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Fe(III)-DOXYCYCLINE COMPLEXES WITH DIIMINE LIGANDS: SYNTHESES, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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Three new iron(III) complexes of doxycycline *viz*: $[Fe(dox)_2Cl]Cl_2$ (**1**), $[Febpy(dox)Cl]Cl_2$ (**2**) and $[Fephen(dox)Cl]Cl_2$ (**3**), where dox is doxycycline, bpy is 2,2′-bipyridine and phen is 1,10-phenanthroline, were synthesized and characterized by elemental analysis, electronic absorption, FT-IR, and electrospray ionization mass spectroscopy. Doxycycline and the polypyridyl ligands behave as bidendate ligands; the polypyridyl ligands coordinate through the two diimine nitrogen atoms and doxycycline through enolate and diketoamide oxygen atoms of ring A in a five-coordinate system with chloride atom in the axial position. Their antibacterial and antiplasmodial activities against chloroquine-sensitive *Plasmodium falciparum* NF54 and their interaction with calf thymus (CT) DNA using electronic titration were investigated. The three complexes showed good activity against strains of *Staphylococcus aureus* and *Klebsiella pneumonia*. The complexes bind moderately to CT DNA with binding constants of 5.6×10^4 and 4.8×10^4 for complexes **2** and **3**, respectively.

Keywords: doxycycline; iron(III); diimine; antibacterial; DNA binding; antiplasmodial

Fe(III)-ДОКСИЦИКЛИНСКИ КОМПЛЕКСИ СО ДИИМИНСКИ ЛИГАНДИ: СИНТЕЗИ, КАРАКТЕРИЗАЦИЈА И БИОЛОШКИ СВОЈСТВА

Синтетизирани се три нови комплекси на железо(III) со доксициклин, имено: $[Fe(dox)_2Cl]Cl_2$ (**1**), $[Febpy(dox)Cl]Cl_2$ (**2**) и $[Fephen(dox)Cl]Cl_2$ (**3**), каде dox е доксициклин, bpy е 2,2′-бипиридин и phen е 1,10-фенантролин, кои се карактеризирани со елементна анализа, електронска апсорпција, FT-IR и масена спектроскопија со електронспреј јонизација. Доксициклинот и лигандите на полипиридил се однесуваат како бидентатни лиганди; лигандите полипиридил се координираат преку двата диимински азотни атоми, додека доксициклинот преку енолатните и дикетоамидните кислородни атоми на прстенот А во петочлен координатен систем со хлорниот атом во аксијална положба. Со електронска титрација беше испитано и нивното антибактереиско и антиплазмоидно дејство врз хлорохинон осетливите *Plasmodium falciparum* NF54, како и нивната интеракција со DNA од телешки тимус (CT). Трите комплекси покажаа добро дејство врз *Staphylococcus aureus* и *Klebsiella pneumonia*. Комплексите умерено се сврзуваат со CT DNA со константи на сврзување, соодветно, од $5,6 \times 10^4$ and $4,8 \times 10^4$ за комплексите **2** и **3**.

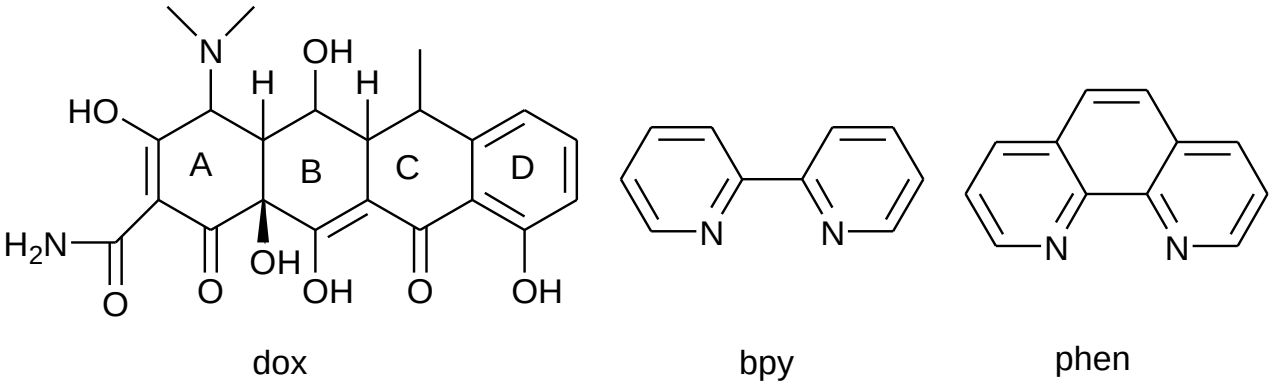
Клучни зборови: доксициклин; железо(III); диимин; антибактериски; DNA сврзување; антиплазмоиден

1. INTRODUCTION

Among the tetracyclines, doxycycline is an antibiotic with pleiotropic properties [1–3]. Doxycycline alone or in combination with another compound has been widely used for treatment of diverse antibacterial infections. Besides the use of doxycycline as broad spectrum antibiotic, new uses

have emerged including its use as slow-acting but effective anti-malarial agent [4–5], its potential in matrix metalloproteinase (MMP) inhibition [6] and in anticancer therapy [7–10].

The binding of tetracyclines and chemically modified tetracyclines (CMTs) to metal ions has been implicated in their biological activities [11–13]. DNA strand breakage by tetracycline alone and the role of metal ions in tetracycline-induced strand cleavage have been demonstrated [14]. DNA binding, cleavage and cytotoxicity of two new ternary copper(II) complexes of 1,10-phenanthroline with doxycycline and tetracycline have also been reported [15–16]. Bipyridine (bpy) (Scheme 1) and other polypyridyl derivatives, both as ligands and as transition metal complexes, play active roles in the normal functioning of a number of biological systems [17]. Such bioactivities of the free polypyridyl ligands are usually associated with the sequestering of trace metals at the active site and the active species are always the resulting metal complexes.



Scheme I. Ligands used

The inertness and structural versatility of polypridyl ligand complexes have been used to probe their interactions with DNA and the formation of non-covalent adducts with essential proteins in cells [15–16]. Chemical properties such as hydrophobicity and redox potentials of polypyridyl ligands have also been exploited to obtain complexes with cytotoxic and enzyme-inhibition properties [19]. The diimine chelating ligands such as 2,2′-bipyridine (bpy) and 1,10-phenanthroline (phen) generally bind moderately strongly to Fe(III) giving stable complexes and their homoleptic and mixed diimine ligand complexes have been reported for various applications, including DNA interactions [20–22]. Tetracyclines are also avid iron chelators and form moderately stable complexes with Fe^{3+} by acting as an O,O-donor ligand [23]. The ability of tetracyclines to remove iron from human holotransferrin has been demonstrated [24]. The complexing of iron to bleomycin [16] has been implicated in bleomycin-DNA interactions and the interaction of some Fe^{III}-diimine complexes with DNA has also been reported [25–26]. Iron, often as complexes, plays many vital roles in biology and medicine [27–29].

These significant bioactivities of iron(III) and tetracycline complexes prompted us to synthesize three iron(III) complexes of doxycycline namely $[Fe(Dox)_2Cl]Cl_2$ (**1**), $[Febpy(Dox)Cl]Cl_2$ (**2**) and $[Fephen(Dox)Cl]Cl_2$ (**3**), where dox is doxycycline, bpy is 2,2′-bipyridine and phen is 1,10-phenanthroline. Herein, we report spectroscopic analyses of the complexes, their antibacterial potentials against *Staphylococcus aureus* and *Klebsiella pneumonia* strains, their antiplasmodial activity and their interaction with calf thymus DNA (CT DNA) as evidenced by electronic titration of complexes **2** and **3**. This work is part of our effort targeted towards synthesizing metal complexes of biologically relevant ligands for various antibacterial and antimalarial applications, [30–33].

2. EXPERIMENTAL

2.1. *Materials and methods*

All reagents and solvents were of analytical grade and used without further purification. Doxycycline hyclate was sourced from Neimeth International Pharmaceuticals Plc, Lagos, Nigeria and solutions were freshly prepared to ensure stability; 1,10-phenanthroline monohydrate and iron(III) chloride were obtained from S. D. Fine Chemicals Ltd., India and used as received. Chloroquine diphosphate was obtained from Sigma.

UV/Vis spectra were recorded on a Jasco UV-vis spectrophotometer. Infrared spectra were recorded in the range of 4,000-400 cm^{-1} on samples pressed into KBr pellets. Elemental analyses were taken on Elementar Analysen Systeme Vario® MICRO VI 6.2 GmbH. Melting points were taken on Jenway digital melting point apparatus and were uncorrected. Electrospray mass spectra were recorded on a THERMO-Finningan-LCQ Advantage max ion trap mass spectrometer. A 10 μl methanol solution of the sample was introduced into the ESI source through Finnigan surveyor autosampler. Methanol-water (90 : 10) was used as the mobile phase and flowed at a rate of 250 μl/min by MS pump. The ion spray voltage was set at 5.3 KV and the capillary voltage was 34 V. The MS scan was 2.5 min and the spectra printouts were averaged of over 10 scans at the peak top in Total Ion Chromatogram (TIC).

2.2. *Synthesis of the complexes*

2.2.1. *Preparation of* $[FeDox_2Cl]Cl_2 \cdot 6H_2O$ (**1**)

0.041 g (0.25 mmol) of anhydrous ferric chloride was dissolved in 10 ml of methanol and 0.256 g (0.5 mmol) of doxycycline hyclate was added. The resulting brown solution was stirred for 4 h, filtered and allowed to evaporate slowly at room temperature precipitating brown powder after a few days. The brown powder was washed with acetone and dried in a vacuum desiccator. *Anal.* Calc. for $C_{44}H_{60}Cl_3N_4O_{22}Fe$: C, 45.59; H, 5.22; N, 4.83. Found: C, 45.35; H, 5.61; N, 4.80. FT-IR (KBr, ν/cm^{-1}): 3205, 1610, 1562, 1445, 1323, 1240, 1214, 1169, 1128, 1038, 1001, 935, 865, 824, 803, 706, 659.

2.2.2. *Preparation of* $[FebpyDoxCl]Cl_2$ (**2**)

0.128 g (0.25 mmol) of doxycycline hyclate was added to 0.041 g (0.25 mmol) anhydrous ferric chloride in 10 ml methanol and the resulting dark brown solution was stirred for 1 h. 39.5 mg (0.25 mmol) of 2,2-bipyridine was added and the solution stirred for additional 2 hours to obtain an orange precipitate. The resulting precipitate was filtered and dried in vacuum desiccator. *Anal.* Calc. for $C_{32}H_{32}Cl_3N_4O_8Fe$: C, 45.06; H, 4.96; N, 6.57. Found: C, 45.06; H, 4.09; N, 6.59. UV-Vis (H_2O, nm): 231, 284, 348, 368, 422, 518. FT-IR (KBr, ν/cm^{-1}): 3286, 3209, 3088, 3060, 1604, 1582, 1494, 1442, 1241, 1214, 1170, 1127, 1060, 1038, 995, 933, 882, 824, 804, 763, 732, 708, 674, 653, 617, 582, 546, 507, 442, 417.

2.2.3. *Preparation of* $[FephenDoxCl]Cl_2 \cdot 5H_2O$ (**3**)

0.128 g (0.25 mmol) doxycycline hyclate was added to 0.0401 g (0.25 mmol) anhydrous ferric chloride in 10 ml aqueous methanol and the resulting dark brown solution stirred for 30 min. 51 mg (0.25 mmol) of 1,10-phenanthroline was added and the solution was stirred for additional 2 hours to obtain an orange precipitate. The orange precipitate was filtered and dried in vacuum desiccator. *Anal.* Calc. for $C_{34}H_{42}Cl_3N_4O_{13}Fe$: C, 46.57; H, 4.83; N, 6.39. Found: C, 46.06; H, 4.69; N, 6.59. UV-Vis (H_2O, nm): 225, 267, 323, 362, 401, 510. FT-IR (KBr, ν/cm^{-1}): 3555, 3275, 2877, 1979, 1587, 1518, 1446, 1429, 1384, 1325, 1244, 1219, 1170, 1141, 1130, 1105, 1039, 1001, 933, 873, 830, 823, 802, 768, 725, 680, 660, 615, 584, 545, 503, 452, 447, 432.

2.3. *Antimicrobial susceptibility testing*

Antibacterial susceptibility testing was carried out to compare the antibacterial activities of the complexes with the parent ligand, doxycycline. The *in vitro* antibacterial activities of all compounds were tested by standard well diffusion method using nutrient agar media. A sterile swab was used to inoculate the organism on the plate. The plate was left for some time so that the inocula would diffuse into the media. A sterile needle was used to make 6 mm wells uniformly on the surface of the plates. Three wells were made in the seeded plates and were labelled well I, II and III, respectively. Different concentrations of the test solution (5 % aqueous DMSO) were introduced into the wells using a sterile syringe. The plates were then set aside for 1 hour at room temperature to allow proper diffusion of the test solution to occur. All the plates were incubated at 37 ºC for 24–48 hours before bacteria and zones of inhibition were detected. A zone of clearance round each well indicated inhibition and the diameter of such zones were measured in millimeters (mm).

2.4. *Antiplasmodial study*

Samples of all compounds were tested in triplicate against chloroquine-sensitive (NF54) strains of *Plasmodium falciparum*. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified procedure of Trager and Jensen [35]. Quantitative assessment of *in vitro* antiplasmodial activity was determined with the parasite lactate dehydrogenase

assay using a modified method of Makler and Hinrichs [36]. A 20 mg ml^{-1} stock solution in 100 % DMSO of the test samples were prepared and stored at –20 °C. Further dilutions were prepared on the day of the experiment. Chloroquine diphosphate (CQDP) was used as the reference drug. A full dose-response experiment was performed for all compounds to determine the concentration inhibiting 50 % of parasite growth (IC_{50}-value). Samples were tested at a starting concentration of 100 μg ml^{-1}, which was then serially diluted twofold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 μg ml^{-1}. The reference drug (CQDP) was tested at a starting concentration of 1000 ng ml^{-1}. The highest concentration of the solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The IC_{50}-values were obtained using a non-linear dose response curve fitting analysis via Graph Pad Prism v. 4.0 software.

2.5. *DNA binding study by electronic absorption titration*

The electronic absorption spectra of 20 μM of the complexes with increasing concentration of nucleotide (5 μM – 150 μM) were recorded in Tris HCl buffer following reported procedures [37–38]. Absorption readings were taken after 20 minutes of adding DNA to the metal complexes so as to allow the solutions to equilibrate. Binding constants of complexes were estimated using the equation 1 [39–40].

$$\frac{[\mathrm{DNA}]}{[\varepsilon a-\varepsilon b]}=\frac{[\mathrm{DNA}]}{[\varepsilon b-\varepsilon f]}+1/Kb[\varepsilon-\varepsilon f] \qquad (1)$$

where [DNA] is the concentration of DNA in base pairs, ε_a is the extinction coefficient observed for the MLCT absorption band at the given DNA concentration, ε_f is the extinction coefficient of the free (unbound) complex and ε_b is the extinction coefficient of the fully bound complex. The ratio of the slope $\frac{1}{[\varepsilon a-\varepsilon f]}$ and the intercept $\frac{1}{Kb[\varepsilon b-\varepsilon f]}$ obtained from a plot of [DNA] / [ε_a-ε_f] versus [DNA] gives the intrinsic binding constant [40]. 1 mmol stock solutions of the complexes **1, 2, 3** were prepared in Tris HCl buffer.

3. RESULTS AND DISCUSSION

3.1. *Synthesis and characterization*

The complexes were obtained in good yield and are stable in solid state and in solution at ambient conditions. UV-Vis, FT-IR and elemental analysis data are in agreement with the proposed structures for the complexes (Scheme **2**). The ultraviolet and visible spectra of the complexes are presented in Figure 1. There was no change in the UV-visible spectra of the complexes after dissolution for many hours, demonstrating stability and integrity of these complexes in solution. The FT-IR spectra are given in the supplementary material.

The electrospray ionization mass spectrum of complex **3** was also taken in methanol. The mass spectrum (Fig. 2) shows that the complex has main peaks at 860.1 and 968.8 which corresponds to [Fe+dox+2phen] and [Fedoxphen$_2$]Cl_3, respectively.

To establish the coordination sites of doxycycline to Fe^{III} we obtained the infrared spectra of doxycycline and the complexes **1**, **2** and **3**. The FTIR spectra of the complexes were then compared to that of doxycycline as assigned in previous published literature [15, 31, 33].

Amide(I) absorption of doxycycline at 1678 cm^{-1} appeared at about 1610 cm^{-1} for complex **1** and 1604 cm^{-1} for complex **2**, while amide(II) absorption band at 1520 of ring A carbonyl stretching ν (C=O) are absent in all the complexes indicating participation of ring A carbonyl oxygen in coordination to Fe^{III}. Two bands at 1244 and 1219 cm^{-1} assigned to δ (NH_2) and ν(C-NH_2) of doxycycline are essentially unchanged in the new complexes, indicating that the amino group is not involved in coordination in the complexes.

The bands that appear at 1494 cm^{-1} in complex **2** and 1518 in complex **3** are due to the phenanthroline and bipyridine C=C coordination to iron(III) in complexes **2** and **3**, respectively. Carbonyl stretching ν (C=O) on ring C at 1616 cm^{-1} in doxycycline shifted to 1562, 1582 and 1587 cm^{-1} in complexes **1**, **2** and **3**, respectively.

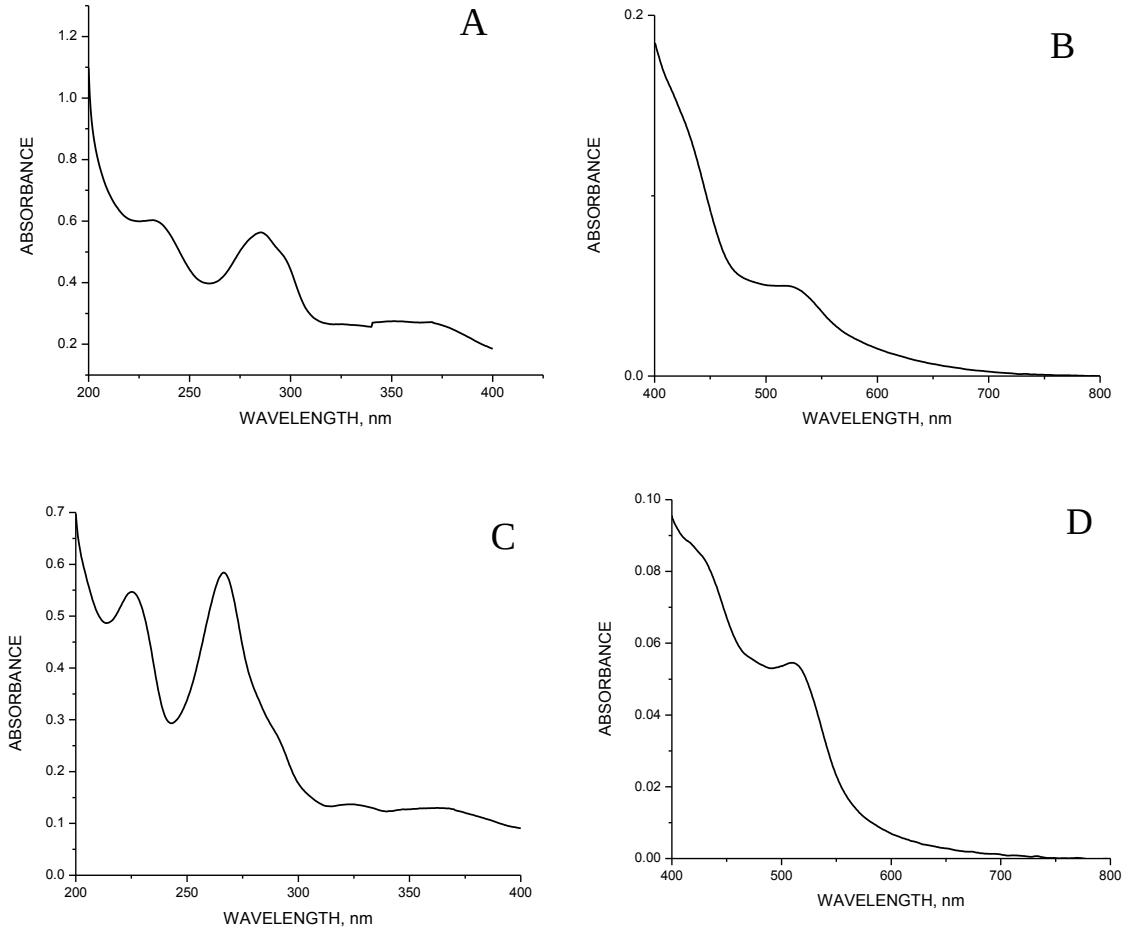


Fig. 1. UV and visible spectra of aqueous solutions of complexes **2** (A and B) and **3** (C and D) at 20 μM; l = 1 cm

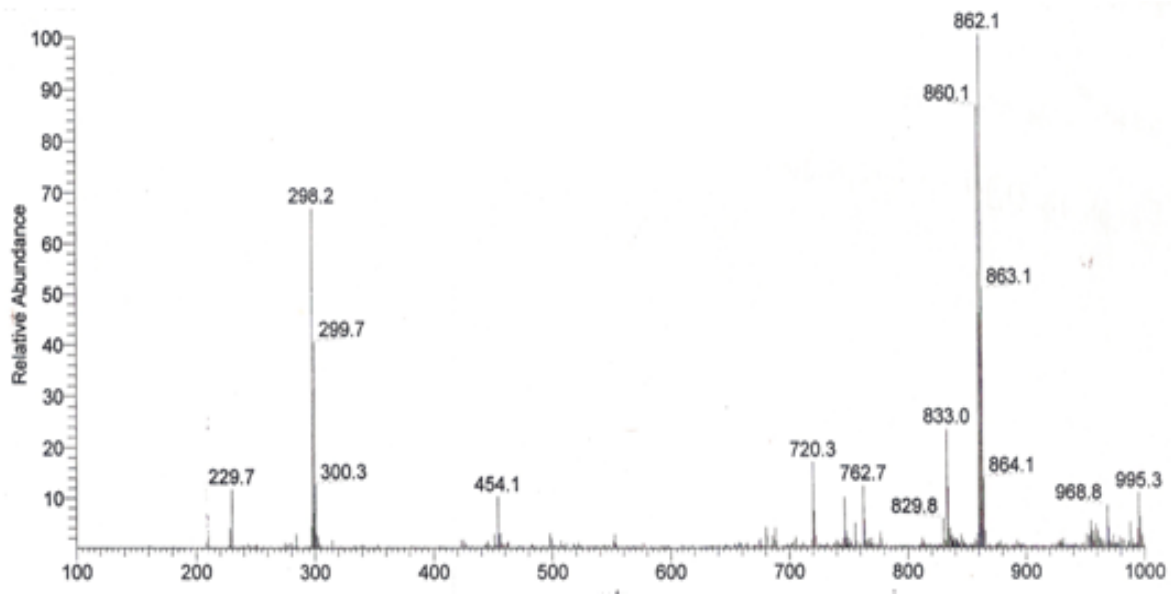
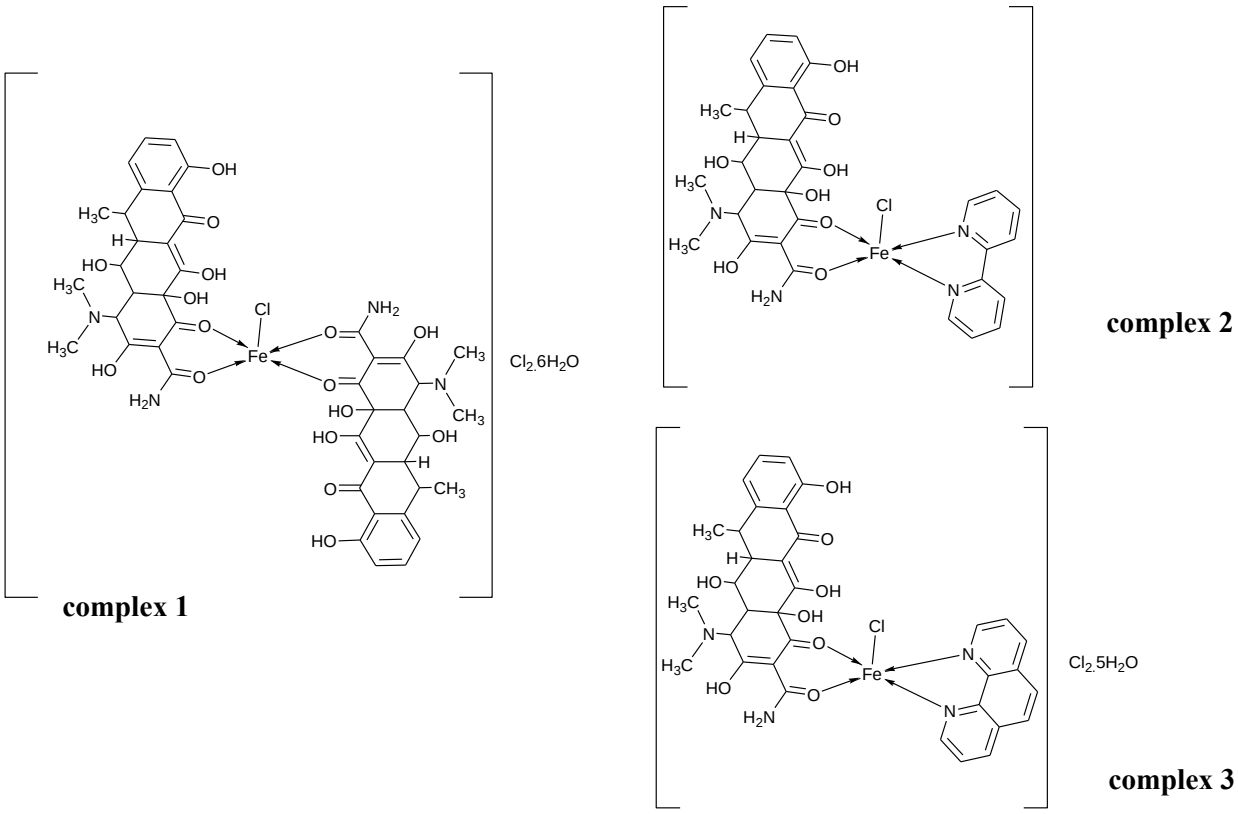


Fig. 2. Electrospray ionization mass spectrum of complex **3**



Scheme II. Proposed structures of complexes **1**, **2** and **3**

T a b l e 1

Physicochemical properties of the complexes

S/N	Complex molecular formula	Melting point /ºC	Color
1	$[FeDox_2Cl]Cl_2 \cdot 6H_2O$ [1]	203–205	Brown
2	$[FebpyDoxCl]Cl_2$ [2]	205–206	Brown
3	$[FephenDoxCl]Cl_2 \cdot 5H_2O$ [3]	196, dec	Orange

3.2. *Antimicrobial studies*

3.2.1. *Antibacterial susceptibility testing*

Antimicrobial susceptibilities of the complexes were tested on *Staphylococcus aureus* and *Klebsiella pneumonia* as a function of concentration. *Staphylococcus aureus* and *Klebsiella pneumonia* are pathogens that cause respiratory and urinary tract infections in humans [41]. Four concentrations of the complexes were taken, i.e. 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml and 2.0 mg/ml. The susceptibility of the strains of bacteria towards the complexes were determined by measuring the size of inhibition diameter and compared to that of doxycycline, a standard antibiotic and also the parent ligand. All experiments were performed in triplicate and the standard deviations were negligible. The antibacterial susceptibility tests of the complexes against *Staphylococcus aureus* and *Klebsiella pneumonia* are presented in Table 2. The antibacterial activities of the three complexes were in the same range with the parent ligand, doxycycline. The presence of the ancillary polypyridyl ligands did not contribute significantly to the antibacterial activities of the complexes. The coordination of iron alone to doxycycline in the complexes did not improve their antibacterial activity but rather reduced it though to a small extent.

T a b l e 2

Antibacterial activity of doxycycline and its complexes against Klebsiella pneumonia and Staphylococcus aureus

AGENTS	**ZONE OF INHIBITION /mm * against *Klebsiella pneumonia***				**ZONE OF INHIBITION /mm * *Staphylococcus aureus***			
	0.5 mg	1.0 mg	1.5 mg	2.0 mg	0.5 mg	1.0 mg	1.5 mg	2.0 mg
1	10 ± 0.2	11 ± 0.1	13 ± 0.1	13 ± 0.2	13 ± 0.3	14 ± 0.2	15 ± 0.1	17 ± 0.2
2	12 ± 0.5	15 ± 0.7	16 ± 0.3	16 ± 0.4	10 ± 0.6	12 ± 0.4	13 ± 0.5	14 ± 0.3
3	13 ± 0.5	14 ± 0.4	15 ± 0.5	16 ± 0.2	12 ± 0.6	13 ± 0.4	16 ± 0.5	17 ± 0.3
Doxycycline hyclate	11 ± 0.6	12 ± 0.4	13 ± 0.5	15 ± 0.3	15 ± 0.3	15 ± 0.5	16 ± 0.4	17 ± 0.2
Cefotaxime— 30 μg [**42**]	31.04				18.91			
Chloramphenicol—30 μg [**42**]	24.69				15.54			
Streptomycin— 10 mcg/disc [**43**]	19 + 1.4				25.5 + 0.5			

*Values are average of triplicate determinations and standard deviations were negligible

3.2.2. *Antiplasmodial activity study*

The relative activity as the IC_{50} [parental compound/IC_{50} (metal complex)] presented in Table 3 showed that FephenDox (**3**) was the most active among all the complexes, though only in the same range with doxycycline, the parent compound from which it was derived, while complex **1** showed no activity in the concentration range tested. The presence of iron in the complexes did not add to the antibacterial activity of the complexes but rather reduced it to a small extent. Complexation of iron alone reduced the antiplasmodial activity of complex **1** to a large extent, while it can be said that the presence of ancillary polypyridyl ligands compensated for this reduced antiplasmodial activity and that a more planar the polypyridyl ligand improves the antiplasmodial activity of the complex. Iron(III) chloride has been reported to reduce biological activity of tetracyclines [44].

This suggests that the antiplasmodial activity of the complexes could be associated with the interaction of the planar aromatic rings of the complexes with the parasitic DNA. Complex **3** which bears 1,10-phenathroline which is more planar than 2,2′-bipyridine in complex **2** exerted the higher activity between the complexes. However, the activities of all the complexes were lower than that of chloroquine diphosphate.

T a b l e 3

Antiplasmodial activity of doxycycline and its complexes

Compound	**IC_{50} /μg/ml ***	**Relative activity to Dox /IC_{50}**
1	>100 ± ND	ND
2	39 ± 10.8	0.26
3	11 ± 5.5	0.91
Dox hyclate	10 ± 0.7	
Chloroquine diphosphate (CQDP)	0.02 ± 0.004	500

*Values are average of triplicate determinations. ND = not determined

3.3. *DNA binding*

Monitoring the changes in absorption spectra of metal complexes upon the addition of increasing amounts of DNA is one of the most widely used methods for determining overall binding constants of metal complexes to DNA [38]. Upon the addition of calf thymus DNA to **2** and **3**, there was a decrease in molar absorptivity (hypochromism) of the absorption bands of the complexes indicating interaction of the complexes to DNA. To quantitatively compare the binding strength of these complexes, their intrinsic binding constants with CT-DNA were obtained by monitoring the changes in absorption bands (Fig. 3) with increasing concentration of DNA using equation 1 and were found to be 5.6×10^4 and 4.8×10^4 for complexes **2** and **3**, respectively.

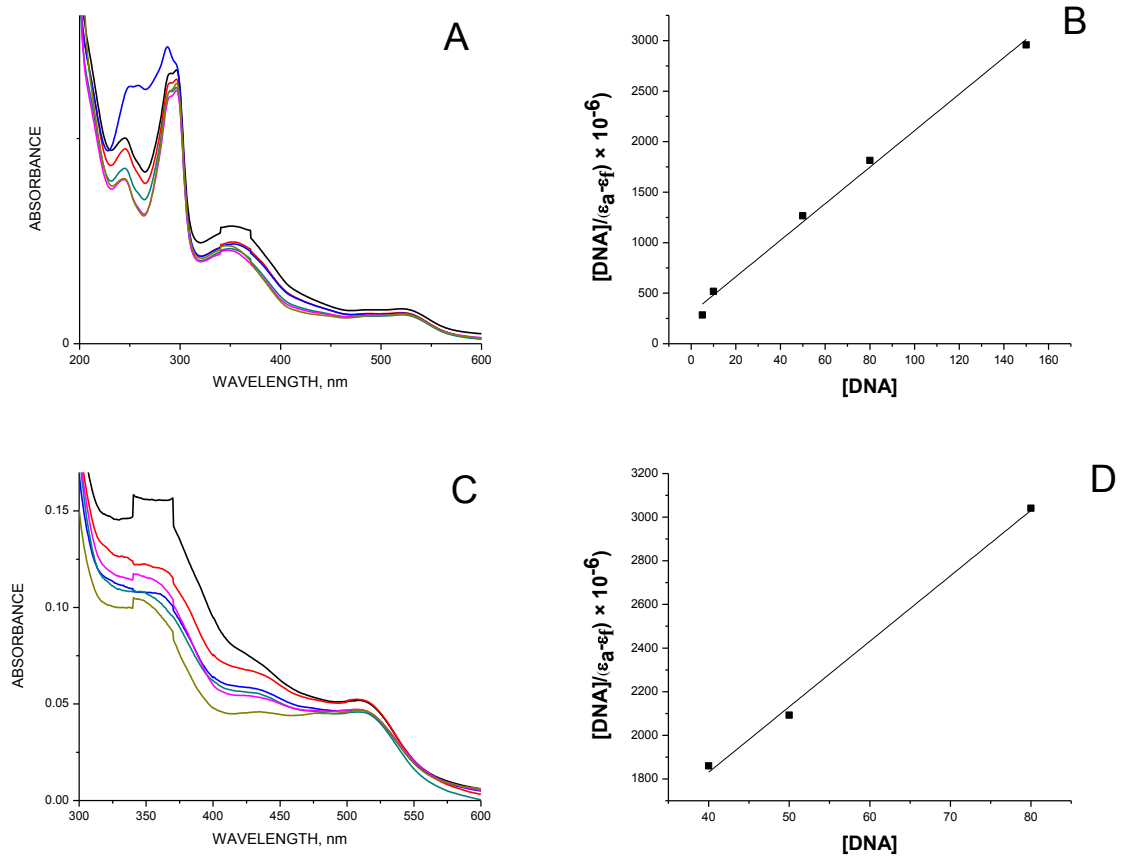


Fig. 3. Graphs showing the electronic absorption spectra of 20 μM complexes **2** (A) and **3** (C) with increasing concentrations of CT-DNA in 50 mmol NaCl and 5 mmol Tris HCl buffer at pH 7.2. B and D show a fitting of the absorbance data at 412 nm for complex **2** and 413 nm for complex **3** used to obtain the binding constants.

4. CONCLUSION

Three iron(III) complexes of the antibiotic doxycycline have been synthesized and well characterized by elemental analysis, UV-vis, FT-IR and electrospray mass spectroscopy. The three complexes showed some activity against strains of *Staphylococcus aureus* and *Klebsiella pneumonia*. The ancillary polypyridyl ligands did not contribute implicitly to the antimicrobial activities of these complexes. In addition, the coordination of iron in the complexes reduced their antibacterial activity, while the ancillary polypyridyl ligands compensated for the reduced antiplasmodial activity. The more planar the polypyridyl ligand, the higher the antiplasmodial activity of the complex. The complexes were also found to interact with DNA with binding constants 5.6×10^4 and 4.8×10^4 for complexes **2** and **3**, respectively.

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