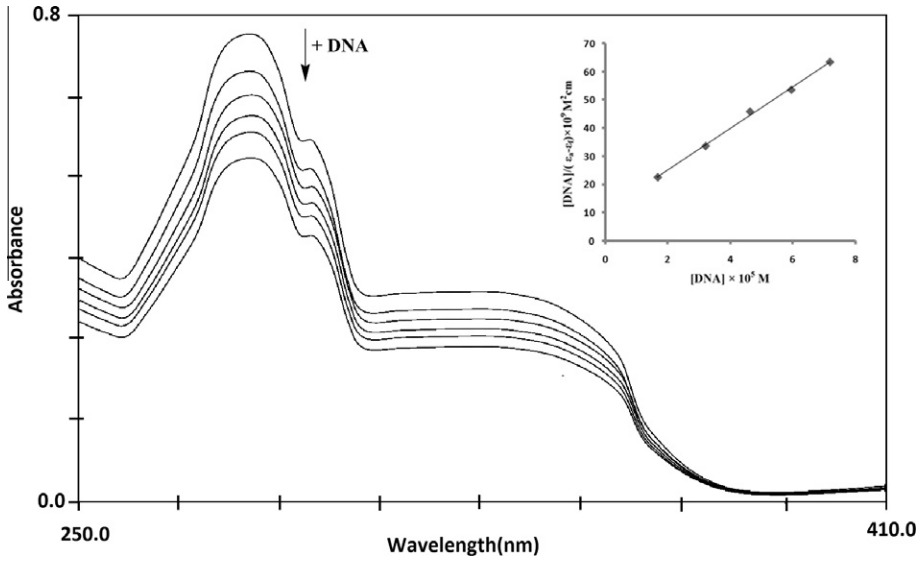


Fig. 2. Electronic absorption spectra of [Cu(NFL)(L^3)Cl] in phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.2) in the absence and presence of increasing amount of DNA. Inset: Plot of [DNA]/($\varepsilon_a - \varepsilon_f$) vs. [DNA]. Arrow shows the absorbance change upon increasing the DNA concentration.

Table 3
The binding constants (K_b) of the complexes.

Complexes	K_b (M^{-1})	% hypochromism
Norfloxacin	2.43×10^3	6.86
[Cu(NFL)(L^1)Cl]	5.06×10^4	18.19
[Cu(NFL)(L^2)Cl]	6.03×10^4	12.40
[Cu(NFL)(L^3)Cl]	7.19×10^4	5.67
[Cu(NFL)(L^4)Cl]	3.47×10^4	5.99
[Cu(NFL)(L^5)Cl]	1.03×10^4	15.38
[Cu(NFL)(L^6)Cl]	3.68×10^3	5.13
[Cu(NFL)(L^7)Cl]	5.85×10^3	5.88

of the complexes are affected. This results in hypochromism with a bathochromic shift. The extent of the binding strength of the complexes is quantitatively determined by calculating the intrinsic binding constants K_b of the complexes. This is done by monitoring the change in absorbance at various concentrations of DNA (Fig. 2). From the plot of [DNA]/($\varepsilon_a - \varepsilon_f$) versus [DNA] (inset in Fig. 2), the K_b values of the complexes were determined and were found to be in the range 3.68×10^3–7.19×10^4 (Table 3). The K_b values of all the complexes are higher than that of norfloxacin ($2.43 \times 10^3\ M^{-1}$). These values are much lower than those of classical intercalators, e.g. ethidium bromide ($7.16 \times 10^5\ M^{-1}$), but are comparable to those of [Ni(erx)$_2$(H_2O)$_2$], [Ni(erx)$_2$(phen)], [Ni(erx)$_2$(bipy)] (1.63–4.09 × $10^4\ M^{-1}$), [66] [Zn(oxo)(bipy)Cl], [Zn(oxo)$_2$(phen)] (5.91 and $9.60 \times 10^4\ M^{-1}$) [67] and [SPFX–Mg] ($7.558 \times 10^3\ M^{-1}$) [68]. Therefore, the above results indicate that the complexes may bind with DNA by the intercalative mode. The binding mode is further confirmed by viscosity measurements.

3.5.2. Viscosity titrations

The binding modes of the Cu(II) complexes were further investigated by viscosity measurements. Viscosity measurements are sensitive to DNA length change, and are regarded as the least ambiguous and most critical test for measuring the binding mode of DNA in solution. A classical intercalative mode demands that the DNA helix must lengthen as base pairs are separated to accommodate the binding ligand, which leads to an increase in DNA viscosity [69]. The effects of the complexes, EtBr and NFLH on the viscosity of DNA are shown in Fig. 3. EtBr increases the relative specific viscosity by lengthening the DNA double helix through the intercalation mode. With increasing the amounts of the complexes, the relative viscosity

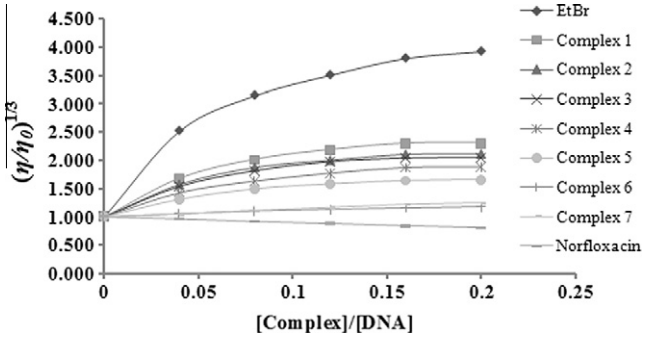


Fig. 3. Effect on the relative viscosity of DNA under the influence of increasing amounts of the complexes at 27 ± 0.1 °C in phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.2).

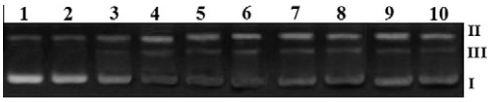


Fig. 4. Photogenic view of the cleavage of pUC19 DNA (300 μg/mL) with a series of copper(II) complexes (200 μM) using 1% agarose gel containing 0.5 μg/mL ethidium bromide. All the reactions were incubated in TE buffer (pH 8) in a final volume of 15 μL, for 24 h at 37 °C: lane 1, DNA control; lane 2, $CuCl_2 \cdot 2H_2O$; lane 3, norfloxacin; lane 4, [Cu(NFL)(L^1)Cl]; lane 5, [Cu(NFL)(L^2)Cl]; lane 6, [Cu(NFL)(L^3)Cl]; lane 7, [Cu(NFL)(L^4)Cl]; lane 8, [Cu(NFL)(L^5)Cl]; lane 9, [Cu(NFL)(L^6)Cl]; lane 10, [Cu(NFL)(L^7)Cl].

of DNA increases steadily, which is similar to that of the classical intercalative complex [Ru(bpy)$_2$ ppd]$^{+2}$ {ppd = pteridino[6,7-f]-[1,10]phenanthroline-11,13(10H,12H)-dione} [70]. This is similar to the behavior of EtBr, which indicates that the complexes bind with DNA through the classical intercalation mode. The increase in the degree of viscosity may depend on the affinity to DNA, and follows the order: EtBr > **3** > **2** > **1** > **4** > **5** > **7** > **6**.

3.5.3. Gel electrophoresis; photo quantisation technique

Agarose gel electrophoresis was used as a base for monitoring the plasmid DNA cleavage reaction. Circular plasmid DNA, when subjected to electrophoresis, undergoes a relatively fast migration

Table 4
Complex mediated DNA cleavage data by gel electrophoresis.

Lane	Compound	Form I (SC)	Form II (OC)	Form III (LC)	% cleavage
1	Control	88	12	–	–
2	$CuCl_2{\cdot}2H_2O$	84	16	–	4.55
3	Norfloxacin	61	27	12	30.68
4	$[Cu(NFL)(L^1)Cl]$	30	52	18	65.91
5	$[Cu(NFL)(L^2)Cl]$	27	56	17	69.32
6	$[Cu(NFL)(L^3)Cl]$	24	57	19	72.73
7	$[Cu(NFL)(L^4)Cl]$	31	53	16	64.77
8	$[Cu(NFL)(L^5)Cl]$	35	51	14	60.23
9	$[Cu(NFL)(L^6)Cl]$	33	55	12	62.50
10	$[Cu(NFL)(L^7)Cl]$	39	50	11	55.68

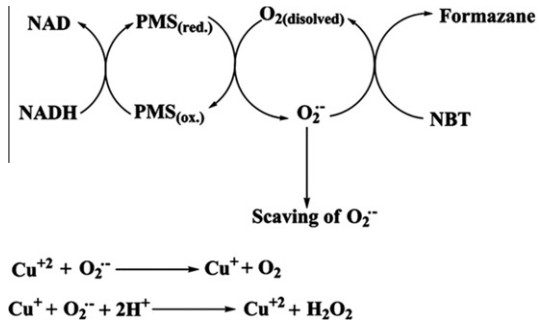
for the intact supercoil form (SC; Form I). Scission on one strand (nicking) will relax the supercoil to generate a slower moving open circular form (OC; Form II). If both strands are cleaved, a linear form (LC; Form III) that migrates between Form I and Form II will be generated [71]. Data of the cleavage study obtained from Fig. 4 are presented in Table 4. Complex **3** shows the maximum cleavage ability compared to all the synthesized complexes. All the complexes show a higher DNA cleavage ability compare to NFLH and the metal salt.

3.6. Brine shrimp assay

A general bioassay that appears capable of detecting a broad spectrum of the bioactivity present in a complex is the brine shrimp lethality bioassay. The technique is easily mastered, is of little cost and utilizes a small amount of test material. The aim of this method is to provide a front-line screen that can be backed up by more specific and more expensive bioassays after isolating the active compounds. The brine shrimp lethality bioassay (BSLA) is a development in the assay procedure of a bioactive compound, which indicates cytotoxicity as well as a wide range of pharmacological activities (such as anticancer, antiviral, insecticidal, pesticidal, AIDS, etc.) of the compound. All the synthesized compounds were screened for their cytotoxicity (brine shrimp bioassay) using the protocol of Meyer et al. [43]. From the data recorded in Table 5,

Table 5
Effect of compounds on brine shrimp lethality bioassay.

Compound	Concentration ($\mu g\ mL^{-1}$)	Log(Conc.)	No. of nauplii taken	No. of nauplii dead	% of mortality	$LC_{50}(\mu g\ mL^{-1})$
Norfloxacin	10	1	10	1	10	
	50	1.698	10	2	20	
	100	2	10	3	30	194.98
	150	2.176	10	4	40	
	200	2.301	10	5	50	
	240	2.380	10	6	60	
$[Cu(NFL)(L^1)Cl]$	2	0.301	10	1	10	
	4	0.602	10	3	30	
	8	0.903	10	5	50	6.93
	10	1	10	6	60	
	12	1.079	10	7	70	
	16	1.204	10	8	80	
$[Cu(NFL)(L^2)Cl]$	2	0.301	10	1	10	
	4	0.602	10	3	30	
	8	0.903	10	6	60	6.36
	10	1	10	7	70	
	12	1.079	10	8	80	
	16	1.204	10	9	90	
$[Cu(NFL)(L^3)Cl]$	2	0.301	10	2	20	
	4	0.602	10	4	40	
	8	0.903	10	6	60	5.24
	10	1	10	7	70	
	12	1.079	10	8	80	
	16	1.204	10	9	90	
$[Cu(NFL)(L^4)Cl]$	2	0.30	10	1	10	
	4	0.602	10	2	20	
	8	0.903	10	4	40	7.82
	10	1	10	6	60	
	12	1.079	10	7	70	
	16	1.204	10	8	80	
$[Cu(NFL)(L^5)Cl]$	2	0.301	10	1	10	
	4	0.602	10	2	20	
	8	0.903	10	4	40	8.1
	10	1	10	5	50	
	12	1.079	10	7	70	
	16	1.204	10	8	80	
$[Cu(NFL)(L^6)Cl]$	2	0.301	10	0	0	
	4	0.602	10	1	10	
	8	0.903	10	2	20	11.96
	10	1	10	4	40	
	12	1.079	10	5	50	
	16	1.204	10	7	70	
$[Cu(NFL)(L^7)Cl]$	2	0.301	10	0	0	
	4	0.602	10	2	20	
	8	0.903	10	4	40	11.3
	10	1	10	5	50	
	12	1.079	10	6	60	
	16	1.204	10	7	70	



Scheme 3. Mechanism for SOD-like activity performed using the NBT/NADH/PMS system.

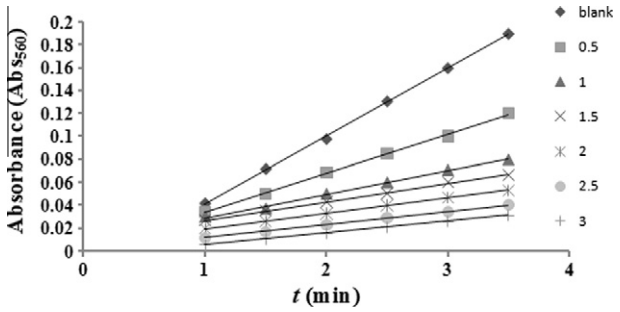


Fig. 5. Absorbance values (Abs_{560}) as a function of time (t) plotted for varying concentrations of complex **3** from 0.5 to 3 μM, for which good straight lines are observed.

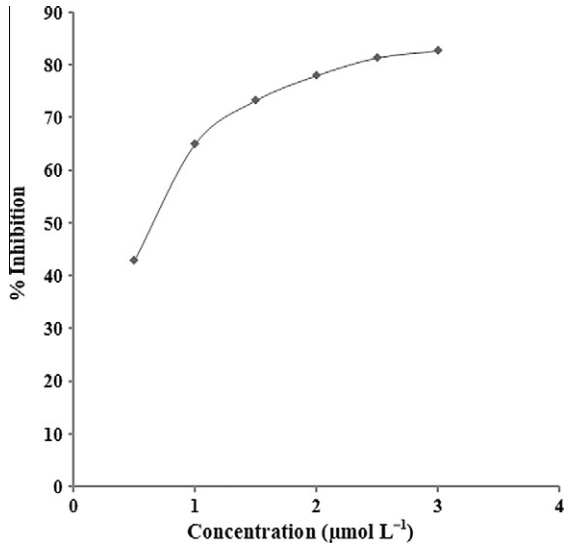


Fig. 6. Plot of percentage of inhibiting NBT reduction with an increase in the concentration of complex **3**.

complex **3** is the most potent amongst all the compounds. The order of potency of compounds is: EtBr > **3** > **2** > **1** > **4** > **5** > **7** > **6**. All the complexes show better cytotoxic activity than norfloxacin. The variation in the BSLA results may be due to the difference in the amount and kind of cytotoxic substances. The tested copper(II) complexes have strong cytotoxic activities, but this investigation is a primary one and further tests are required to investigate the actual mechanism of the cytotoxicity and the probable effects on higher animal models and on cancer cell lines. It suggests that the complexes can be used as potent cytotoxic agents with the hope of adding to the arsenal of weapons used against the fatal disease cancer.

Table 6
The IC_{50} values of the complexes.

Complexes	IC_{50} (μM)
Norfloxacin	161.1
[Cu(NFL)(L^1)Cl]	0.784
[Cu(NFL)(L^2)Cl]	0.713
[Cu(NFL)(L^3)Cl]	0.653
[Cu(NFL)(L^4)Cl]	0.876
[Cu(NFL)(L^5)Cl]	1.145
[Cu(NFL)(L^6)Cl]	1.344
[Cu(NFL)(L^7)Cl]	1.319
[Ni(sf)$_2$(bipy)]	28.30[a]
[Ni(erx)$_2$(bipy)]	26.54[a]
[Ni(oxo)$_2$(bipy)]	27.45[a]
[Ni(flmq)$_2$(bipy)]	12.69[a]

[a] In mM.

3.7. SOD-like activity

The SOD-like behavior was checked using the NBT/NADH/PMS system. PMS gets reduced by the hydrogen donor NADH. The reduced PMS generates $O_2^{\cdot-}$ from dissolved O_2. NBT is reduced by $O_2^{\cdot-}$, which results in a linear accumulation of blue formazan with an increase in the absorbance at 560 nm. SOD or a model compound when present in the reaction medium scavenges $O_2^{\cdot-}$, resulting in a decrease in the formation of formazan. The mechanism is shown in Scheme 3. The percentage inhibition of formazan formation at various concentrations of the complexes as a function of time is calculated by measuring the absorbance at 560 nm, and the results are plotted to give a straight line (Fig. 5). With an increase in the concentration of the tested compounds, a decrease in slope (m) is observed. The percentage inhibition of the reduction of NBT is plotted against the concentration of the complex (Fig. 6). The compounds exhibit SOD-like activity at biological pH, with IC_{50} values in the range 0.653–1.344 μM (Table 6). The IC_{50} values of the copper(II) complexes are better than those of the Ni(II) complexes recently reported [72].

4. Conclusions

The data of magnetic behavior and electronic spectral measurement point towards a d^9 system with a distorted octahedral geometry. The antibacterial activity of norfloxacin is changed upon coordination with the copper(II) ion. From the viscosity data of the complexes, it is clear that all the complexes show a classical intercalative mode of binding. Complex **3** is bound more strongly than the other complexes. The absorption titration data are in good accordance with the viscosity titration curves. All the complexes exhibit good cytotoxic activity. The DNA cleavage study of pUC19 shows that all the complexes have a high cleavage ability compared to the metal salt and the drug. Upon determination of the antioxidant activity in the NBT/NADH/PMS system, complex **3** shows the highest scavenging ability for the oxygen radical.

Conflict of interest

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.poly.2012.03.050.

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