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Feature article

Pyrazolone incorporating amino acid metallointercalators as effective DNA targets: Synthesis and *in vitro* biocidal evaluation

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ABSTRACT

Three novel mixed-ligand complexes using pyrazolone derivative (4-chloro-benzylidene-4-aminoantipyrine) as primary ligand and L-methionine as co-ligand, were synthesized and characterized by physico-chemical analytical techniques. The DNA interaction of these complexes was investigated by electronic absorption spectroscopy, viscosity, cyclic voltammetry and gel electrophoresis measurements. The results indicate that the complexes bind to DNA through intercalation and act as efficient cleaving agents. The *in vitro* antibacterial and antifungal assay indicates that these complexes are good antimicrobial agents against various pathogens.

Contents

Exploring and designing novel molecules capable of interacting with nucleic acids and triggering apoptosis are currently two of the most promising strategies for researchers to discover novel DNA-targeted anticancer drugs for chemotherapy [1–4]. Amino acids can perform a variety of beneficial functions in our body. Methionine is an essential amino acid and a powerful antioxidant. It contains the health-promoting substance sulfur, which is found primarily in meat, eggs, fish, and dairy products. The coordination of amino acids to transition metals occurs *via* a chelate binding mode involving both carboxylate and amine groups. Mixed ligand transition metal complexes, which strongly bind and cleave DNA can also exhibit prominent anticancer activities and regulate apoptosis [5–9]. Synthesis, structural studies and few other properties of mixed ligand complexes formed with amino acids have been reported [10–13]. The desire for an in-depth understanding of the rules that lead to the formation of systems of different nuclearities, together with their pharmacological activity as well as their interesting electrical and magnetic properties, makes research on the coordination chemistry of pyrazolone derivatives even more attractive. Moreover, Schiff bases play an imperative role in bioinorganic chemistry as they exhibit incredible biological activity.

In recent years, Schiff bases of 4-aminoantipyrine transition metal complexes and their derivatives have been extensively examined due to their wide applications in various fields such as antifungal, antibacterial, analgesic, sedative, antipyretic, anti-inflammatory agents [14] and DNA binding properties [15–17]. Modern tentative research shows promising results with regard to their anti-tumor actions [18]. Therefore, much of the attention has been targeted on the design of metal-based complexes, which can bind and cleave DNA. Literature survey proves that no studies on the synthesis and characterization of mixed ligand complexes of methionine have been reported.

Bearing these facts in mind, herein, based on our recent endeavors we have focused on the synthesis and structural determination of 4-aminoantipyrine based Schiff base, derived from 4-aminoantipyrine and 4-chlorobenzaldehyde, with L-methionine and its Cu(II), Ni(II) and Zn(II) complexes. Binding propensity of these mixed ligand complexes with DNA has also been investigated using electronic absorbance spectroscopy, viscosity measurement and cyclic voltammetry techniques. Moreover, the capability of these complexes having nitrogen and oxygen donors to induce DNA cleavage in the presence of H_2O_2 has been scrutinized. The *in vitro* antimicrobial evaluation of present complexes is also explored. These complexes may give an opportunity to provide routes towards rational drug design as well as means to widen perceptive chemical probes for DNA.

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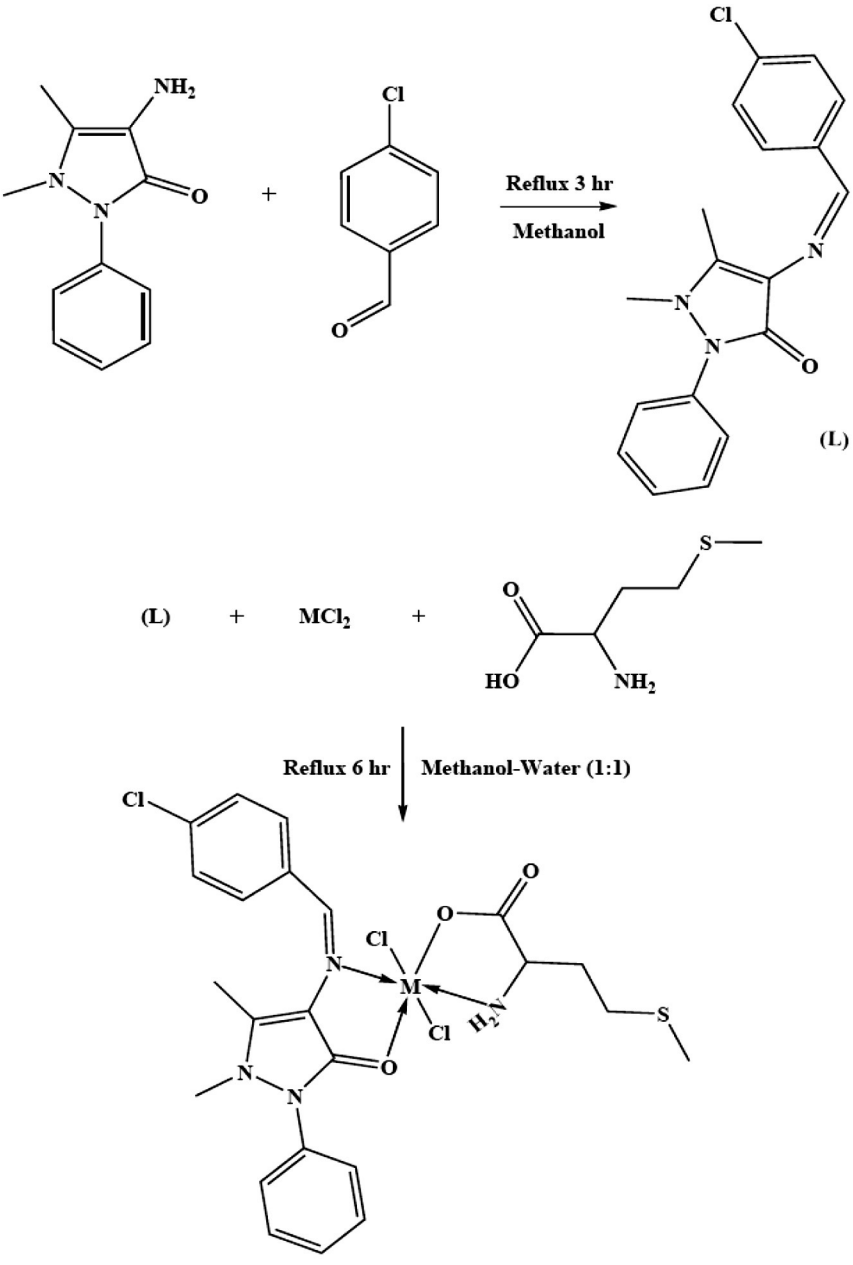
The Schiff base ligand, L (condensation of 4-aminoantipyrine and *p*-chlorobenzaldehyde) and its mixed-ligand [L-Met (*i.e.*, L-methionine)] Cu(II), Ni(II) and Zn(II) complexes were prepared by a typical procedure [19–24]. The materials and methods for the newly synthesized mixed-ligand complexes and their EPR spectral characterization were depicted as S1, S2 and S3 (Supplementary files). The complexes were found to be air stable. The ligand is soluble in common organic solvents but their complexes are soluble only in DMF, and DMSO. We acquired the probable composition of complexes as $[ML(Met)Cl_2]$ which were confirmed through elemental analyses, magnetic susceptibility, NMR, UV, IR, EPR spectra and molar conductivity measurements since no single crystals suitable for X-ray determination could be isolated (Scheme 1).

The mode and propensity of binding of metal complexes to CT DNA have been studied by different techniques. Thus, we propose to study the nature and extent of DNA binding of the complexes with CT DNA by using absorption spectroscopy, cyclic voltammetry, and viscosity measurements. The stability of our complexes in Tris–HCl buffer solution has been studied by observing the UV–Vis spectra and estimating the molar conductivities at different time intervals for any possible change [25]. The tested compounds were prepared in DMF and for experiments, they were freshly diluted in Tris–HCl buffer system. Then the UV–Vis spectra were recorded and molar conductivities were measured at different time intervals. From the results (Fig. S1), it is seen that there is no change in the absorption bands and their molar conductance values for the freshly prepared solutions which indicates that our complexes are sufficiently stable in Tris–HCl buffer environment even after reaching DNA.

As the crucial pharmacological target of many antitumor drugs, DNA and DNA binding activities of metal complexes have been an inkling of prevailing significance for the growth of efficient metal based chemotherapeutic drugs. Therefore, the potential binding ability of complex to CT-DNA was characterized by UV spectroscopy. The absorption



Scheme 1. Synthesis of Schiff base ligand and its mixed-ligand metal complexes.

spectra of [CuL(Met)Cl_2] in the absence and presence of CT-DNA at different concentrations are given in Fig. 1.

As the concentration of CT-DNA increases, the absorption spectra show apparent hypochromism effect and red shift in the MLCT bands. The extent of the hypochromism in the metal-to-ligand charge transfer (MLCT) band is commonly consistent with the strength of intercalative interaction.

With increasing DNA concentrations, the absorption band at 341.0 nm of the Ni(II) complex exhibits hypochromism of 20.40%; the absorption band at 343.1 nm of the Cu(II) complex appears hypochromism of 29.20% and the absorption band at 342.1 nm of the Zn(II) complex appears hypochromism of 22.00%. The red shifts of Ni(II), Cu(II), and Zn(II) complexes are shifted to 2.3, 2.5, and 2.7 nm (Table 1) respectively. The hypochromism observed for the transition band indicates strong binding of the metal complexes to DNA. After 24 h, the spectra were again recorded and observed that the same results were obtained which confirm the stability of our complex–DNA system. Thus, the synthesized complexes have significantly greater intercalating properties due to the presence of primary ligand and two coordinated chlorides [26]. All the metal complexes showed decrease in absorption intensity (hypochromism) with a slight red shift which is due to the intercalative binding between DNA and metal complexes.

Optical photophysical probes generally provide necessary, but not sufficient clues to support a binding model. An emblematical intercalative molecular interaction causes a momentous increase in viscosity of the DNA solution due to the increase in separation of the base pairs at the intercalation sites and hence an increase in the overall DNA length. The effect of mixed-ligand complexes on the viscosity of DNA depicted in Fig. 2 shows sturdy increase in the viscosity of the DNA with the addition of increasing amounts of the complexes. The values of relative specific viscosity $(\eta/\eta_0)^{1/3}$, where η and η_0 are the specific viscosities of DNA in the presence and absence of the complexes, were determined and plotted against values of $1/R$ (R = [DNA] / [Complex]). A petite to huge increase in and the ability of the complexes to increase the viscosity of DNA follows the order EB > [CuL(Met)Cl_2] > [NiL(Met)Cl_2] > [ZnL(Met)Cl_2]. The increase in viscosity of DNA by EB is the highest among all the complexes. Cu(II) complex interacts more strongly compared to others.

Optical photophysical probes generally provide necessary, but not sufficient clues to support a binding model. To authenticate the intercalative binding, viscosity experiments were performed. An emblematical intercalative molecular interaction causes a momentous increase in viscosity of the DNA solution due to the increase in separation of the base pairs at the intercalation sites and hence an increase in the overall DNA length. Viscosity measurements perceptibly show that all the complexes can intercalate between adjacent DNA base pairs, causing an extension in the helix and thus augment the viscosity of DNA, and that the complexes can intercalate a huge more sturdily and acutely than the free ligand. The results reveal that the presence of the complexes increases the relative viscosity of the DNA solution. The results obtained from the viscosity experiments validate those obtained from the spectroscopic studies. Such behavior is consistent with other intercalators (*i.e.*, EB), which increase the relative specific viscosity for the lengthening of the DNA double helix resulting from intercalation. The results obviously indicate that both the complexes intercalate between adjacent DNA base pairs, causing an extension in the helix thereby increasing the viscosity of DNA [27].

Table 1
Electronic absorption spectral properties of Cu(II), Ni(II) and Zn(II) mixed-ligand complexes.

Compound	λ max		Δλ		
	Free	Bound	(nm)	[a]H%	[b]$K_b \times 10^5$ (M^{-1})
[CuL(Met)Cl_2]	343.0	335.5	2.5	29.20	2.29
[NiL(Met) Cl_2]	341.0	338.7	2.3	20.40	1.85
[ZnL(Met)Cl_2]	342.1	344.8	2.7	22.00	1.17

[a] $H\% = [(A_{free} - A_{bound}) / A_{free}] \times 100\%$.
[b] K_b = Intrinsic DNA binding constant determined from the UV–Vis absorption spectral titration.

The application of electrochemical methods to the study of metallo-intercalation and coordination of transitional metal complexes to DNA provides a useful complement to the previously used methods of investigation, such as UV–vis spectroscopy and viscosity measurements. The electrochemical behavior of complex is well known, and is strongly influenced by the electrode material. A well defined and sensitive peak observed from the solution of the complex with a GC electrode was used in this investigation [28]. The cyclic voltammetric behavior of all the complexes has been examined in the absence and presence of DNA in 5 mM Tris–HCl/50 mM NaCl buffer solution as the supporting electrolyte. Electrochemical data for the Cu(II), Ni(II), and Zn(II) complexes are shown in Table 2. No new redox peaks appeared after the addition of CT DNA to each complex, but the current intensity of all the peaks decreased significantly, suggesting the existence of an interaction between each complex and CT DNA. The decrease in current intensity can be explained in terms of an equilibrium mixture of free and DNA-bound complex to the electrode surface [28].

The cyclic voltammograms of the copper complex in the absence and presence of different amounts of DNA are shown in Fig. 3. In the absence of CT DNA, the first redox cathodic peak appeared at −0.156 V for

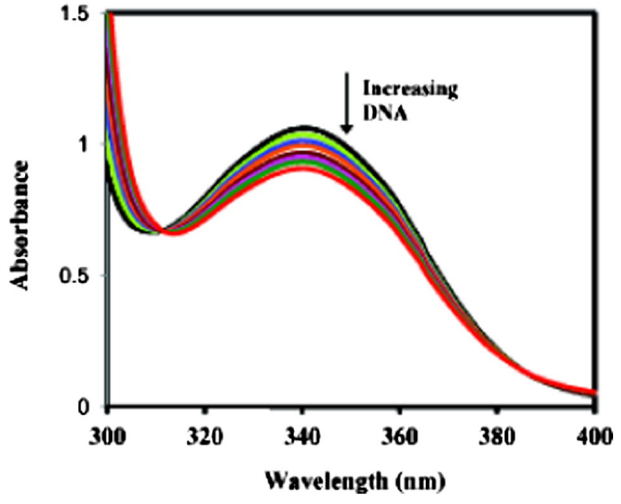


Fig. 1. Absorption spectrum of [CuL(Met)Cl_2] complex in buffer pH = 7.2 at 25 °C in the presence of increasing amount of DNA. Arrow indicates the changes in absorbance upon increasing the DNA concentration.

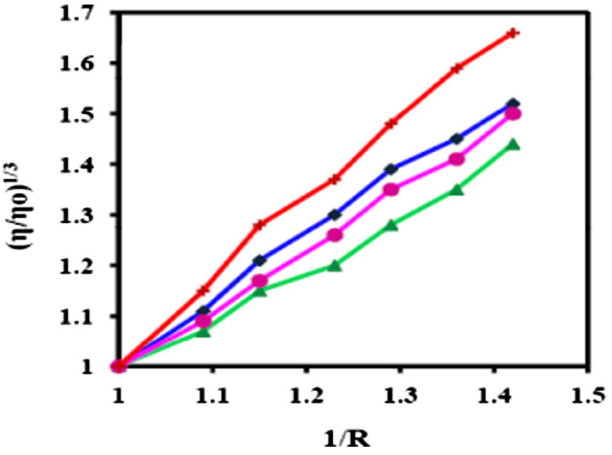


Fig. 2. Effect of increasing amounts of [EB] (×), [CuL(Met)Cl2] (■),[NiL(Met)Cl_2] (●) and [ZnL(Met)Cl_2] (▲) on the relative viscosity of DNA. $1/R$ = [complex]/[DNA] or [EB]/[DNA].

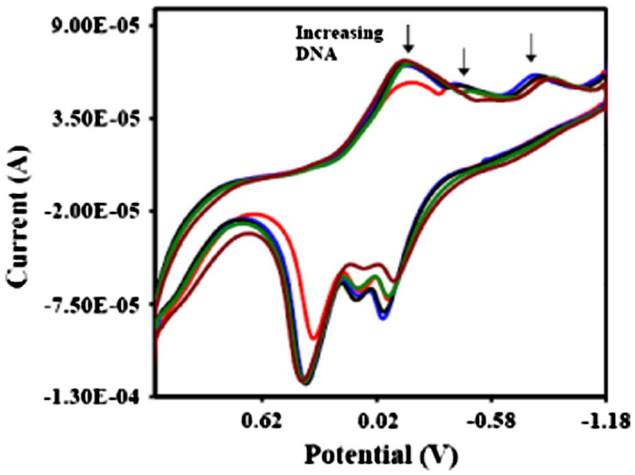


Fig. 3. Cyclic voltammogram of [CuL(Met)Cl_2] in buffer pH = 7.2 at 25 °C in the presence of increasing amount of DNA. Arrow indicates the changes in voltammetric currents upon increasing the DNA concentration.

Cu(III) → Cu(II) (Ep_a = 0.426 V, Ep_c = −0.156 V, ΔEp = 0.57 V, and $E_{1/2}$ = 0.135 V); in the second redox couple, the cathodic peak appeared at −0.408 V for Cu(II) → Cu(I) (Ep_a = 0.154 V, Ep_c = −0.408 V, ΔEp = 0.54 V, and $E_{1/2}$ = −0.126 V); and in the third redox couple, the cathodic peak appeared at −0.836 V for Cu(I) → Cu(0) (Ep_a = 0.008 V, Ep_c = −0.836 V, ΔEp = 0.83 V, and $E_{1/2}$ = −0.404 V). The Ip_a/Ip_c ratios for these three redox couples were 1.56, 1.27, and 1.20, respectively, which indicate that reaction of the complex on the glassy carbon electrode surface is a quasi-reversible redox process. During the incremental addition of CT DNA to the complex, the redox couples caused a less negative shift in $E_{1/2}$ and decrease of ΔEp (Table 2). The Ip_a/Ip_c values also decreased in the presence of DNA (Table 2).

For Ni(II) → Ni(I) the redox couple cathodic peak appeared at −0.539 V in the absence of CT DNA (Ep_a = −0.064 V, Ep_c = −0.539 V, ΔEp = 0.47 V, and $E_{1/2}$ = −0.298 V). The Ip_a/Ip_c ratio was approximately unity. This indicates the quasi-reversible redox process of the metal complex. During incremental addition of CT DNA to the complex, the redox couple caused a negative shift in $E_{1/2}$ and a decrease in ΔEp. Finally a quasi-reversible transfer process with the redox couple [Zn(II) → Zn(0)] was observed for the Zn(II) complex. The cathodic peak appeared at −0.532 V in the absence of DNA (Ep_a = −0.065 V, Ep_c = −0.532 V, ΔEp = 0.464 V, and $E_{1/2}$ = −0.295 V). The Ip_a/Ip_c ratio was 1.03. This indicates the quasi-reversible redox process of the metal complex. Incremental addition of DNA to the Zn(II) complex resulted in a slight decrease in the current intensity and negative shift of the oxidation peak potential. The resulting minor changes in the current and potential are indicative of diffusion of the metal complexes bound to the large, slowly diffusing DNA molecule [29].

Table 2
Electrochemical parameters for interaction of DNA with Cu(II), Ni(II) and Zn(II) complexes.

Compound	Redox couple	$E_{1/2}$(V)[a]		ΔEp(V)[b]		Ip_a/Ip_c
		Free	Bound	Free	Bound	
[CuL(Met)Cl_2]	Cu(III) → Cu(II)	0.135	0.123	0.57	0.59	1.46
	Cu(II) → Cu(I)	−0.126	−0.144	0.54	0.56	1.27
	Cu(I) → Cu(0)	−0.404	−0.416	0.83	0.85	1.20
[NiL(Met)Cl_2]	Ni(II) → Ni(I)	−0.298	−0.341	0.472	0.523	0.95
[ZnL(Met)Cl_2]	Zn(II) → Zn(0)	−0.295	−0.358	0.464	0.376	1.03

Data from cyclic voltammetric measurements:
[a] $E_{1/2}$ is calculated as the average of anodic (E_{Pa}) and cathodic (Epc) peak potentials; $E^1_{1/2} = E_{Pa} + E_{pc}/2$.
[b] $\Delta Ep = E_{pa} - E_{pc}$.

Many biologically active compounds used as drugs possess modified pharmacological and toxicological potentials when administered in the form of metal-based compounds. Since the novel ligand and its transition metal complexes exhibit good DNA binding affinity, it is considered worthwhile to investigate their other biological activities, such as antibacterial and antifungal activity. The *in vitro* antimicrobial activity of the ligand and its complexes was investigated against the sensitive two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacterial strains and for *in vitro* antifungal activity against *Aspergillus niger*, *Rhizoctonia bataticola*, *Candida albicans* and *Aspergillus flavus*, by disc diffusion method. The minimum inhibitory concentration (MIC) values of the compounds are listed in Tables 3 and 4. The DMF control showed no activity against any microbial strain. The activity of the complexes has been compared with the activity of common standard antibiotics, Kanamycin and Clotrimazole which are shown in Fig. 4a and b (Tables 3 and 4).

Inspection of these data reveals that the newly prepared Schiff base and its metal complexes showed an amazing effect against these microbial strains. Such increased activity of the complexes can be explained on the basis of Overtone's concept [30] and Tweedy's Chelation theory [31]. According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favors the passage of only the lipid soluble materials due to which liposolubility is an important factor, which controls the antimicrobial activity. On chelation, the polarity of the metal ion will be reduced to a greater extent due to the overlap of the ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Further, it increases the delocalization of π-electrons over the whole chelate ring and enhances the lipophilicity of the complexes. This increased lipophilicity enhances the penetration of the complexes into lipid membranes and blocking of the metal binding sites in the enzymes of microorganisms. These complexes also disturb the respiration process of the cell and thus block the synthesis of the proteins that restricts further growth of the organisms.

Furthermore, the mode of action of the compound may involve formation of a hydrogen bond through the azomethine group with the active center of cell constituents, resulting in interference with the normal cell process. Also, the increase in lipophilicity enhances the penetration of Schiff base and its metal complexes into the lipid membrane and thus confines further growth of the organism [32].

A comparative study of the ligand and its complexes (MIC values) indicates that complexes exhibit higher antimicrobial activity than the free ligand (Fig. 4a and b). From the MIC values, it was found that the [CuL(Met)Cl_2] was more potent among the other investigated complexes. It may be attributed to the atomic radius and the electronegativity of Cu(II) ions. Current studies reveal that the high atomic radius and electronegative metal ions in their metal complexes exhibit high antimicrobial activity. Higher electronegativity and large atomic radius decrease the effective positive charges on the metal complex molecules which facilitates their interaction with the highly sensitive cellular membranes towards the charged particle.

DNA is the pharmacologic target of many drugs currently in clinical use or in advanced clinical trials. Small molecules that bind genomic DNA have proven to be effective anticancer, antibiotic, and antiviral therapeutic agents [33–35]. Agarose gel electrophoresis assay is a useful method to investigate various binding modes of small molecules to supercoiled DNA. Thus, suitably designed metal complexes, after binding to DNA, can induce several changes in the DNA conformation, such as bending, 'local denaturation' (overwinding and underwinding), intercalation, micro loop formation and subsequent DNA shortening, leading to a decrease in the molecular weight of the DNA. The photograph, as shown in Fig. 5, shows bands with different bandwidths compared to the control and this is the differentiating criteria for binding and cleavage abilities of the complexes with pUC19 DNA in this study. DNA cleavage was controlled by relaxation of the super coiled circular form of pUC19 into the open circular form and linear form. The general

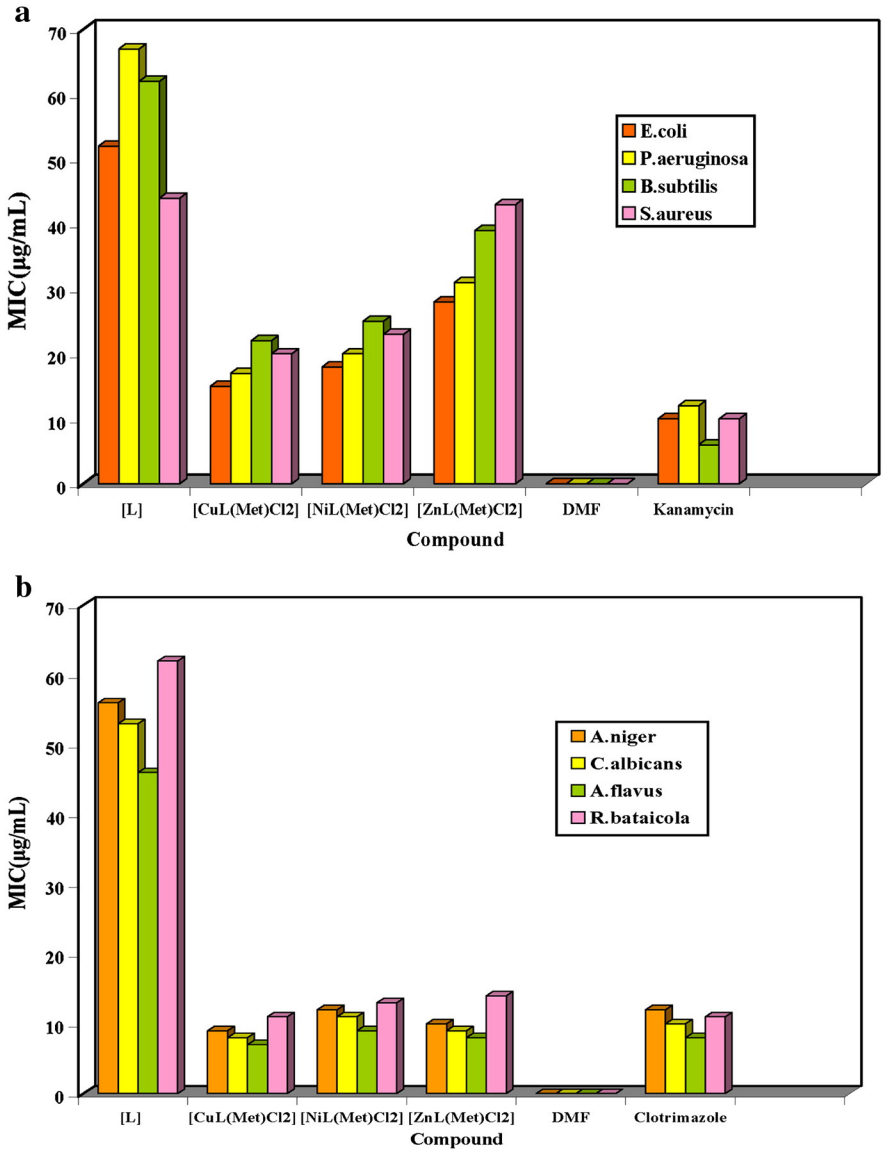


Fig. 4. a. Minimum inhibitory concentration of the synthesized compounds against the growth of bacteria (µg/mL). b. Minimum inhibitory concentration of the synthesized compounds against the growth of fungi (µg/mL).

oxidative mechanisms proposed account for DNA cleavage by hydroxyl radicals *via* abstraction of a hydrogen atom from sugar units and predict the release of specific residues arising from transformed sugars, depending on the position from which the hydrogen atom is removed [36]. When circular plasmid DNA is run on horizontal gel using electrophoresis, the super coiled form will migrate first (Form I). If the strand of the selected DNA is cleaved by interaction with the metal complexes, the supercoils will relax to produce an open circular form (Form II) that moves slower than Form I, whereas with cleavage of both strands to a linear form (Form III) the resulting system will migrate in between the above two forms. A control experiment using DNA alone does not show any significant cleavage of DNA, even after a long exposure time (Lane 1). Further, when pUC19 DNA is allowed to interact with the complexes, a substantial decrease in the intensities of the bands (Lanes 3–7) for the metal bound DNA as compared to the untreated control DNA is

Table 3
Minimum inhibitory concentration of the synthesized compounds against the growth of bacteria (µg/mL).

No.	Compound	MIC (µg/mL)			
		E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
1	[L]	52	67	62	44
2	[CuL(Met)Cl_2]	15	17	22	20
3	[NiL(Met)Cl_2]	18	20	25	23
4	[ZnL(Met)Cl_2]	28	31	39	43
5	DMF	_	_	_	_
6	Kanamycin[a]	10	12	06	10

[a] Kanamycin is used as the standard.

Table 4
Minimum inhibitory concentration of the synthesized compounds against the growth of fungi (µg/mL).

S. No	Compound	MIC(µg/mL)			
		A. niger	*C. albicans*	*A. flavus*	*R. bataicola*
1	[L]	56	53	46	62
2	[CuL(Met)Cl_2]	9	8	7	11
3	[NiL(Met)Cl_2]	12	11	9	13
4	[ZnL(Met)Cl_2]	10	9	8	14
5	DMF	_	_	_	_
6	[a]Clotrimazole	12	10	8	11

[a] Clotrimazole is used as the standard.

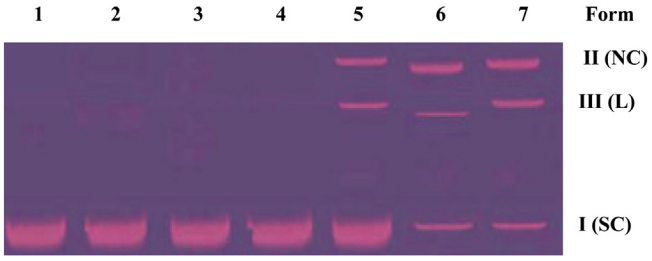


Fig. 5. Changes in the agarose gel electrophoretic pattern of pUC19 DNA (0.3 mg) by metal(II) complexes (0.30 mmol L^{-1}) in the presence of H_2O_2 (100 mmol L^{-1}) in 50 mmol L^{-1} Tris–HCl/50 mmol L^{-1} NaCl buffer (pH 7.2): Lane 1, DNA alone; Lane 2, DNA + H_2O_2; Lane 3, DNA + [L] + H_2O_2; Lane 4, DNA + [CuL(Met)Cl_2]; Lane 5, DNA + [CuL(Met)Cl_2] + H_2O_2; Lane 6, DNA + [NiL(Met)Cl_2] + H_2O_2 and Lane 7, DNA + [ZnL(Met)Cl_2] + H_2O_2 respectively.

observed, which suggests an intercalative binding mode for the complexes (Fig. 5).

These experimental facts demonstrated that a permutation of both the metal complexes and H_2O_2 is required to show effective cleavage of pUC19 DNA. The "chemical nuclease" activity of the [CuL(Met)Cl_2] complex is due to formation of a reactive copper-(I) species that activates molecular oxygen to form a hydroxyl radical and/or copper-oxo species that cleaves DNA by abstraction of the deoxyribose sugar hydrogen atom [37]. Similarly, [NiL(Met)Cl_2] and [ZnL(Met)Cl_2] complexes oxidatively cleave DNA in the presence of H_2O_2. The results suggest that adequate binding of the ligand and all the metal complexes causes change in the conformation of pUC19 DNA. Thus, all the complexes reveal an incredible DNA cleavage activity in the presence of H_2O_2 with efficiency depending upon the mode of DNA binding as determined by the nature of the imine ligand. It is believed that the superior cleavage ability of complexes is due to the reaction of metal ions with H_2O_2, which produces diffusible hydroxyl radicals or molecular oxygen at ease, which in turn damage DNA through Fenton-type chemistry [38].

In summary, novel mononuclear mixed ligand complexes using 4-aminoantipyrine based Schiff base ligand and methionine (co-ligand) have been synthesized and characterized by spectral and analytical data. The IR, electronic transition and NMR studies suggest octahedral structure for Cu(II), Ni(II) and Zn(II) complexes with two chlorine atoms in the coordination sphere. Binding induces changes in absorption along with viscosity measurements revealing that the ligand and all the complexes could intercalatively bind to DNA. Anti-microbial activity study shows that the complexes reveal good biological activity against different microorganisms. The DNA cleaving activity of metal complexes with pUC19 DNA under aerobic conditions shows more pronounced activity in the presence of an oxidant. The investigation of these issues is a current goal of our groups and will be properly developed in the future. Nonetheless, the data observed thus far provide a compelling rationale for the clinical development of [CuL(Met)Cl_2] as a potential anticancer drug. Therefore, [CuL(Met)Cl_2] complex exhibits most potent activity which warrants further investigation. Current studies are ongoing in our laboratory in order to gain a better insight in the mechanism of action and water solubility of these complexes, which may be helpful for the design of new metal-based anti-tumor agents.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.inoche.2013.09.028.

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[19] A methanolic solution of (20 mL) 4-aminoantipyrine (0.01 mol) was added to a methanolic solution of *p*-chlorobenzaldehyde (0.01 mol). The resultant mixture was refluxed for ca. 3 h. The solid product formed (4-chloro-benzylidene-4-aminoantipyrine) was filtered, washed, dried and recrystallized from methanol.
[20] [$C_{18}H_{16}ClN_3O$]. Yield: 86%. FT-IR (KBr disc): 1,624 (HC = N), 1,717 υ(C = O) cm^{-1}; MS: m/z 326. ^{1}H NMR (DMSO-d_6) δppm: 7.0–7.5 (phenyl multiplet), 8.8 (s,-CH = N), 2.6 (s,-OCH_3), 2.2 (s,-C-CH_3), 1.5 (s,N-CH_3); ^{13}C NMR (DMSO-d_6): d = 112.37, 118.95, 125.11, 129.05, 131.46, 136.10, 142.12, (aromatic C), 166.15 (CH = N), 158.56 (C = O) ppm; Anal.Calc. for $C_{18}H_{16}ClN_3O$: C, 66.36; H, 4.95; Cl, 10.88; N, 12.90; Found: C, 66.31; H, 4.88; Cl, 10.83; N, 12.86%. λ_{max} in DMF 39,225 and 27,521 cm^{-1}.
[21] To a methanolic solution of Schiff base (0.01 mol) was heated under reflux with the methanolic solution of metal(II) chloride (0.01 mol) in methanol (20 mL) for *ca.* 3 h. To the above mixture, 0.01 mol of L-methionine dissolved in 1:1 water–methanol (40 mL) was added to a hot methanolic solution (30 mL) of KOH was added and the reflux was continued for *ca.* 2–3 h. The resultant product was washed and recrystallized with methanol. The solid product formed was filtered, washed with methanol and dried in vacuo.
[22] [CuL(Met)Cl_2]. Yield: 75%. FT-IR (KBr disc): 3,220 (NH_2), 1,695 υ(C = O), 1,597 (HC = N), 1,610 υ_{asy}(COO), 1,406 υ_{sy}(COO); 475 (M = O), 448 (M = N), 356 (M = Cl) cm^{-1}; MS: m/z 610. Anal.Calc. for $C_{23}H_{27}Cl_3N_4O_3SCu$: C, 45.33; H, 4.47; Cl, 17.45; N, 9.19; S, 5.26; Cu, 10.43. Found: C, 45.26; H, 4.39; Cl, 17.37; N, 9.11; S, 5.18; Cu, 10.35%. $\Lambda m \times 10^{-3}$(Ω^{-1} $mol^{-1}cm^2$) 5.45; μ_{eff} (BM) 1.98; λ_{max} in DMF 37,593, 43,482 and 13,268 cm^{-1}.
[23] [NiL(Met)Cl_2]. Yield: 72%. FT-IR (KBr disc): 3,189 (NH_2), 1,689 υ(C = O), 1,574 (HC = N), 1,592 υ_{asy} (COO), 1,385 υ_{sy}(COO); 463 (M = O), 442 (M = N), 324 (M = Cl) cm^{-1}; MS: m/z 605. Anal.Calc. for $C_{23}H_{27}Cl_3N_4O_3SNi$: C, 45.69; H, 4.50; Cl, 17.59; N, 9.27; S, 5.30; Ni, 9.71%. Found: C, 45.54; H, 4.43; Cl, 17.46; N, 9.16; S,

5.22; Ni, 9.63%. $\Lambda m \times 10^{-3}(\Omega^{-1}\,mol^{-1}\,cm^2)$ 1.28; μ_{eff} (BM) 3.08; λ_{max} in DMF 14,509, 15,567 and 22,784 cm^{-1}.

[24] [ZnL(Met)Cl_2]. Yield: 81%. FT-IR (KBr disc): 3,148 (NH_2), 1,686 υ(C = O), 1,541 (HC = N), 1,586 υ_{asy}(COO); 1,378 υ_{sy}(COO); 452 (M = O), 426 (M = N) 305 (M = Cl) cm^{-1}; MS: m/z 612. ^{1}H NMR (DMSO-d_6) δppm: 6.9–7.2 (phenyl multiplet), 8.6 (s, −CH = N) 2.6 (s, $-CH_2$); ^{13}C NMR (DMSO-d_6): d = 112.76, 119.25, 125.86, 129.79, 131.85, 136.92, 142.68, (aromatic C) 21.28–46.47 (Met C), 60.83 (C-NH_2), 174.67 (COO^-), 157.25 (C = O), 165.47 (CH = N); Anal.Calc. for $C_{23}H_{27}Cl_3N_4O_3SZn$: C, 45.19; H, 4.45; Cl, 17.40; N, 9.17; S, 5.25; Zn, 10.70%. Found: C, 45.07; H, 4.37; Cl, 17.31; N, 9.06; S, 5.18; Zn, 10.62%. $\Lambda m \times 10^{-3}$ $(\Omega^{-1}\,mol^{-1}\,cm^2)$ 12.32; λ_{max} in DMF 29,438, 33,109 cm^{-1}.

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